

# **Linking Metabolism to Epigenetics: Chromatin Remodeling and Circadian Control**

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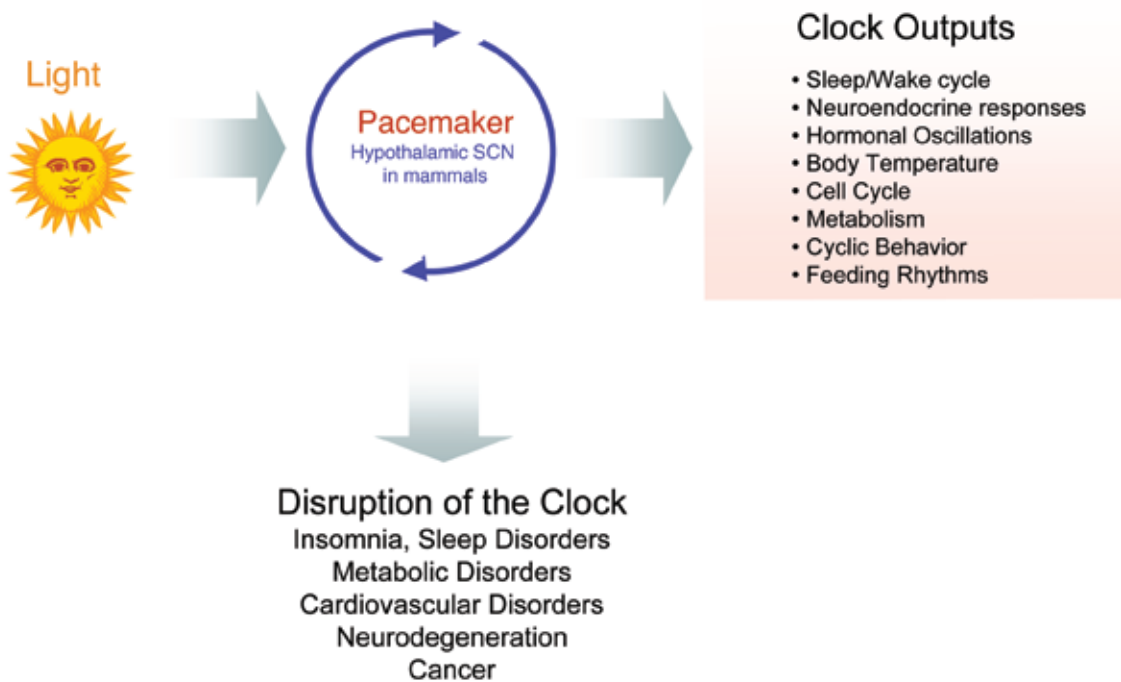
## Introduction to Circadian Rhythms

Neurons are cells that are submitted to an exceptional variety of stimuli and are able to convert them into high-order functions, such as storing memories, controlling behavior, and governing consciousness. These unique properties are based on highly plastic processes, which intimately depend on the complex molecular machinery that controls gene expression. Evidence is accumulating that neuronal functions have more than a solely genetic basis. Epigenetic control, which largely involves events of chromatin remodeling, appears to govern some of the more distinctive features of neuronal responses (Borrelli et al., 2008).

Circadian rhythms of 24 h govern a number of fundamental physiological functions in almost all organisms, from prokaryotes to humans (Dunlap, 1999; Cermakian and Sassone-Corsi 2000; King and Takahashi, 2000; Young and Kay, 2001; Reppert and Weaver, 2002). The circadian clocks are intrinsic time-tracking systems with which organisms can anticipate environmental changes and adapt themselves to the appropriate time of day. In mammals, circadian rhythms are generated in pacemaker neurons within the suprachiasmatic nuclei

(SCN) of the hypothalamus and are entrained by environmental cues, principally light. Disruption of these rhythms can have a profound influence on human health and has been linked to insomnia, depression, coronary heart diseases, various neurodegenerative disorders, and cancer (Fu and Lee, 2003; Hastings et al., 2003) (Fig. 1).

