Animal Cells and Systems
Vol. 16, No. 1, February 2012, 1–10

Taylor & Francis
Taylor & Francis Group

Posttranslational and epigenetic regulation of the CLOCK/BMAL1 complex in the mammalian

Yool Lee and Kyungjin Kim*

Seoul National Universisty, School of Biological Sciences, 504 dong 222 ho, 599 Gwanak-ro, Gwanak-gu, Seoul, 151-747, Republic of Korea

(*Received 18 May 2011; accepted 5 July 2011*)

Most living organisms synchronize their physiological and behavioral activities with the daily changes in the environment using intrinsic time-keeping systems called circadian clocks. In mammals, the key molecular features of the internal clock are transcription- and translational-based negative feedback loops, in which clock-specific transcription factors activate the periodic expression of their own repressors, thereby generating the circadian rhythms. CLOCK and BMAL1, the basic helix-loop-helix (bHLH)/PAS transcription factors, constitute the positive limb of the molecular clock oscillator. Recent investigations have shown that various levels of posttranslational regulation work in concert with CLOCK/BMAL1 in mediating circadian and cellular stimuli to control and reset the circadian rhythmicity. Here we review how the CLOCK and BMAL1 activities are regulated by intracellular distribution, posttranslational modification, and the recruitment of various epigenetic regulators in response to circadian and cellular signaling pathways.

Keywords: circadian clock; post-translational modification; epigenetic regulation; BMAL1; CLOCK

Introduction

Circadian clocks are intrinsic, time-tracking systems that most living organisms have developed through undergoing a long period of 24 hr light/dark cycles as a result of Earth's rotation around its axis. These internal clocks allow organisms to adapt their physiology and behavior to the daily changes which take place in the environment (Schibler and Sassone-Corsi 2002). In mammals, the circadian rhythm is controlled by a central clock in the suprachiasmatic nucleus (SCN), which adjusts the internal time to an environmental light stimulus (Bass and Takahashi 2010). Recently, peripheral organs, tissues, and even individual cells have been found to possess self-sustaining clock oscillators, which are entrained by various neural and humoral signals from the SCN as well as certain physiological activities and environmental stimuli independently of the central clock (Stratmann and Schibler 2006; Wijnen and Young 2006; Dibner et al. 2010).

Both central and peripheral oscillators are believed to utilize common molecular 'gears and circuitry' for circadian rhythm generation (Reppert and Weaver 2002). The molecular clockwork is based on the interplay between transcriptional activators and repressors that generate a negative feedback loop for core clock gene expression. CLOCK and BMAL1 heterodimerize to form an active transcription complex that binds to the promoter E-box motifs (CACGTG) present in the *Per* and *Cry* genes so as to initiate transcription. Once PER and CRY proteins reach a critical concentration in the cytoplasm, they form a complex that translocates to the nucleus to repress CLOCK/BMAL1-mediated transcription of their own genes through direct protein-protein interaction (Cermakian and Sassone-Corsi 2000). The primary feedback loop is further stabilized by an additional transcriptional loop in which CLOCK/BMAL1 induces the transcription of certain nuclear receptors such as *Rev-erbα* and *Rorα* which, in turn, repress and activate *Bmal1* transcription via a binding and unbinding of the ROR-responsive element (RORE), respectively (Gallego and Virshup 2007). Notably, the CLOCK/BMAL1 heterodimer also activates the transcription of many other clock-controlled genes (CCGs), which activation, in turn, drives the circadian rhythmicity of various physiological functions, such as food intake, hormonal synthesis and release, and metabolism (Wijnen and Young 2006; Eckel-Mahan and Sassone-Corsi 2009). In addition to positive and negative transcriptional feedback-loops, the circadian oscillator is fine-tuned by various levels of post-transcriptional regulation, including intracellular localization, post-translational modification, the stability of the clock proteins and chromatin remodeling (Gallego and Virshup 2007; Duguay and Cermakian 2009; Mehra et al. 2009) (Figure 1). Further regulation of clock proteins by intra- and extracellular signaling is likely to play an important role in the physiological

*Corresponding author. Email: kyungjin@snu.ac.kr

ISSN 1976-8354 print/ISSN 2151-2485 online

http://dx.doi.org/10.1080/19768354.2011.603749
http://www.tandfonline.com

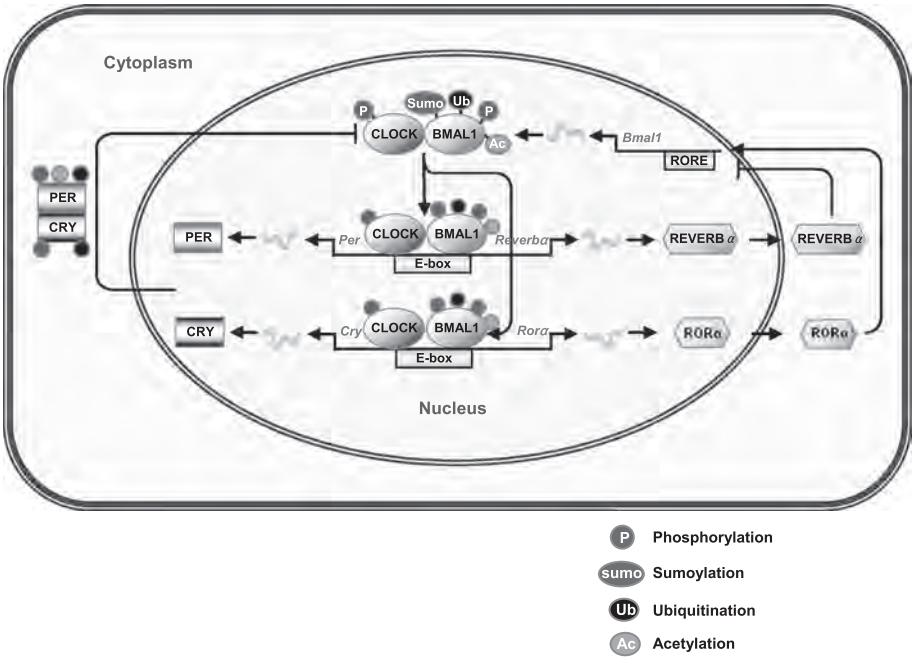

Figure 1. Molecular clockwork in mammals. The molecular clock is driven by a transcriptional-translational feedback loop in which clock-specific transcription factors activate periodic expression of their own repressors. CLOCK and BMAL1 form an active transcription factor complex to increase the transcription of *Period* (*Per*), *Cryptochrome* (*Cry*). When the levels of the PER and CRY proteins increase in the cytoplasm, they dimerize and translocate to the nucleus to repress CLOCK/BMAL1-mediated transcription. Various protranslational modifications such as phosphorylation (P), sumoylation (Sumo), ubiquitination (Ub) and acetylation (Ac) underlie the fine-tuning of the molecular feed-back loop. Furthermore, the CLOCK/BMAL1 heterodimer also induces the transcription of the nuclear receptors *Rev-erbα* and *Rorα* which, in turn, repress and activate *Bmal1* transcription, respectively, thereby constituting an additional feedback loop.

control and resetting of circadian rhythmicity (Bass and Takahashi 2010; Asher and Schibler 2011).

