Mammalian circadian clock and metabolism – the epigenetic link

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Summary

Circadian rhythms regulate a wide variety of physiological and metabolic processes. The clock machinery comprises complex transcriptional–translational feedback loops that, through the action of specific transcription factors, modulate the expression of as many as 10% of cellular transcripts. This marked change in gene expression necessarily implicates a global regulation of chromatin remodeling. Indeed, various descriptive studies have indicated that histone modifications occur at promoters of clock-controlled genes (CCGs) in a circadian manner. The finding that CLOCK, a transcription factor crucial for circadian function, has intrinsic histone acetyl transferase (HAT) activity has paved the way to unraveling the molecular mechanisms that govern circadian chromatin remodeling. A search for the histone deacetylase (HDAC) that counterbalances CLOCK activity revealed that SIRT1, a nicotinamide adenin dinucleotide (NAD^+)-dependent HDAC, functions in a circadian manner. Importantly, SIRT1 is a regulator of aging, inflammation and metabolism. As many transcripts that oscillate in mammalian peripheral tissues encode proteins that have central roles in metabolic processes, these findings establish a functional and molecular link between energy balance, chromatin remodeling and circadian physiology. Here we review recent studies that support the existence of this link and discuss their implications for understanding mammalian physiology and pathology.

Key words: Chromatin, Epigenetics, Metabolism

Introduction

All life forms have evolved by adapting to the light-dark cycle generated by the rotation of the earth on its axis. Most of them developed an internal clock that controls their circadian (from the latin 'circa diem', meaning about a day) rhythms. In mammals, the internal clock exhibits significant plasticity that enables it to contribute to body homeostasis, regulating a wide variety of physiological and metabolic processes. Importantly, ample epidemiological evidence shows that disruption of the circadian rhythm in humans has a profound impact on many physiological functions and is also related to several pathologies, including insomnia, depression, cardiovascular disorders and cancer (Bunney and Bunney, 2000; Sahar and Sassone-Corsi, 2009).

A major stride in understanding the molecular basis of circadian rhythms was the identification (Konopka and Benzer, 1971) and cloning of the first clock genes in *Drosophila melanogaster* (Bargiello and Young, 1984; Reddy et al., 1984). Since these discoveries were made, other crucial regulators have been identified. In mammals, the core components of the clock molecular machinery (Fig. 1) operate in almost all cells of the body through a complex network of transcriptional–translational loops and modulate the expression of specific target genes and their products, thus allowing their expression to oscillate in a 24-hour rhythm. Recent studies revealed that dynamic chromatin remodeling has a crucial role in the circadian regulation of gene expression. Here we discuss the close links between the core components of the circadian clock, chromatin remodeling and cellular metabolism. In light of recent findings, we focus on the dual role of the clock system in these processes, in which it acts as both a sensor and a mediator of changes in the intracellular metabolic state.

Circadian rhythms in mammals

The circadian clock is a highly conserved system that enables organisms to adapt to common daily changes, such as the day-night cycle and food availability. This system controls a wide variety of physiological functions, including sleep-wake cycles, body temperature, hormone secretion, locomotory activity and feeding behaviour (Schibler and Sassone-Corsi, 2002).

In mammals, the anatomical structure in the brain that governs circadian rhythms is a small area consisting of ~15,000 neurons localized in the anterior hypothalamus, called the suprachiasmatic nucleus (SCN) (Ralph et al., 1990; Welsh et al., 1995). This 'central pacemaker' receives signals from the environment and coordinates the oscillating activity of peripheral clocks, which are located in almost all tissues (Morse and Sassone-Corsi, 2002; Schibler and Sassone-Corsi, 2002; Yamazaki et al., 2000; Yoo et al., 2004). One important feature of the circadian clocks is that they are self-sustained; circadian oscillations that are intrinsic to each cell can occur autonomously without the need for an environmental signal. However, because the period of these oscillations is not exactly 24 hours, the endogenous clock needs to be synchronized by external cues, a process called entrainment. External cues (also known as zeitgebers) reset the system daily and thereby prevent the endogenous clock from free-running out of phase. The predominant external cue of the central clock is light (Quintero et al., 2003). In mammals, specialized cells in the retina detect the light signal that is then transmitted to the SCN via the retino-hypothalamic tract (RHT) (Cermakian and Sassone-Corsi, 2002; Freedman et al., 1999; Gooley et al., 2001). At the level of SCN neurons, the light signal stimulates a cascade of signaling pathways that lead to the activation of a transcriptional