The molecular mechanism of the circadian clock is based on interlocked transcriptional–translational feedback loops, as revealed by molecular and genetic analyses in *Drosophila* and mammals (Dunlap, 1999; Cermakian and Sassone-Corsi 2000; King and Takahashi, 2000; Young and Kay, 2001; Reppert and Weaver, 2002). To date, various core circadian-clock genes have been identified in mammals: *Clock*, *Bmal1*, *casein kinase I epsilon (CK1e)*, *cryptochromes 1 and 2 (Cry1, Cry2)*, *Period 1, 2 and 3 (Per1, Per2, Per3)*, and *Rev-erb-a*. Interaction of clock proteins occurs via the PAS domains (named after the proteins PER-ARNT-SIM), which provide heterodimerization surfaces. The *Clock* and *Bmal1* genes encode basic-helix-loop-helix (bHLH)–PAS transcription activators, which heterodimerize and induce the expression of *Per* and *Cry* genes by binding to E-box elements (CACGTG) present in their promoters. Once the PER and CRY



**Figure 1.** Schematic model of the circadian clock (pacemaker) and its input signals, the most prominent being light. The light signaling directly influences neurons in the central clock in the hypothalamic SCN, thereby modulating the self-sustained clock circadian regulation. The outputs of the circadian system include a large array of physiological, metabolic, and neuronal functions. Disruption of clock function may cause dramatic pathophysiological effects, including neurodegeneration and cancer.

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proteins are synthesized, they form heterodimeric complexes, which in turn translocate to the nucleus and inhibit CLOCK-BMAL1-mediated transcription through direct protein-protein interactions (Reppert and Weaver, 2002).

The result of these complex regulatory pathways is that the messenger RNAs (mRNAs) and protein levels of most circadian genes (except *Clock* and *CKIε*) oscillate with a 24 h period. Importantly, the CLOCK-BMAL1 heterodimer regulates the transcription of many clock-controlled genes (CCGs), which in turn influence a wide array of physiological functions external to the oscillatory mechanism. This genetic influence mediates the output function of the clock, thereby controlling food intake, hormonal synthesis and release, body temperature, metabolism, and other functions. Remarkably, 10-15% of all mammalian transcripts undergo circadian fluctuations in their expression levels (Akhtar et al., 2002; Duffield et al., 2002; Panda et al., 2002), indicating that a global circadian chromatin remodeling system must operate.

### Plasticity in Circadian Regulation by Chromatin Remodeling

How is the oscillatory expression of clock-controlled genes regulated so that transcription-permissive chromatin states are dynamically established in a circadian, time-specific manner? The first evidence that chromatin remodeling may be intimately implicated in circadian control was obtained almost 10 years ago, by studying histone modifications induced by a light pulse in neurons of the suprachiasmatic nucleus (Crosio et al., 2000). Subsequently, the activation of CCGs by CLOCK:BMAL1 was correlated to circadian changes in histone acetylation at their promoters (Etchegaray et al., 2003; Curtis et al., 2004; Naruse et al., 2004; Nakahata et al., 2007).

A finding that paved the way toward understanding how circadian chromatin remodeling could occur was that CLOCK induces histone acetylation (Doi et al., 2006). The carboxy-terminal glutamine-rich region of CLOCK, a region implicated in transactivation function (Allada et al., 1998; Gekakis et al., 1998), displays a significant sequence homology with the carboxy-terminal domain of ACTR, a domain previously shown to have histone acetyltransferase (HAT) activity (Chen et al. 1997). Our analyses indicated that CLOCK could have intrinsic enzymatic HAT activity, and we did establish this premise using an in-gel HAT assay. This biochemical assay is ideal for unequivocally determining that a protein exhibits HAT function.

In our experiment, we immunoprecipitated a Myc-mCLOCK fusion protein expressed in cultured cells and used it in in-gel HAT assays with a mixture of purified histones as substrate (Brownell et al., 1999). Proteins resolved by SDS-PAGE were subjected to in-gel enzymatic reaction. Detection of histones [<sup>14</sup>C]-acetylated *in situ* demonstrated that acetylation took place specifically in a position corresponding to where the Myc-CLOCK protein had migrated. As control, it is helpful to use truncated and/or mutated forms of the protein suspected to have HAT activity. In this experiment, also to rule out the possibility that a contaminant HAT comigrating with Myc-CLOCK would be responsible for the acetylation, we used an N-terminally truncated mCLOCK (Myc-mCLOCKΔN) protein in the in-gel HAT assay. This truncated CLOCK protein lacks the N-terminal residues 1–242 but has an intact C-terminal region and still displays efficient HAT activity in the gel.