Here, we briefly review the structural and functional features of CLOCK/BMAL1 transcription factors and also describe in detail how each of the transcriptional activators is functionally regulated in response to circadian and cellular signaling in terms of intracellular distribution, posttranslational modification and the recruitment of chromatin remodeling factors.

CLOCK/BMAL1 as a circadian transcriptional factor

In mammals, CLOCK is the first core clock protein to have been shown to play a central role in the maintenance of circadian rhythmicity. Initially, the Clock mutant mice screened by an ethylnitrosourea (ENU) mutagenesis method displayed arrhythmic behavior as a result of the dominant negative effect of mutant CLOCK protein (CLOCK-$\triangle$19) lacking exon 19 in its C terminal region (Vitaterna et al. 1994; King et al. 1997). Phylogenetic studies have also indicated that CLOCK contains the basic-helix-loop-helix (bHLH) domain and two Per-Arnt-Sim (PAS) domains, which are required for DNA binding and heterodimerization, respectively (Gu et al. 2000) (Figure 2). Following the cloning of CLOCK, many efforts have been made to characterize its partner, which was identified as another bHLH-PAS protein, called MOP3 or BMAL1, using yeast two-bybrid screens (Hogenesch et al. 1997, 1998; Ikeda and Nomura 1997; Gekakis et al. 1998). The essential role of BMAL1 in circadian clock regulation is further demonstrated by generating BMAL1 specific knockout mice that exhibit arrhythmic locomoter activity under both entrained and constant conditions (Bunger et al. 2000). Meanwhile, intensive genetic and molecular analyses further demonstrated that CLOCK and BMAL1 require each other to function as a positive transcription factor; this transcription factor binds to E-box elements in the promoter region of clock-controlling genes which encode their own repressors (*Periods* and *Cryptochromes*) and transcriptional regulators (*Rev-erbα* and *Rorα*), thereby constituting circadian transcriptional feedback loops (Gekakis et al. 1998; Hogenesch et al. 1998; Shearman et al. 2000).

The accompanying structural and functional studies which have been carried out have also contributed to a detailed understanding of the

transcriptional role of CLOCK/BMAL1 in generating the molecular clock work. CLOCK has a glutamine-rich (Q-rich) region containing a poly-glutamine (Poly Q) stretch (amino acids 751–769) in its C-terminal domain, which is characteristic of the activation domains in many transcription factors (Figure 2). Upon further sequence analysis in a comparison with related proteins, the carboxyl-terminal glutamine-rich region was found to contain a conserved acetyl-CoA binding motif that is required for histone acetyltransferase (HAT) enzyme activity of CLOCK, underscoring the transactivational function of the protein (Doi et al. 2006). Similarly, BMAL1 possesses a transcriptional activation domain (TAD) in its C-terminus, where transcriptional coactivators such as the CREB binding protein (CBP) bind and activate E-box dependent transcription (Takahata et al. 2000). Intriguingly, intensive mutational analyses revealed that the BMAL1 C-terminal TAD associates with CRY1, thereby mediating negative transcriptional feed-back (Kiyohara et al. 2006; Sato et al. 2006). These findings open the possibility that the BMAL1 in the C-terminus has dual antagonistic functions and serves as a molecular switch for the circadian gene transcription controlled by the CLOCK/BMAL1 heterodimer.

Importantly, the global circadian role of CLOCK/BMAL1-mediated transcription was suggested by the extensive microarray analyses of the mouse transcriptome which showed that up to 10% of all of the mammalian transcripts follow a circadian rhythm in their pattern of expression (Akhtar et al. 2002; Duffield et al. 2002; Panda et al. 2002). In addition, transcriptional profiling of live and skeletal tissues from *Clock* mutant mice recently identified an abundance of CLOCK-regulated transcripts which are related to the cell cycle, cell proliferation and other cellular functional activities (Oishi et al. 2003; McCarthy et al. 2007; Miller et al. 2007). In parallel with these large-scale genomics approaches, several molecular and functional studies have more specifically unraveled the key roles played by CLOCK/BMAL1 in transcriptional regulation of the clock output genes involved in adipogensis, tumorigenesis, the immune response, development and various aspects of metabolism (Duguay and Cermakian 2009; Bass and Takahashi 2010). These findings are underscored by the accumulating reports that CLOCK mutant and/or BMAL1 knock-out mice exhibit various physiological defects, such as obesity, diabetes, cancer and premature aging (Kondratov and Antoch 2007; Eckel-Mahan and Sassone-Corsi 2009).

Poststranslational regulation of CLOCK/BMAL1 activity

Besides the characterization of CLOCK/BMAL1 as a circadian transcriptional complex, a number of biochemical and cell-based studies have contributed to a detailed understanding of how it is that the CLOCK/BMAL1 association confers the transcriptional potential on the complex through various levels of post-translational regulation, such as its sub-cellular distribution, posttranslational modification and interaction with other transcriptional cofactors.