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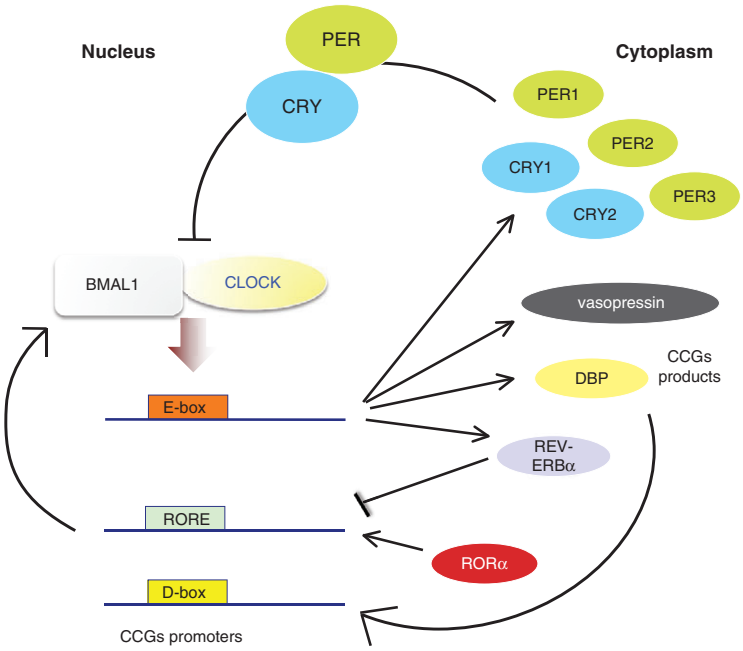

Fig. 1. Schematic representation of the transcriptional–translational loops regulating circadian rhythms in mammals. The positive regulators CLOCK–BMAL1 activate genes with E-box elements in their promoters; these are commonly indicated as clock-controlled genes (CCGs). Among the CCGs are also the genes encoding the CRY and PER proteins that act as negative regulators of their own transcription. Most CCGs encode essential regulators of hormonal and metabolic control; here, vasopressin is shown as example. Additional loops of the circadian machinery involve other transcription factors, whose expression is primarily activated by CLOCK–BMAL1. These are DBP and REV-ERBα, which control the expression of genes with D-Box and RORE elements in their promoters (Reppert and Weaver, 2002).

program that involves immediate early genes and clock-controlled genes (CCGs) (Loros et al., 1989). These gene expression events are associated with specific histone modifications that lead to chromatin remodeling (Crosio et al., 2000). Peripheral tissues also contain functional circadian oscillators that are self-sustained at the single cell level, but they do not respond to light-dark cycles and appear to require other physiological stimuli in order to sustain their circadian rhythms.

Importantly, lesion of the SCN in rodents disrupts the circadian periodicity in peripheral tissues and SCN transplantation into SCN-ablated and thus arrhythmic animals restores this dysfunction (Lehman et al., 1987; Ralph et al., 1990). Additional experiments, in which the transplantation approach of fibroblasts was applied to peripheral tissues demonstrates a hierarchical dominance of the SCN over peripheral clocks (Pando et al., 2002). To date, however, the means by which the SCN communicates with the other tissues to sustain and synchronize their cycles is still not clear. Several observations support the idea that communication might be exerted by a combination of neuronal signals through the autonomic nervous system and humoral factors, of which glucocorticoids and retinoic acid are the most likely candidates (Antle and Silver, 2005; Green et al., 2008). Furthermore, peripheral rhythms in mammals are affected by other SCN-independent stimuli (Yoo et al., 2004). Although light is the main stimulus that entrains the central pacemaker, peripheral clocks themselves can be entrained by food (Stokkan et al., 2001), probably through modifications of hormonal secretion or metabolite availability. Conversely, restricted access to food can reset the phase of peripheral oscillators with little, if any, effects on the central pacemaker in the SCN (Damiola et al., 2000).