In a series of experiments using cultured cells that recapitulate circadian clock regulation, it was shown that the HAT function of CLOCK is essential for the circadian control of CCGs (Doi et al., 2006). This system is based on mouse embryonic fibroblast (MEF) cells derived from homozygous *Clock* mutant (*c/c*) mice (Pando et al., 2002). Because *Clock* is essential for circadian rhythm, as expected, MEF *c/c* cells show no cyclic expression of clock genes (Pando et al., 2002), whereas ectopic expression of CLOCK is able to rescue the circadian phenotype. On the contrary, ectopic expression of a HAT-deficient CLOCK failed to restore the circadian gene expression, demonstrating the essential role of CLOCK's HAT activity (Doi et al., 2006). These findings underscore the importance of chromatin remodeling in circadian regulation and reveal the molecular pathways by which such essential control is achieved.

### Acetylation of nonhistone proteins

Acetylation of nonhistone proteins is achieved by various HATs (Glozak et al., 2005; Zhang and Dent, 2005) and is demonstrated to have profound physiological significance. In a survey aimed at identifying proteins that might be acetylated rhythmically *in vivo*, we analyzed various clock proteins, such as BMAL1, CLOCK, and PER1, in the mouse liver at different circadian times. While, as expected, these proteins oscillate in terms of abundance and phosphorylation levels (Lee et al., 2001; Matsuo et al., 2003), acetylation of BMAL1 displays a robust acetylation with a circadian peak at ZT 15 (Hirayama et al., 2007). Significantly, the other

clock proteins are not acetylated. Ongoing studies in our laboratory on a variety of nuclear proteins and transcription factors indicate that BMAL1 is one of the few substrates for CLOCK, underscoring the specificity of the assay. Importantly, CLOCK is directly responsible for BMAL1 acetylation in cultured mammalian cells (Grimaldi et al., 2007; Hirayama et al., 2007).

To identify the site of CLOCK-mediated acetylation, we generated several mutant proteins with Lys>Arg substitutions in the putative acetylated sites. All mutants displayed acetylated levels comparable with wild-type BMAL1, with the exception of K537, a highly conserved residue among all vertebrate BMAL1s. By using MEFs, with an approach analogous to the one described above, we could also demonstrate that acetylation at K537 is critical for circadian function (Grimaldi et al., 2007; Hirayama et al., 2007).

This finding suggests that CLOCK may have several putative targets and that their identification is likely to provide significant clues about the neuronal pathways influenced by the circadian clock. In this respect, another protein may play a relevant regulatory function: NPAS2. This is an alternative partner of BMAL1, whose structure is loosely similar to CLOCK (Reick et al., 2001). Interestingly, NPAS2 displays a neuronal-specific distribution, being abundant in the forebrain areas, including the cortex, hippocampus, striatum, amygdala, and thalamus (Garcia et al., 2000). Although it is yet unclear whether NPAS2 may have acetyltransferase activity, its function as a substitute for CLOCK in the dimerization with BMAL1 confers on it a potential role in indirectly regulating CLOCK's HAT activity.

### The Flip Side of Acetylation: SIRT1, a Circadian Histone Deacetylase

A hallmark of chromatin remodeling factors is that they may have both positive and negative enzymatic activities, functions that we have previously described as “writers” and “erasers” of specific histone modifications (Borrelli et al., 2008). Thus, as soon as CLOCK was found to be a HAT, the quest for its counterbalancing histone deacetylase (HDAC) had begun.

CLOCK expression is not rhythmic (Gekakis et al., 1998), whereas its induced acetylation is, indicating that its chromatin remodeling activity is critical for circadian physiology (Doi et al., 2006). There are four classes of HDACs, and the subdivision is based on their protein structure (Yang and Seto, 2008). SIRT1

belongs to the family of sirtuins that constitutes the so-called class III HDACs. These are the only HDACs whose enzymatic activity is NAD<sup>+</sup>-dependent and that have been intimately linked to the control of metabolism and aging (Bordone and Guarente, 2005). SIRT1 directly associates with CLOCK and functions as a rheostat in modulating the acetylation state of histone H3 and BMAL1 (Nakahata et al., 2008). These observations are relevant to establishing a direct molecular coupling between circadian control and energy metabolism (Fig. 2).