1. Intracellular distribution

CLOCK and BMAL1 exhibit a circadian oscillation pattern in their intracellular distribution in mouse liver, suprachiasmatic nucleus and cultured fibroblasts (as well as in the suprachiasmatic nucleus) (Kondratov et al. 2003; Tamaru et al. 2003). Interestingly, CLOCK is predominantly immunostained in the cytoplasm when ectopically overexpressed, whereas it colocalizes with BMAL1 in the nucleus upon the coexpression of the proteins (Kondratov et al. 2003; Kwon et al. 2006). On further mutational analysis, BMAL1 was shown to contain functional nuclear localization signals (NLS) and nuclear export signals (NES), so that CLOCK is acquired to be localized in the nucleus to function as a

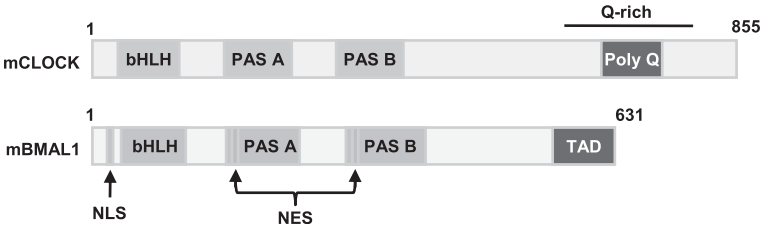

Figure 2. Schematic representation of the functional domains present in the mouse CLOCK and BMAL1 protein. Both CLOCK and BMAL1 contain a basic helix-loop-helix (bHLH) DNA binding domain and two PER-ARNT-SIM (PAS) dimerization domains (PAS-A, PAS-B). The C-terminal domain of CLOCK has a poly-glutamine repeat (Poly Q; amino acids 751–768), which is characteristic of activation domains in any transcription factors. BMAL1 also contains a transcriptional activation domain (TAD) in its C-terminus as well as a functional nuclear localization signal (NLS) adjacent to bHLH, and two nuclear export signals (NES) within PAS-A and PAS-B, respectively.

transcriptional activator together with the nuclear/cytoplasmic shuttling partner (Kwon et al. 2006) (Figure 2). In addition to the findings on circadian regulation, recent biochemical experiments revealed that heterodimerization and nuclear translocation of CLOCK with BMAL1 is rapidly induced within one hour by serum shock, a previously well-known clock synchronizing stimulus (Balsalobre et al. 1998; Shim et al. 2007). More recently, microscopic analysis using bimolecular fluorescence complementation (BiFC), a novel technique for imaging protein-protein interactions in intact cells, further visualized the binding-induced nuclear translocation of CLOCK/BMAL1 by means of the protein kinase C pathway upon stimulation in living cells (Lee et al. 2010). The functional significance of these observations is underscored by previous results that serum shock promotes the binding of both CLOCK and BMAL1 to E-box elements of the *Per1* promoter, *Per1* mRNA expression and eventually phase shifts of the circadian rhythm in cultured fibroblasts (Jung et al. 2003; Shim et al. 2007).

2. Phosphorylation

Previously, extensive in vivo studies on mouse liver extracts showed that most of the core clock proteins exhibit circadian patterns of phosphorylation generated by the cell-autonomous clock (Lee et al. 2001). In particular, the phosphorylation of CLOCK was found to be induced by dimerization with BMAL1, which leads to not only its nuclear localization, but also its degradation during the course of transcriptional activation. (Kondratov et al. 2003). In the subsequent bioinfomatic and biochemical studies, a conserved serine cluster within the CLOCK protein termed a 'phosphor-degron' was identified as responsible for BMAL1 dependent phosphorylation and degradation (Spengler et al. 2009). In the phosphor-degron region, GSK-3β phosphorylates CLOCK for degradation specifically at S427, with S431 acting as the BMAL1 dependent phospho-priming site, thereby increasing the transcriptional activity of the CLOCK/BMAL1 complex (Spengler et al. 2009). In addition, CLOCK was also shown to be phosphorylated by Ca^{2+}-dependent protein kinase C so as to induce acute *Per1* expression via dimerization with BMAL1 during clock resetting in serum-shocked fibroblasts (Shim et al. 2007). Interestingly, studies have shown that cryptochromes, negative regulators, impair CLOCK phosphorylation and degradation, thus suggesting the transactivational effect of phosphorylation (Kondratova et al. 2006b; Kwon et al. 2006; Dardente et al. 2007). On the other hand, Fukada and his colleagues recently reported that CLOCK phosphorylation at Ser38 and S42 by CIPC, a recently reported CLOCK-interacting negative regulator, occurs in a BMAL1-dependent manner and leads to the suppression of E-box-dependent transcription (Zhao et al. 2007; Yoshitane et al. 2009). This evidence suggests that phosphorylation mediates the diverse transcriptional roles of CLOCK in a site-specific manner for clock gene expression.

Similar to CLOCK, BMAL1 is also subject to multiple phosphorylation events directed by distinct kinases, which regulate its transcriptional activities. For example, phosphorylation by casein kinase Iε (CKIε) has been reported to activate BMAL1-mediated transcription, while phosphorylation by MAPK inhibits it (Eide et al. 2002; Sanada et al. 2002). In vitro and in vivo studies have also shown that CK2α rhythmically phosphorylates BMAL1 at Ser90 so as to regulate its nuclear accumulation and circadian gene expression (Tamaru et al. 2009). More recently, extensive proteomic analysis demonstrated that GSK-3β phosphorylates BMAL1 specifically on Ser17 and Thr21, which phosphorylation, in turn, facilitates its ubiquitylation and the degradation required for the maintenance of stable clock oscillation (Sahar et al. 2010). On the other hand, similar to CLOCK protein, the CRY repressors stabilize BMAL1 by impairing its phosphorylation, which in turn blocks transactivation of the clock genes coupled with degradation of the transcription complex (Kondratov et al. 2006b; Kwon et al. 2006; Dardente et al. 2007). Interestingly, a receptor for activated C kinase-1 (RACK1) was found to stimulate the phosphorylation of BMAL1 by PKCalpha as this protein is recruited into the nuclear BMAL1 complex during the negative feedback phase of the circadian cycle (Robles et al. 2010). Taken together, these results suggest that phosphorylation plays an important role in mediating the sub-cellular localization and stability of both CLOCK and BMAL1 in a dimerization-dependent manner, as well as interaction with the related kinases and co-regulators during the circadian and signal-regulated transcription of clock genes.