Another important environmental cue is temperature (Roenneberg and Merrow, 2005). Temperature compensation is one of the most prominent features of the circadian system as it allows the integration of moderate variations in ambient temperature that do not affect the period length of circadian oscillation. Nevertheless, low-amplitude temperature cycles can synchronize the circadian clocks in peripheral tissues in mammals independently of the central clock (Brown et al., 2002).

The molecular clock machinery

The clock machinery is based on a group of complex transcriptional–translational feedback loops (Fig. 1) (Glossop and Hardin, 2002; Morse and Sassone-Corsi, 2002; Reppert and Weaver, 2002). In mammals, the proteins circadian locomotor output cycles kaput (CLOCK) and brain and muscle ARNT-like protein 1 (BMAL1) form the positive branch of the feedback loop (Gekakis et al., 1998; Vitaterna et al., 1994). These proteins are members of the family of basic-helix-loop-helix (bHLH)-PAS transcription factors that heterodimerize through their PER–ARNT–SIM (PAS) domains and bind to DNA elements, the so-called E-boxes with the nucleotide sequence CACGTG. E-boxes are among the most frequent promoter elements in mammalian genomes and are present in either one or multiple copies in the regulatory regions of CCGs. Among the CLOCK–BMAL1 target genes are the period genes (*Per1*, *Per2* and *Per3*) and cryptochrome genes (*Cry1* and *Cry2*). PER and CRY proteins form a heterodimeric repressor complex that translocates into the nucleus to inhibit CLOCK–BMAL1-mediated activation of CCGs – that include *Per* and *Cry* genes (Darlington et al., 1998; Griffin et al., 1999; Kume et al., 1999; Sato et al., 2006). A cyclic proteolytic degradation of the repressor complex has an essential role in the maintenance of the amplitude of the circadian oscillation. Degradation occurs upon phosphorylation of the PER proteins by casein kinase Iε (CKIε) and recruitment of the specialized F-box protein FBXL3 (Busino et al., 2007; Godinho et al., 2007; Siepka et al., 2007). These events are thought to allow the CLOCK–BMAL1 activator complex to start a new transcriptional cycle within 24 hours (Eide et al., 2005).

Gene expression profiles obtained from microarray studies have revealed that 10–15% of all transcripts in different tissues display circadian oscillation (Akhtar et al., 2002; Duffield et al., 2002; Panda et al., 2002; Storch et al., 2002). Considering that the overlap of circadian genes in various tissues is limited, the proportion of genes that are expressed in a rhythmic manner is actually much higher. Among these, many are genes directly regulated by CLOCK–BMAL1 through E-boxes contained in their promoters,

such as vasopressin (*Avp*) or D site albumin promoter-binding protein (*Dbp*) (Jin et al., 1999; Ripperger et al., 2000; Ripperger and Schibler, 2006). Other genes encode transcription factors that, in turn, regulate the cyclic expression of additional CCGs. This mode of regulation occurs through binding of these transcription factors to regulatory consensus sequences that differ from E-boxes, such as D-boxes or receptor-related orphan receptor (ROR) response elements (ROREs) (Ueda et al., 2005).

One aspect that has been poorly investigated thus far is the tissue specificity of the clock machinery. As the panel of CCGs is significantly different in the SCN compared with that in peripheral tissues, and similarly also varies among different peripheral tissues, it is expected that stringent rules of cell-type-specific transcriptional control apply to the circadian system. A good example for such a tissue-specific expression is neuronal PAS-domain protein 2 (NPAS2), a functional analogue of CLOCK that is mainly expressed in the forebrain. NPAS2 heterodimerizes with BMAL1 and activates transcription through E-boxes with an efficacy analogous to CLOCK (Debruyne et al., 2006). However, the extent of overlap among CLOCK–BMAL1 and NPAS2–BMAL1 target genes remains unclear.