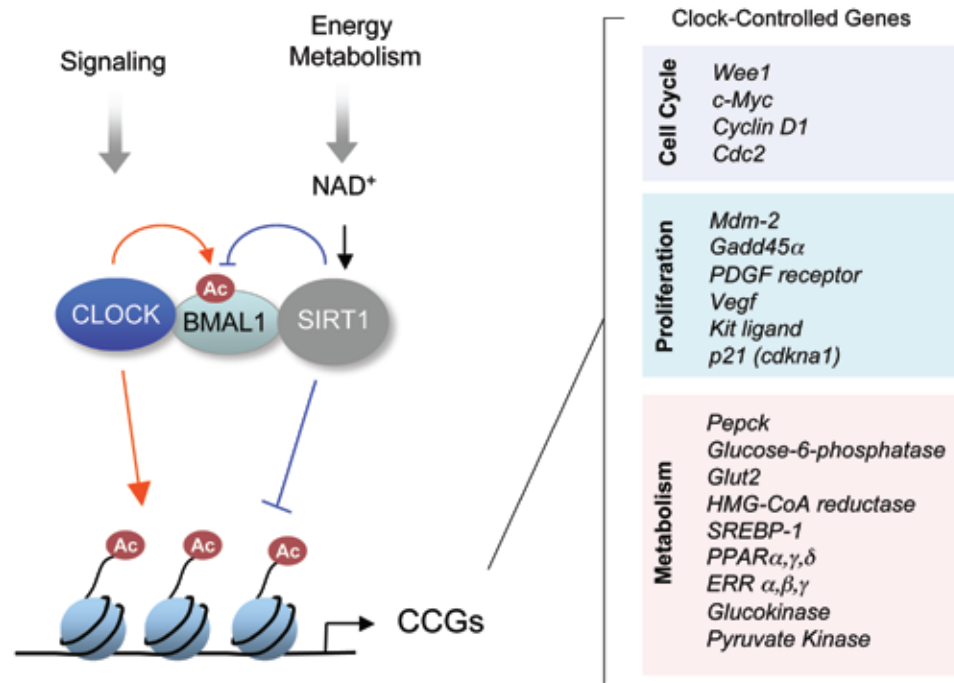
The CLOCK–SIRT1 complex is, in fact, regulated by the NAD<sup>+</sup>/nicotinamide balance in the cell (Nakahata et al., 2008). This finding provides a novel perspective on the links that have been historically reported between circadian rhythms, metabolism, and cellular reduction-oxidation pathways (Wijnen and Young, 2006; Collis and Boulton, 2007). The finding that SIRT1 acts as a rheostat in the context of circadian acetylation is of interest because it may be linked to other, recently described functions of this regulator in aging and neurodegeneration (Gan and Mucke, 2008). For example, inhibitors of SIRT1 rescue  $\alpha$ -synuclein-mediated toxicity in animal models of Parkinson's disease (Outeiro et al., 2007). As the role of dopamine in neurotoxicity and neuroprotection is established (Bozzi and Borrelli, 2006), the link between dopamine-mediated signaling and the circadian machinery (Yujnovsky et al., 2006) will take on new significance. Furthermore, SIRT1 has been found to contribute to the redox-dependent fate of neural progenitors (Prozorovski et al., 2008).

### The Central Role of Metabolites in Chromatin Remodeling

Accumulating evidence shows that the enzymatic machinery that elicits histone modifications operates under the control of a variety of neuronal stimuli. These stimuli link physiological variations to modulated chromatin remodeling, and thereby to controlled gene expression. One important consideration relates to the intracellular pathways involved in marking these posttranslational modifications (Borrelli et al., 2008). Interestingly, all of the modifications use physiological metabolites. This indicates that the dynamic process of chromatin remodeling may “sense” cellular metabolism and changes in energy levels (Table 1), which are highly controlled and essential to functioning neuronal responses.

The example of SIRT1 is paradigmatic because its enzymatic activity is under the control of metabolic cofactors and inhibitors. While NAD<sup>+</sup> is SIRT1's

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**Figure 2.** Schematic model of CLOCK-mediated histone acetylation and its regulation by the NAD<sup>+</sup>-dependent histone deacetylase SIRT1. The HAT function of CLOCK regulates promoters of CCGs and clock genes (such as *Per1*) by inducing locally open organization of the chromatin. CCGs include a large number of genes involved in cellular metabolism, proliferation, and cell cycle (examples are indicated), underscoring the critical function of the CLOCK–SIRT1 complex in neurodegeneration and physiological control. Strikingly, the expression of 15% of all mammalian transcripts oscillates in a circadian manner. CLOCK acetylates H3 and BMAL1, its natural heterodimerization partner, in order to regulate promoters of CCGs. Acetylation by CLOCK is thought to elicit chromatin remodeling by inducing a transcription-permissive state. The acetyltransferase enzymatic activity of CLOCK has a dual function: It regulates the circadian machinery by targeting both histone and nonhistone proteins. We envisage a scenario in which circadian control of chromatin remodeling by CLOCK may be influenced by the dynamic assembly of a multiprotein regulatory complex. SIRT1 associates with CLOCK and, in response to the metabolic changes in intracellular NAD levels, modulates CCGs by virtue of its HDAC enzymatic activity. Thus, metabolic, nutritional, and environmental cues modulate the circadian machinery via chromatin remodeling.