3. Sumoylation

sumoylation has also been shown to regulate the function and stability of circadian transcription factors. Sassone-Corsi and his collegues showed that BMAL1 is modified by a small ubiquitin-related modifier protein (SUMO) at lysine 259 in a CLOCK-dependent manner in vivo (Cardone et al. 2005). Mutation of the target lysine residue (BMAL1 K259R) prevents sumoylation and stabilizes the protein, which in turn impairs the normal circadian rhythm of clock gene expression. Recently, BMAL1 was further shown to be mainly modified by SUMO2/3, which is a prerequisite for its ubiquitination leading to proteasomal degradation during the transactivation

stage (Lee et al. 2008). Given that phosphorylation also primes BMAL1 for its ubiquitin-mediated degradation as mentioned above, the interplay between phosphorylation and sumoylation seems to underlie the transcription-coupled degradation of BMAL1 (Lee et al. 2008; Sahar et al. 2010). Notably, sumoylation localizes BMAL1 to the promyelocytic leukemia (PML) nuclear body, a well-recognized sub-nuclear compartment wherein a variety of SUMO-conjugated transcription factors and co-regulators, including CBP and RNA polymerase II (Pol II), are recruited to form the transcriptional apparatus (Zhong et al. 2000; Lee et al. 2008). Consistent with this observation, a recent study showed that CLOCK also localizes at the PMA bodies with BMAL1 and thus enables herpes simplex virus gene expression (Kalamvoki and Roizman 2010). Taken together, these findings suggest that sumoylation is likely to play a crucial role in the relocation of the CLOCK/BMAL1 complex into sub-nuclear foci for transcriptional activation of its target genes.

4. Acetylation

Histone acetylation and deacetylation modify the chromatin structure and thus regulate gene transcription by increasing or decreasing the DNA accessibility of the transcriptional apparatus (Vaissiere et al. 2008). Circadian rhythms have also been found to be regulated by dynamic chromatin remodeling. For example, the rhythmic acetylation of histones H3/H4 in the chromatin region spanning the promoters of the clock genes (*Per1*, *Per2*, and *Cry1*) is coupled with their circadian transcription (Etchegaray et al. 2003; Naruse et al. 2004). Strikingly, CLOCK itself is found to possess certain HAT activity which catalyzes the acetylation of histones H3 and H4 and hence the regulation of the circadian expression of clock genes (Doi et al. 2006). The HAT activity of CLOCK is further enhanced in the presence of BMAL1. Besides targeting histones, CLOCK also acetylates BMAL1, its non-histone heterodimeric partner, specifically at lysine 537 (Hirayama et al. 2007). Interestingly, the acetylation of BMAL1 by CLOCK promotes CRY recruitment, thereby inhibiting its transcriptional activity without altering its subcellular localization, interaction with CLOCK, phosphorylation, or stability (Hirayama et al. 2007). These findings suggest that the acetyltransferase activity of CLOCK plays a dual role in both the transcriptional activation (via histones) and repression (through BMAL1) phases of the circadian gene expression.

Epigenetic regulation

As CLOCK itself functions as a HAT enzyme in complex with BMAL1, the CLOCK/BMAL1 complex recruits various transcriptional cofactors, which are mostly related to chromatin remodeling for circadian and signal-regulated gene expression. Here we briefly review the epigenetic regulation involved in the circadian clock machinery.

1. CBP/p300, a histone acetyltransferase (HAT)

The CREB binding protein (CBP) and its close relative p300 possess histone acetyltransferase (HAT) activity (Vo and Goodman 2001). The transcriptional coactivators interact with the CLOCK/BMAL1 heterodimer for its transcriptional potentiation in cell culture when over-expressed (Takahata et al. 2000). Consistent with the earlier observation, Etchegaray and coworkers reported that CLOCK/BMAL1 exhibits circadian binding with the transcriptional coactivators in mice liver extracts (Etchegaray et al. 2003). Interestingly, the in vivo coimmunoprecipitation experiments showed that p300, rather than CBP, was markedly associated with CLOCK during the enhanced CLOCK/BAML1-mediated transcription which contributes to the rhythmic histone acetylation of the *Per* and *Cry* promoters (Etchegaray et al. 2003). In addition, Curtis et al. (2004) showed that CLOCK/BMAL1 associates with other histone acetyltransferases such as the p300/CBP associated factor (PCAF) and ACTR in vascular tissues. However, the major cofactor for rhythmic histone acetylation is not clear, particularly since CLOCK was shown to possess HAT activity, as mentioned above (Doi et al. 2006). Besides the circadian coactivator recruitment, it was shown that CBP is phosphorylated and recruited to the CLOCK/BMAL1 complex via D2R receptor signaling, thereby activating *Per1* gene expression in cultured retinal neurons (Yujnovsky et al. 2006). Our recent studies using bimolecular fluorescence complementation assay (BiFC) revealed that CBP, but not p300, is specifically recruited to BMAL1 so as to control the induction of *Per1* gene transcription shortly after serum stimuli. Previously, the transcriptional coactivation by CBP, in spite of its functional overlap with p300, occurred in a signal-dependent manner, mainly via phosphorylation (Kalkhoven 2004). Therefore, it is likely that CBP, unlike p300, has a unique role in signal-regulated transcriptional activation, probably via phosphorylation-dependent interaction with CLOCK/BMAL1.

2. SIRT1, a histone deacetylase (HDAC)

In recent years, a growing body of evidence has come to suggest a close relationship between the circadian clock and metabolism. The molecular link between clockwork and metabolism was first suggested by an in vitro electrophoretic mobility shift assay (EMSA) study showing that the DNA binding activity of CLOCK/BMAL1 and NPAS2/BMAL1 dimers is inhibited by oxidized NAD+ and enhanced by reduced NAD(H) (Rutter et al. 2001). The involvement of NAD cofactors in the clockwork was further highlighted by recent studies showing that SIRT1, a mammalian homolog of the yeast Sir2 having histone deacetylase (HDAC) activity dependent on NAD+, transduces the metabolic signals to the molecular clock machinery. In the liver and fibroblasts, SIRT1 exhibits not only rhythmic expression levels, but also rhythmic enzyme activity (Asher et al. 2008; Nakahata et al. 2008). Furthermore, SIRT1 is recruited to the CLOCK/BMAL1 complex at the E-box elements in the clock gene promoter via interacting with CLOCK (Nakahata et al. 2008). This accounts for the increases of SIRT1 activity in parallel with histone H3 acetylation in the promoters of clock genes and with high BMAL acetylation (Nakahata et al. 2008). More recently, two groups reported simultaneously that the cellular levels of NAD+ are rhythmic as a result of the circadian control of the enzyme nicotinamide phosphoribosyltransferase (NAMPT), a rate-limiting component in the NAD+ salvage pathway (Nakahata et al. 2009; Ramsey et al. 2009). This highlights a reciprocal feedback relationship between the molecular clock and energy metabolism; the oscillatory levels of NAD+, which are rate-limited by the clock-controlled expression of *Nampt*, provide a key metabolic cue for the circadian expression of core clock genes by affecting the CLOCK/BMAL1 DNA binding or HDAC activity of SIRT1 in complex with the heterodimer.