Additional transcriptional–translational regulatory loops contribute to the function of the circadian clock. Specifically, the transcription factors reverse erythroblastosis virus-α (REV-ERBα) and retinoic acid receptor-related orphan receptor-α (RORα) are nuclear receptors that constitute an additional loop and therefore have an essential role in driving rhythmic *Bmal1* expression. These two transcription factors bind to ROREs in the *Bmal1* promoter and either activate (in the case of RORα binding) or repress (by binding to REV-ERBα) *Bmal1* transcription (Preitner et al., 2002; Sato et al., 2004). Completion of this feedback loop occurs through transcription of the gene encoding REV-ERBα that is directly activated by CLOCK–BMAL1 and through its expression with robust rhythmicity.

The effect of post-translational modifications on circadian control

A variety of regulatory processes are superimposed on the canonical transcriptional–translational loop that constitutes the core of the circadian machinery. It has been shown that control of circadian clock components operates not only at the post-transcriptional level (Cermakian and Sassone-Corsi, 2000; So and Rosbash, 1997), but also at the level of protein stability (Sahar et al., 2010) and through intracellular localization (Takano et al., 2004; Vielhaber et al., 2001). In particular, post-translational modifications (PTMs) of clock proteins are important for ensuring the maintenance of circadian rhythms, as they can modulate the activity and turnover of major clock components (Gallego and Virshup, 2007). Various PTMs have been described for most clock proteins, and many of these occur in a time-specific manner. In this section, we do not present an exhaustive survey of these modifications, but rather discuss representative examples that have been linked to specific circadian functions.

Phosphorylation

Phosphorylation of clock proteins has a prominent role in the circadian system of different organisms. Many clock proteins contain several putative phosphoacceptor sites for a number of possible candidate kinases (Hirayama and Sassone-Corsi, 2005). A paradigmatic example of the importance of this modification for circadian function is the phosphorylation of PER proteins by CKIε. This kinase has been closely linked to circadian rhythms, as the phenotype of the hamster *tau* mutant, which is characterized by impaired circadian rhythmicity, is caused by a mutation in the gene encoding CKIε (Lowrey et al., 2000). CKIε-mediated phosphorylation of PER proteins targets them for ubiquitin-mediated degradation, thereby controlling the duration of their function (Eide et al., 2005; Gallego and Virshup, 2007). Further evidence for the importance of CKIε comes from *Drosophila*, in which drastic disruptions in circadian rhythms are caused by mutations in the fly ortholog of CKIε, *Doubletime* (*dbt*) (Kloss et al., 1998; Price et al., 1998).

CKIε also phosphorylates and activates BMAL1 (Eide et al., 2002). BMAL1 is a target for various kinases and displays a remarkable profile of circadian phosphorylation in a range of tissues and in serum-shocked synchronized fibroblasts. Glycogen synthase kinase 3β (GSK3β) has recently been shown to phosphorylate mammalian BMAL1 and to control its stability and activity (Sahar et al., 2010). Interestingly, the *Drosophila* ortholog of GSK3, *Shaggy* (*sgg*), also phosphorylates the fly clock proteins PERIOD and TIMELESS (Martinek et al., 2001). Moreover, a high-throughput approach recently demonstrated that inhibition of GSK3β expression and activity leads to a shortening of the circadian period (Hirota et al., 2008). The involvement of GSK3β in circadian physiology is of interest as this kinase has been implicated in the molecular mechanism of lithium-based therapy of depression (Doble and Woodgett, 2003), a syndrome in humans linked to disruption of circadian rhythms (Bunney and Bunney, 2000; Perreau-Lenz et al., 2007). Finally, in addition to BMAL1, GSK3β phosphorylates other clock proteins, including CRY2 (Harada et al., 2005), PER2 (Iitaka et al., 2005), REV-ERBα (Yin et al., 2006) and CLOCK (Spengler et al., 2009), resulting in different effects on the protein stability of each substrate.

BMAL1 is also targeted by other kinases, for example by mitogen-activated protein kinases (MAPKs) (Sanada et al., 2002) and CK2α (Tamaru et al., 2009). An important site of CK2α activity is the serine residue at position 90 (S90) in BMAL1, the mutation of which leads to disruptions in circadian rhythmicity (Tamaru et al., 2009). The ortholog of CK2 kinase in *Drosophila* also exerts a crucial circadian function by phosphorylating PER and regulating its nuclear entry (Akten et al., 2003; Lin et al., 2002; Lin et al., 2005). Thus, most clock components are modified by highly controlled phosphorylation events that are mediated by a number of kinases. However, the interplay between these regulatory kinases still needs to be deciphered (Fig. 2).