PTM	Metabolite
Phosphorylation	ATP/ADP
Methylation	SAM/SAH, FAD/FADH <sub>2</sub>
Acetylation	Acetyl-CoA/CoA, NAD/NADH Acetyl-ADP-ribose
Ubiquitination/Sumoylation	Glucose?
Glycosylation	UDP-GlcNAc/UDP

**Table 1.** The importance of posttranslational modifications (PTMs) of histones in chromatin remodeling is well established. One critical facet of these modifications is that they are elicited by specific enzymatic activities that depend on the intracellular levels of essential metabolites; these metabolites sense cellular metabolism, nutrients, and energy levels in the cell. PTMs target specific sites on the histone tails, indicating that the transient states of chromatin remodeling are under the dynamic regulation of cellular physiology (Borrelli et al., 2008).

natural cosubstrate, the oxidated form NADH and the by-product of NAD<sup>+</sup> consumption, nicotinamide (NAM), repress the activity of SIRT1 (Bordone and Guarente, 2005), generating an enzymatic feedback loop on the HDAC function of this enzyme. Indeed, it has been established that fluctuations in NAD<sup>+</sup> or changes in the NAD<sup>+</sup>/NADH ratio and nicotinamide concentrations directly influence SIRT1 function. Two main systems determine NAD<sup>+</sup> levels in the cell: the *de novo* biosynthesis from tryptophan and the NAD<sup>+</sup> salvage pathway.

A critical step of this latter pathway is controlled by the enzyme nicotinamide phosphoribosyltransferase (NAMPT), also known as visfatin or PBEF (Rongvaux et al., 2003). NAMPT catalyzes the first step in the biosynthesis of NAD<sup>+</sup> from nicotinamide. In mammalian cells, NAMPT slows down senescence and promotes survival during genotoxic stress (Anderson et al., 2002; Revollo et al., 2004; van der Veer et al., 2007; Yang et al., 2007). Importantly, *Nampt* gene expression is dynamic because it is inducible by various agents and responds to specific cellular stresses (e.g., DNA damage) and nutrients. This resilience indicates that its control is central to governing the intracellular NAD<sup>+</sup>:NAM balance. Interestingly, SIRT1 and CLOCK protein levels do not seem to oscillate (Nakahata et al., 2008), whereas the acetylation of the targets of CLOCK-mediated HAT function (e.g., K14 of histone H3 and K537 of BMAL1) is circadian (Hirayama et al., 2007; Nakahata et al., 2008). Thus, one possibility is that circadian oscillation would be determined by oscillating levels of intracellular NAD<sup>+</sup>. Indeed, intracellular NAD<sup>+</sup> levels cycle with a 24 h rhythm, and this oscillation is driven by the circadian clock. CLOCK:BMAL1 regulates the circadian expression of the *Nampt* gene in concert with SIRT1, which thereby contributes to the circadian synthesis of its own coenzyme (Nakahata et al., 2009). Using the specific inhibitor of NAMPT enzyme activity, FK866, researchers have also demonstrated that NAMPT is required to modulate circadian gene expression as well as BMAL1 circadian acetylation (Nakahata et al., 2009).

These results uncover two self-regulating, interlocking systems, in which a classical transcription circadian loop is coupled to an enzymatic feedback loop (Fig. 2). In addition, at least one other enzyme may be critically influenced by oscillatory NAD<sup>+</sup> levels: the poly(ADP-ribose) polymerase-1 (PARP-1), which utilizes NAD<sup>+</sup> as a coenzyme to exert its role in recovery from DNA damage. A functional interplay between SIRT1 and PARP-1 has been reported (Kolthur-Seetharam et al., 2006).

The implications of these findings are myriad. First, they reveal an intriguing link between cellular metabolism and circadian physiology (Eckel-Mahan and Sassone-Corsi, 2009). In addition, they highlight possible molecular pathways that could bridge the circadian machinery to various diseases. Finally, they may open up novel avenues for pharmacological intervention.

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