3. MLL1, a histone methyltransferase (HMT)

Besides histone acetylation, another important epigenetic mark for chromatin remodeling is histone methylation (Vaissiere et al. 2008). The methylation of histones, unlike acetylation, which is correlated with transcriptional activation in general, is associated with either activation or repression depending on the sites and degree (mono-, di- or trimethylation at the ε-amino group of lysine residues) of modification (Klose and Zhang 2007). Generally, it has been shown that trimethylation of histone 3 at Lys4 (H3K4me3) is closely linked to transcriptional activation, whereas di-methylated H3K4 (H3Kme2) occurs at both inactive and active euchromatic genes (Kouzarides 2002; Santos-Rosa et al. 2002; Schneider et al. 2004). The first clue of the link between histone methylation and the circadian clock was provided by a biochemical study showing that WD40-repeat protein 5, a subunit of (a) histone methyltransferase, is found in a complex with mPER1 (Brown et al. 2005). This was taken to indicate that mPER1 mediates recruitment of the methyltransferase in the CLOCK/BMAL1/PER complex in the repression phase of circadian transcription. Remarkably, extensive ChIP experiments revealed that rhythmic binding of CLOCK/BMAL1 to multiple E-box motifs drives dynamic chromatin changes via both acetylation at Lys9 of histone H3 (H3-K9 Ac) and trimethylation at Lys4 of histone H3 (H3K4me3) accompanying circadian *Dbp* transcription (Ripperger and Schibler 2006). Although CLOCK was thought to be a HAT enzyme which acetylates histone H3-K9, a major factor involved in histone methylation still remained in question. In this context, Sassone-Corsi and coworkers recently found that mixed-lineage leukemia 1 (MLL1), a mammalian homolog of *Drosophila* trithorax, acts as a key histone methyltransferase which induces rhythmic trimethylation of histone H3 at Lys4 (H3K4me3) via complex formation with CLOCK-BMAL1 in accompaniment with H3 acetylation (Katada and Sassone-Corsi 2010). Notably, MLL1 seems to be recruited to CLOCK/BMAL1 through an interaction with CLOCK as it failed to associate with CLOCK $\triangle$19. Given that CLOCK $\triangle$19 also cannot bind SIRT1, these findings suggest that CLOCK associates with MILL1 to form a histone modifying complex by replacing SIRT1 in its commonly shared binding region, thereby leading to both H3 acetylation and H3K4 tri-methylation in the active phase of circadian transcription (Nakahata et al. 2008; Katada and Sassone-Corsi 2010). Based on these findings, the impaired coactivator or corepressor recruitment of the mutant CLOCK protein seems to provide functional and molecular reasons for the circadian defects displayed by mutant *clock*/*clock* mice that previously had been obscure (Vitaterna et al. 1994; King et al. 1997).

Conclusion

Over the past 20 years, significant progress has been made in identifying the key molecular components and mechanisms of the mammalian clockwork using various genetic and molecular techniques. In addition to the establishment of the circadian transcriptional feedback loop, intensive studies have been focused on the post-translational regulation of the core clock proteins and signal transduction pathways involved in the control of circadian rhythmicity. Particularly, CLOCK and BMAL1, the core components of the circadian clock,

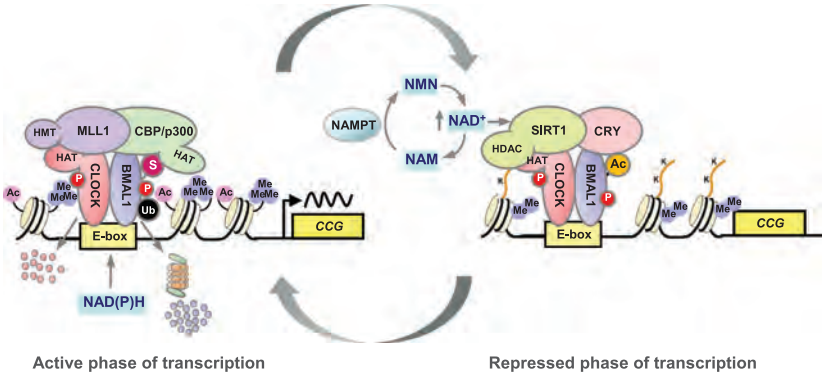

Figure 3. Hypothetical model of the interplay among the posttranslational and epigenetic regulators of CLOCK/BMAL1 in the circadian clock machinery.During the transactivation stage, CLOCK and BMAL1 undergo multiple posttranslational modifications, including phosphorylation (P) and sumoylation (S), so as to become subject to ubiquitin (U)-mediated degradation in a dimerization-dependent manner. In addition, CLOCK/BMAL1 recruits CBP/p300 and MILL1 to form a histone modifying complex, thereby inducing both H3 acetylation and H3K4 trimethylation in the activation phase of circadian transcription. CLOCK/BMAL1 also activates the rhythmic expression of nicotinamide phosphoribosyltransferase (NAMPT), one of the clock controlled genes (CCGs), which converts nicotinamide (NAM) into nicotinamide mononucleotide (NMN) so as to be eventually modified into NAD+ via further enzymatic processes. Increased NAD+ activates SIRT1, which in turn feeds back into the NAD+-salvage pathway by directly regulating NAMPT gene expression together with CLOCK/BMAL1. Meanwhile, the high level of NAD(P)H promotes DNA binding of CLOCK/BMAL1 to the E-box elements of CCGs. On the other hand, BMAL1 is subject to acetylation by CLOCK HAT enzyme activity so as to recruit CRY1, thereby enhancing CRY-mediated negative feedback. In addition, SIRT1 forms a complex with CLOCK/BMAL1 probably replacing MLL1 in the common binding region of CLOCK so as to both deacetylate H3 and block the trimethylation of H3 in the course of transcriptional repression.

have been shown to be regulated interdependently by phosphorylation, sumoylation, ubiquitylation and acetylation as well as interactions with transcriptional coactivators, repressors, and chromatin remodeling factors in order to fine-tune the temporal organization of clock gene expression (Figure 3). Recently, the association of CLOCK/BMAL1 with metabolic cofactors or modulators provided molecular evidence of the close interrelationship between the circadian clock and metabolism, underscoring the important role of the heterodimeric components in mediating the circadian and metabolic cues. From the physiological point of view, these findings also provide further insight into the reasons why CLOCK mutant and BMAL1 knock-out mice display circadian and metabolic defects, such as obesity, diabetes and premature aging (Turek et al. 2005; Kondratov et al. 2006a; Marcheva et al. 2010).