Sumoylation, acetylation and ubiquitylation

Among clock proteins, BMAL1 has been shown to undergo extensive PTMs in addition to phosphorylation. BMAL1 is a substrate for sumoylation (Cardone et al., 2005), ubiquitylation (Kwon et al., 2006; Lee et al., 2008; Sahar et al., 2010) and acetylation (Hirayama et al., 2007). The role of acetylation is discussed further below.

Sumoylation and ubiquitylation are PTMs that directly influence the intracellular pathways that control protein stability. Sumoylation in particular is involved in various cellular processes, such as transcriptional regulation, nuclear transport and protein stability. BMAL1 is sumoylated by the small ubiquitin-like modifier 1 (SUMO-1) at a specific, conserved lysine residue at position 259 (K259) located in the PAS-domains linker. BMAL1 shows a circadian pattern of sumoylation in mouse liver that closely follows its activation. Sumoylation of BMAL1 is controlled by the circadian

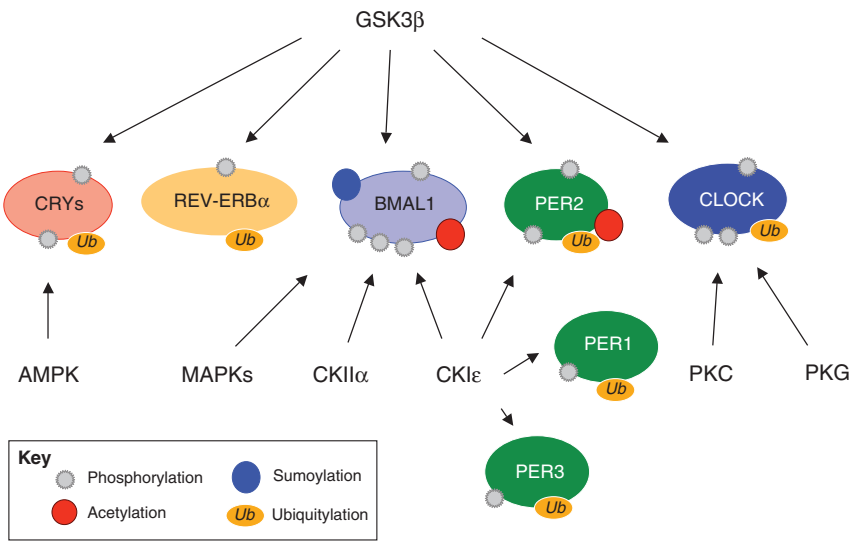

Fig. 2. Clock regulators undergo a large variety of post-translational modifications. Phosphorylation is common and elicited by a number of kinases activated by various signaling pathways (Hirayama and Sassone-Corsi, 2005). GSK3β has been shown to phosphorylate most clock proteins, thereby controlling their stability and subcellular localization. A direct link to protein destabilization was demonstrated for the AMPK-mediated phosphorylation of CRYs (Lamia et al., 2009). Other post-translational modifications include acetylation of BMAL1, a modification that is crucial for circadian rhythmicity (Hirayama et al., 2007). Ubiquitylation and sumoylation have also been described for a number of clock regulators (Akashy et al., 2002; Cardone et al., 2005; Gatfield and Schibler, 2007; Kwon et al., 2006; Lee et al., 2008; Sahar et al., 2010; Yin et al., 2006).

clock and requires CLOCK, the heterodimerization partner of BMAL1. Ectopic expression of a SUMO-1-deficient form of BMAL1 by using a viral vector demonstrated that sumoylation has an important role in the rhythmic expression of BMAL1. Sumoylation of BMAL1 is enhanced by ubiquitin conjugating enzyme 9 (UBC9), an E2 ligase that activates the SUMO pathway. These findings, and the reported BMAL1 ubiquitylation (Lee et al., 2008; Sahar et al., 2010), stress the important role the proteasome has in regulating clock proteins.

A chromatin remodeling clock

Because the fraction of mammalian transcripts that oscillates in a circadian manner is remarkably high (Akhtar et al., 2002; Duffield et al., 2002; Panda et al., 2002; Storch et al., 2002), it follows that there must be an equally widespread program of dynamic changes in chromatin remodeling that accompany and allow for circadian gene expression. What could be defined as the 'circadian epigenome' probably involves cycles of chromatin transitions that allow a highly dynamic chromatin structure with the DNA to be locally and temporally permissive to transcription.

Epigenetic control can be exerted through a variety of mechanisms, including DNA methylation, microRNA-mediated metabolic pathways, histone variants and histone PTMs (Bernstein and Allis, 2005; Berger, 2007; Borrelli et al., 2008; Cheung et al., 2000a; Miranda and Jones, 2007; Strahl and Allis, 2000). The presence of an 'indexing epigenetic code' suggests that the coordinated and progressive combination of these processes allows the epigenome to move from an 'unlocked' transcriptionally active to a 'locked' silenced state, thereby determining the fate and physiology of a given cell (Borrelli et al., 2008).

Histone PTMs are largely responsible for the plasticity of chromatin remodeling (Cheung et al., 2000a). Histones can be modified at multiple amino acid residues and at more than 30 sites within their N-terminal tails. Many different PTMs (acetylation, methylation, phosphorylation, sumoylation, ubiquitylation, ADP-ribosylation and biotinylation) can occur, some involving modification of the same residue but at different times. Therefore, the number of possible PTM combinations is enormous, an observation that inspired the concept of the 'epigenetic code', suggesting that modifications can be coupled functionally and occur interdependently in specific combinations (Strahl and Allis, 2000). For example, acetylation of lysine residue at positions 9 (K9) or 14 (K14) of histone H3 is known to be associated with an open chromatin conformation that leads to active gene transcription (Lee et al., 1993). Phosphorylation of H3 at the nearby serine residue at position 10 (S10) appears to function as a priming event that allows acetylation of K14 with a much higher efficacy (Cheung et al., 2000b; Lo et al., 2000), probably owing to the physical interaction of enzymes that are thought to elicit these combined modifications (Merienne et al., 2001; Lo et al., 2000).

The first study that showed that chromatin remodeling is involved in circadian gene expression demonstrated that a pulse of light, when applied to mice during the subjective night, induces H3 phosphorylation at S10 in the SCN (Crosio et al., 2000). This is an early response, occurring in parallel with other early events, such as transcriptional induction of *Fos* and *Per1* (Crosio et al., 2000). Subsequently, several studies have indicated that histone modifications at CCG promoters occur in a circadian manner (Curtis et al., 2004; Doi et al., 2006; Etchegaray et al., 2003; Naruse et al., 2004). For example, the rhythmic (rather than constitutive) recruitment of CLOCK–BMAL1 to the *Dbp* promoter is associated with the acetylated status of the H3 tail at that locus (Ripperger and Schibler, 2006). In addition to phosphorylation and acetylation, methylation is thought to contribute to regulating circadian transcription. Whereas trimethylation of lysine residue at position 4 of H3 (H3K4 trimethylation), a marker of open chromatin, has been associated with H3K9 acetylation and therefore activation of the *Dbp* promoter, H3K4 dimethylation at the same promoter correlates with the repressive phase of its cycle (Ripperger and Schibler, 2006). Interestingly, PER1-mediated transcriptional repression is enhanced by its association with the methyltransferase complex subunit WDR5 (Brown et al., 2005). Similarly, H3K27 methylation at *Per* promoters by the polycomb group protein EZH2 has been correlated with their repression of the rhythmic transcription (Etchegaray et al., 2006). Although these descriptive analyses did not identify the specific mechanism of circadian chromatin remodeling, they provided a useful starting point for further studies.

The finding that CLOCK has intrinsic histone acetyl transferase (HAT) activity (Doi et al., 2006) revealed the long-sought molecular

link between epigenetic control and the circadian clock. The initial identification of several regions in CLOCK that are homologues to those in the HAT activator of thyroid and retinoid receptors (ACTR) (Chen et al., 1997) suggests that CLOCK is more than merely a transcription factor and, indeed, reveals it to be a new type of DNA-binding HAT (Doi et al., 2006). CLOCK is a bona fide HAT with a preference for acetylating lysine residues in H3 and H4. Importantly, CLOCK-mediated H3K14 acetylation at CCG promoters follows a circadian rhythmicity (Doi et al., 2006; Nakahata et al., 2008). Interestingly, studies of mouse embryonic fibroblasts (MEFs) derived from homozygous *Clock* mutant (*c/c*) mice demonstrate that the HAT activity of CLOCK is essential for circadian gene expression (Doi et al., 2006).