Finally, accumulating evidence suggests that it is likely that there is a complex interplay among the post-translational modifications and cofactor recruitments in response to circadian and other signaling cues. Therefore, it will be necessary to investigate how it is that the various modes of the posttranslational regulation of CLOCK/BMAL1 in combination play a role in controlling the oscillatory mechanism itself and/or in mediating the signaling pathways to the clock. Further studies will clarify the functional significance of the CLOCK/BMAL1-mediated regulation of circadian clock and physiology.

Acknowledgements

This work was supported by the Brain Research Center for the 21st Century Frontier Program in Neuroscience, MEST, Korea.

References

Akhtar RA, Reddy AB, Maywood ES, Clayton JD, King VM, Smith AG, Gant TW, Hastings MH, Kyriacou CP. 2002. Circadian cycling of the mouse liver transcriptome, as revealed by cDNA microarray, is driven by the suprachiasmatic nucleus. Curr Biol. 12:540–550.

Asher G, Schibler U. 2011. Crosstalk between components of circadian and metabolic cycles in mammals. Cell Metab. 13:125–137.

Asher G, Gatfield D, Stratmann M, Reinke H, Dibner C, Kreppel F, Mostoslavsky R, Alt FW, Schibler U. 2008. SIRT1 regulates circadian clock gene expression through PER2 deacetylation. Cell. 134:317–328.

Balsalobre A, Damiola F, Schibler U. 1998. A serum shock induces circadian gene expression in mammalian tissue culture cells. Cell. 93:929–937.

Bass J, Takahashi JS. 2010. Circadian integration of metabolism and energetics. Science. 330:1349–1354.

Brown SA, Ripperger J, Kadener S, Fleury-Olela F, Vilbois F, Rosbash M, Schibler U. 2005. PERIOD1-associated proteins modulate the negative limb of the mammalian circadian oscillator. Science. 308:693–696.

Bunger MK, Wilsbacher LD, Moran SM, Clendenin C, Radcliffe LA, Hogenesch JB, Simon MC, Takahashi JS, Bradfield CA. 2000. Mop3 is an essential component of the master circadian pacemaker in mammals. Cell. 103:1009–1017.

Cardone L, Hirayama J, Giordano F, Tamaru T, Palvimo JJ, Sassone-Corsi P. 2005. Circadian clock control by SUMOylation of BMAL1. Science. 309:1390–1394.

Cermakian N, Sassone-Corsi P. 2000. Multilevel regulation of the circadian clock. Nat Rev Mol Biol. 1:59–67.

Curtis AM, Seo SB, Westgate EJ, Rudic RD, Smyth EM, Chakravarti D, FitzGerald GA, McNamara P. 2004. Histone acetyltransferase-dependent chromatin remodeling and the Vascular Clock. J Biol Chem. 279:7091–7097.

Dardente H, Fortier EE, Martineau V, Cermakian N. 2007. Cryptochromes impair phosphorylation of transcriptional activators in the clock: A general mechanism for circadian repression. Biochem J. 402:525–536.

Dibner C, Schibler U, Albrecht U. 2010. The mammalian circadian timing system: organization and coordination of central and peripheral clocks. Annu Rev Physiol. 72:517–549.

Doi M, Hirayama J, Sassone-Corsi P. 2006. Circadian regulator CLOCK is a histone acetyltransferase. Cell. 125:497–508.

Duffield GE, Best JD, Meurers BH, Bittner A, Loros JJ, Dunlap JC. 2002. Circadian programs of transcriptional activation, signaling, and protein turnover revealed by microarray analysis of mammalian cells. Curr Biol. 12:551–557.

Duguay D, Cermakian N. 2009. The crosstalk between physiology and circadian clock proteins. Chronobiol Int. 26:1479–1513.

Eckel-Mahan K, Sassone-Corsi P. 2009. Metabolism control by the circadian clock and vice versa. Nat Struct Mol Biol. 16:462–467.

Eide EJ, Vielhaber EL, Hinz WA, Virshup DM. 2002. The circadian regulatory proteins BMAL1 and cryptochromes are substrates of casein kinase Iε. J Biol Chem. 277:17248–17254.

Etchegaray JP, Lee C, Wade PA, Reppert SM. 2003. Rhythmic histone acetylation underlies transcription in the mammalian circadian clock. Nature. 421:117–182.

Gallego M, Virshup DM. 2007. Post-translational modifications regulate the ticking of the circadian clock. Nat Cell Biol. 8:139–148.

Gekakis N, Staknis D, Nguyen HB, Davis FC, Wilsbacher LD, King DP, Takahashi JS, Weitz CJ. 1998. Role of the CLOCK protein in the mammalian circadian mechanism. Science. 280:1564–1569.

Gu YZ, Hogenesch JB, Bradfield CA. 2000. The PAS superfamily: sensors of environmental and developmental signals. Annu Rev Pharmacol Toxicol. 40:519–556.

Hirayama J, Sahar S, Grimaldi B, Tamaru T, Takamatsu K, Nakahata Y, Sassone-Corsi P. 2007. CLOCK-mediated acetylation of BMAL1 controls circadian function. Nature. 450:1086–1090.

Hogenesch JB, Chan WK, Jackiw VH, Brown RC, Gu YZ, Pray-Grant M, Perdew GH, Bradfield CA. 1997. Characterization of a subset of the basic-helix-loop-helix-PAS superfamily that interacts with components of the dioxin signaling pathway. J Biol Chem. 272:8581–8593.

Hogenesch JB, Gu YX, Jain S, Bradfield CA. 1998. The basic-helix-loophelix-PAS orphan MOP3 forms transcriptionally active complexes with circadian and hypoxia factors. Proc Natl Acad Sci USA. 95:5474–5479.

Ikeda M, Nomura M. 1997. cDNA cloning and tissue-specific expression of a novel basic helix-loop-helix/PAS protein (BMAL1) and identification of alternatively spliced variants with alternative translation initiation site usage. Biochem Biophys Res Commun. 233:258–264.

Jung H, Choe Y, Kim H, Park N, Son GH, Khang I, Kim K. 2003. Involvement of CLOCK:BMAL1 heterodimer in serum-responsive mPer1 induction. Neuroreport. 14:15–19.