Similar to other HATs (Glozak et al., 2005), CLOCK also elicits the acetylation of non-histone substrates, such as of its heterodimerization partner BMAL1, which is acetylated at a single, highly conserved lysine residue (K537) (Hirayama et al., 2007). This event is crucial for the circadian transcriptional program. Indeed, ectopic expression of wild-type BMAL1, but not of a non-acetylatable BMAL1-K537R mutant, can rescue circadian expression in MEFs that have been derived from *Bmal1*-null mice. Furthermore, BMAL1 acetylation increases the affinity of CLOCK–BMAL1 for the repressor CRY, an association that is crucial for the negative feedback loop of the clock (Hirayama et al., 2007). Whether BMAL1 acetylation is regulated or whether it influences other PTMs of BMAL1 has not been explored yet. Finally, recent reports show that CLOCK can acetylate non-histone proteins other than BMAL1; for example, the glucocorticoid receptor is a target of CLOCK and its acetylation appears to regulate its function (Nader et al., 2009).

These data strongly support the notion that CLOCK is involved in the acetylation of other non-histone targets and, more specifically, of proteins that have key roles in regulating various cellular events – from the cell cycle to metabolic pathways (Fig. 3).

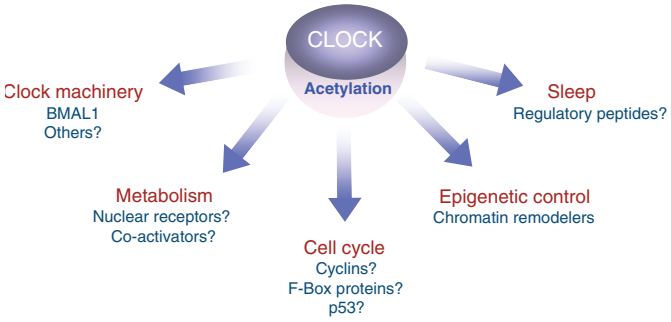

Fig. 3. CLOCK is a HAT. The core circadian regulator CLOCK was identified to have intrinsic HAT activity (Doi et al., 2006) and to acetylate also non-histone proteins, in particular its own dimerization partner BMAL1 (Hirayama et al., 2007). It is tempting to speculate that, similarly to other HATs (Glozak et al., 2005), CLOCK also acetylates other cellular proteins, thereby establishing functional connections to a variety of metabolic pathways as shown here. A substantial proportion of nuclear receptors has been shown to be expressed in a circadian manner (Yang et al., 2006), suggesting that nuclear receptors are regulated by CLOCK-mediated acetylation. Likewise, a number of cell-cycle regulators could be targeted by CLOCK, as some of them are known to be acetylated, for example, p53 (Gu and Roeder, 1997). Also, as CLOCK is in a nuclear complex (see Fig. 4) and thus could associate with other nuclear regulators, it is possible that CLOCK also modifies chromatin remodelers. Finally, the conceptual link between the clock and the sleep-wake cycle could indicate a molecular connection between sleep-related peptides and signaling to CLOCK.

Sirtuins: linking metabolism to epigenetics

A significant feature of all histone PTMs is that they use cellular metabolites as a source for the modifications (Borrelli et al., 2008). Thus, enzymes that mediate chromatin-remodeling events might sense specific metabolic states of individual cells, thereby intimately linking intracellular signaling with histone modifications (Cheung et al., 2000a). With this in mind, it would be important to identify the specific remodeling enzymes that have a crucial role in these processes and to confirm the possibility that chromatin has a central role in adaptation to metabolic fluctuations.