Kalamvoki M, Roizman B. 2010. Circadian CLOCK histone acetyl transferase localizes at ND10 nuclear bodies and enables herpes simplex virus gene expression. Proc Natl Acad Sci USA. 107:17721–17726.

Kalkhoven E. 2004. CBP and p300: HATs for different occasions. Biochem Pharmacol. 68:1145–1155.

Katada S, Sassone-Corsi P. 2010. The histone methyltransferase MLL1 permits the oscillation of circadian gene expression. Nat Struct Mol Biol. 17:1414–1421.

King DP, Zhao Y, Sangoram AM, Wilsbacher LD, Tanaka M, Antoch MP, Steeves TD, Vitaterna MH, Kornhauser JM, Lowrey PL, Turek FW, Takahashi JS. 1997. Positional cloning of the mouse circadian clock gene. Cell. 89:641–653.

Kiyohara YB, Tagao S, Tamanini F, Morita A, Sugisawa Y, Yasuda M, Yamanaka I, Ueda HR, van der Horst GT, Kondo T, Yagita K. 2006. The BMAL1 C terminus regulates the circadian transcription feedback loop. Proc Natl Acad Sci USA. 103:10074–10079.

Klose RJ, Zhang Y. 2007. Regulation of histone methylation by demethylimination and demethylation. Nat Rev Mol Cell Biol. 8:307–318.

Kondratov RV, Antoch MP. 2007. The clock proteins, aging, and tumorigenesis. Cold Spring Harb Symp Quant Biol. 72:477–482.

Kondratov RV, Chernov MV, Kondratova AA, Gorbacheva VY, Gudkov AV, Antoch MP. 2003. BMAL1-dependent circadian oscillation of nuclear CLOCK: posttranslational events induced by dimerization of transcriptional activators of the mammalian clock system. Genes Dev. 17:1921–1932.

Kondratov RV, Kondratova AA, Gorbacheva VY, Vykhovanets OV, Antoch MP. 2006a. Early aging and age-related pathologies in mice deficient in BMAL1, the core componentof the circadian clock. Genes Dev. 20:1868–1873.

Kondratov RV, Kondratova AA, Lee C, Gorbacheva VY, Chernov MV, Antoch MP. 2006b. Post-translational regulation of circadian transcriptional CLOCK(NPAS2)/BMAL1 complex by CRYPTOCHROMES. Cell Cycle. 5:890–895.

Kouzarides T. 2002. Histone methylation in transcriptional control. Curr Opin Genet Dev. 12:198–209.

Kwon I, Lee J, Chang SH, Jung NC, Lee BJ, Son GH, Kim K, Lee KH. 2006. BMAL1 shuttling controls transactivation and degradation of the CLOCK/BMAL1 heterodimer. Mol Cell Biol. 26:7318–7330.

Lee C, Etchegaray JP, Cagampang FR, Loudon AS, Reppert SM. 2001. Posttranslational mechanisms regulate the mammalian circadian clock. Cell. 107:855–867.

Lee J, Lee Y, Lee MJ, Park E, Kang SH, Chung CH, Lee KH, Kim K. 2008. Dual modification of BMAL1 by SUMO2/3 and ubiquitin promotes circadian activation of the CLOCK/BMAL1 complex. Mol Cell Biol. 28:6056–6065.

Lee Y, Lee J, Kwon I, Nakajima Y, Ohmiya Y, Son GH, Lee KH, Kim K. 2010. Coactivation of the CLOCK-BMAL1

complex by CBP mediates resetting of the circadian clock. J Cell Sci. 123:3547–3557.
Lowrey PL, Shimomura K, Antoch MP, Yamazaki S, Zemenides PD, Ralph MR, Menaker M, Takahashi JS. 2000. Positional syntenic cloning and functional characterization of the mammalian circadian mutation tau. Science. 288:483–492.
Marcheva B, Ramsey KM, Buhr ED, Kobayashi Y, Su H, Ko CH, Ivanova G, Omura C, Mo S, Vitaterna MH, Lopez JP, Philipson LH, Bradfield CA, Crosby SD, JeBailey L, Wang X, Takahashi JS, Bass J. 2010. Disruption of the clock components CLOCK and BMAL1 leads to hypoinsulinaemia and diabetes. Nature. 466:627–631.
McCarthy JJ, Andrews JL, McDearmon EL, Campbell KS, Barber BK, Miller BH, Walker JR, Hogenesch JB, Takahashi JS, Esser KA. 2007. Identification of the circadian transcriptome in adult mouse skeletal muscle. Physiol Genomics. 31:86–95.
Mehra A, Baker CL, Loros JJ, Dunlap JC. 2009. Post-translational modifications in circadian rhythms. Trends Biochem Sci. 34:483–490.
Miller BH, McDearmon EL, Panda S, Hayes KR, Zhang J, Andrews JL, Antoch MP, Walker JR, Esser KA, Hogenesch JB, Takahashi JS. 2007. Circadian and CLOCK-controlled regulation of the mouse transcriptome and cell proliferation. Proc Natl Acad Sci USA. 104:3342–3347.
Nakahata Y, Kaluzova M, Grimaldi B, Sahar S, Hirayama J, Chen D, Guarente LP, Sassone-Corsi P. 2008. The NAD+-dependent deacetylase SIRT1 modulates CLOCK-mediated chromatin remodeling and circadian control. Cell. 134:329–340.
Nakahata Y, Sahar S, Astarita G, Kaluzova M, Sassone-Corsi P. 2009. Circadian control of the NAD+ salvage pathway by CLOCK-SIRT1. Science. 324:654–657.
Naruse Y, Oh-hashi K, Iijima N, Naruse M, Yoshioka H, Tanaka M. 2004. Circadian and light induced transcription of clock gene Per1 depends on histone acetylation and deacetylation. Mol Cell Biol. 24:6278–6287.
Oishi K, Miyazaki K, Kadota K, Kikuno R, Nagase T, Atsumi G, Ohkura N, Azama T, Mesaki M, Yukimasa S, Kobayashi H, Iitaka C, Umehara T, Horikoshi M, Kudo T, Shimizu Y, Yano M, Monden M, Machida K, Matsuda J, Horie S, Todo T, Ishida N. 2003. Genome-wide expression analysis of mouse liver reveals CLOCK-regulated circadian output genes. J Biol Chem. 278:41519–41527.
Panda S, Antoch MP, Miller BH, Su AI, Schook AB, Straume M, Schultz PG, Kay SA, Takahashi JS, Hogenesch JB. 2002. Coordinated transcription of key pathways in the mouse by the circadian clock. Cell. 109:307–20.
Ramsey KM, Yoshino J, Brace CS, Abrassart D, Kobayashi Y, Marcheva B, Hong HK, Chong JL, Buhr ED, Lee C, Takahashi JS, Imai S, Bass J. 2009. Circadian clock feedback cycle through NAMPT-mediated NAD+ biosynthesis. Science. 324:651–654.
Reppert SM, Weaver DR. 2002. Coordination of circadian timing in mammals. Nature. 418:935–941.
Ripperger JA, Schibler U. 2006. Rhythmic CLOCK-BMAL1 binding to multiple E-box motifs drivescircadian DBP transcription and chromatin transitions. Nat Genet. 38:369–374.
Ripperger JA, Shearman LP, Reppert SM, Schibler U. 2000. CLOCK, an essential pacemaker component, controls expression of the circadian transcription factor DBP. Genes Dev. 14:679–689.
Robles MS, Boyault C, Knutti D, Padmanabhan K, Weitz CJ. 2010. Identification of RACK1 and protein kinase Calpha as integral components of the mammalian circadian clock. Science. 327:463–466.
Rutter J, Reick M, Wu LC, McKnight SL. 2001. Regulation of clock and NPAS2 DNA binding by the redox state of NAD cofactors. Science. 293:510–514.
Sahar S, Zocchi L, Kinoshita C, Borrelli E, Sassone-Corsi P. 2010. Regulation of BMAL1 protein stability and circadian function by GSK3beta-mediated phosphorylation. PLoS One. 5:e:8561.
Sanada K, Okano T, Fukada Y. 2002. Mitogen-activated protein kinase phosphorylates and negatively regulates basic helix–loop–helix-PAS transcription factor BMAL1. J Biol Chem. 277:267–271.
Santos-Rosa H, Schneider R, Bannister AJ, Sherriff J, Bernstein BE, Emre NC, Schreiber SL, Mellor J, Kouzarides T. 2002. Active genes are tri-methylated at K4 of histone H3. Nature. 419:407–411.
Sato TK, Yamada RG, Ukai H, Baggs JE, Miraglia LJ, Kobayashi TJ, Welsh DK, Kay SA, Ueda HR, Hogenesch JB. 2006. Feedback repression is required for mammalian circadian clock function. Nat Genet. 38:312–319.
Schibler U, Sassone-Corsi P. 2002. A web of circadian pacemakers. Cell. 111:919–922.
Schneider R, Bannister AJ, Myers FA, Thorne AW, Crane-Robinson C, Kouzarides T. 2004. Histone H3 lysine 4 methylation patterns in higher eukaryotic genes. Nat Cell Biol. 6:73–77.
Shearman LP, Sriram S, Weaver DR, Maywood ES, Chaves I, Zheng B, Kume K, Lee CC, van der Horst GT, Hastings MH, Reppert SM. 2000. Interacting molecular loops in the mammalian circadian clock. Science. 288:1013–1019.
Shim HS, Kim H, Lee J, Son GH, Cho S, Oh TH, Kang SH, Seen DS, Lee KH, Kim K. 2007. Rapid activation of CLOCK by Ca2+-dependent protein kinase C mediates resetting of the mammalian circadian clock. EMBO Reports. 8:366–371.
Spengler ML, Kuropatwinski KK, Schumer M, Antoch MP. 2009. A serine cluster mediates BMAL1-dependent CLOCK phosphorylation and degradation. Cell Cycle. 8:4138–4146.
Stratmann M, Schibler U. 2006. Properties, entrainment, and physiological functions of mammalian peripheral oscillators. J Biol Rhythms. 21:494–506.
Takahata S, Ozaki T, Mimura J, Kikuchi Y, Sogawa K, Fujii-Kuriyama Y. 2000. Transactivation mechanisms of mouse clock transcription factors, mClock and mArnt3. Genes Cells. 5:739–747.
Tamaru T, Isojima Y, van der Horst GT, Takei K, Nagai K, Takamatsu K. 2003. Nucleocytoplasmic shuttling and phosphorylation of BMAL1 are regulated by circadian clock in cultured fibroblasts. Genes Cells. 8:973–983.
Tamaru T, Hirayama J, Isojima Y, Nagai K, Norioka S, Takamatsu K, Sassone-Corsi P. 2009. CK2alpha phosphorylates BMAL1 to regulate the mammalian clock. Nat Struct Mol Biol. 16:446–448.
Turek FW, Joshu C, Kohsaka A, Lin E, Ivanova G, McDearmon E, Laposky A, Losee-Olson S, Easton A, Jensen DR, Eckel RH, Takahashi JS, Bass J. 2005. Obesity and metabolic syndrome in circadian Clock mutant mice. Science. 308:1043–1045.
Vaissiere T, Sawan C, Herceg Z. 2008. Epigenetic interplay between histone modifications and DNA methylation in gene silencing. Mutat Res. 659:40–48.

Vitaterna MH, King DP, Chang AM, Kornhauser JM, Lowrey PL, McDonald JD, Dove WF, Pinto LH, Turek FW, Takahashi JS. 1994. Mutagenesis and mapping of a mouse gene, Clock, essential for circadian behavior. Science. 264:719–725.

Vo N, Goodman RH. 2001. CREB-binding protein and p300 in transcriptional regulation. J Biol Chem. 276:13505–13508.

Wijnen H, Young MW. 2006. Interplay of circadian clocks and metabolic rhythms. Annu Rev Genet. 40:409–48.

Yoshitane H, Takao T, Satomi Y, Du NH, Okano T, Fukada Y. 2009. Roles of CLOCK phosphorylation in suppression of E-box-dependent transcription. Mol Cell Biol. 29:3675–3686.

Yujnovsky I, Hirayama J, Doi M, Borrelli E, Sassone-Corsi P. 2006. Signaling mediated by the dopamine D2 receptor potentiates circadian regulation by CLOCK:BMAL1. Proc Natl Acad Sci USA. 103:6386–6391.

Zhao WN, Malinin N, Yang FC, Staknis D, Gekakis N, Maier B, Reischl S, Kramer A, Weitz CJ. 2007. CIPC is a mammalian circadian clock protein without invertebrate homologues. Nat Cell Biol. 9:268–275.

Zhong S, Salomoni P, Pandolfi PP. 2000. The transcriptional role of PML and the nuclear body. Nat Cell Biol. 2:E85–90.