Cells modify their biochemical activities according to transient environmental stimuli, such as food intake and energy expenditure. This occurs through modulating the expression of various metabolic targets – mainly rate-limiting enzymes in central biochemical pathways. By changing the chromatin conformation and the accessibility of transcription factors to genomic regions that encode these central enzymes, cells can adjust enzyme levels according to specific energetic conditions (Feil, 2006). We focus our discussion on histone acetylation, the levels of which are controlled by the concerted enzymatic activity of HATs and histone deacetylases (HDACs) (Strahl and Allis, 2000). As discussed above, previous studies have shown that clock components associate with different HATs and HDACs (Curtis et al., 2004; Etchegaray et al., 2003), providing hints for the mechanisms by which they might exert their temporally regulated function as transcription factors.

Interestingly, it has been found that epigenetic abnormalities are associated with various pathological conditions, including aging, cancer and atherosclerosis (Feinberg, 2007). How these alterations result in metabolic disorders remains largely unclear. However, accumulating evidence of the effect of caloric restriction on regulating gene expression, chromatin structure and genome stability is providing important information in this respect (Heydari et al., 2007; Vaquero and Reinberg, 2009). Caloric restriction is considered the most consistent non-pharmacological intervention that increases lifespan (Canto and Auwerx, 2009) and alters gene expression at the level of chromatin. Accumulating evidence suggests that a specific class of HDACs, the sirtuins, mediates the caloric restriction response on the chromatin level in organisms ranging from yeast to mammals (Haigis and Guarente, 2006; Longo and Kennedy, 2006). SIRT1 is the mammalian ortholog of yeast Sir2 (silencing information regulator), a mediator of transcriptional silencing at repeat DNA sequences in yeast genomes, such as mating-type loci, telomeres and ribosomal DNA (rDNA) (Brachmann et al., 1995). SIRT1 is the best-characterized member of the sirtuin family, which includes seven members that are broadly conserved between species. All sirtuins possess ADP-ribosyltransferase and deacetylase activities, which constitutes the class III of HDACs (Tanny et al., 1999; Imai et al., 2000). Both enzymatic activites of sirtuins require NAD^+ as cofactor. SIRT1 converts NAD^+ into nicotinamide to either remove the acetyl group from its substrate or to attach a molecule of ADP-ribose to the acetylated residue. Because of its dependency on the ratio between NAD^+ and NADH (Lin et al., 2004), SIRT1 is considered a sensor of metabolic changes that enables cells to survive in conditions of reduced nutrient availability. Indeed, SIRT1-knockout mice display defective metabolism and partial unresponsiveness to caloric restriction (Boily et al., 2008; Chen et al., 2005), whereas SIRT1

overexpression protects mice from high-fat-diet-induced metabolic disease and induces a phenotype similar to that observed in calorie-restricted mice (Bordone et al., 2007; Banks et al., 2008; Pfluger et al., 2008).

SIRT1 is thought to exert its functions mainly, but not exclusively, at the level of chromatin. The preferential targets of SIRT1 activity are H3K9 and H4K16 (Vaquero et al., 2004). At these sites, SIRT1-mediated deacetylation promotes the formation of facultative heterochromatin and thereby induces gene silencing. In this respect, another sirtuin of interest is SIRT6, which is the only other sirtuin family member that is localized in the nucleus and associates with chromatin (Michishita et al., 2008). Recent data indicate that SIRT6 is directly involved in controlling glucose metabolism (Zhong et al., 2010).

Importantly, SIRT1 also targets several non-histone substrates, including proteins with prominent roles in cell-cycle regulation, such as p53 and NF-κB, in DNA repair, for example nibrin (NBS1) and Ku70, and in the metabolism of glucose and lipids, such as the fork head transcripton factors FOXOs, the peroxisome proliferator-activated receptor γ (PPARγ), the PPARγ coactivator 1α (PGC-1α) and liver X receptor (LXR) (Li et al., 2007; Luo et al., 2001; Motta et al., 2004; Picard et al., 2004; Rodgers et al., 2005; Vaziri et al., 2001; Yeung et al., 2004). Therefore, as described further below, SIRT1 is a central physiological and molecular link that connects chromatin remodeling to circadian and metabolic processes.

How the circadian system senses metabolism

Before food consumption, most organisms display a series of physiological and behavioral changes that enable them to 'anticipate' timed meals. Many of these effects, including increased body temperature, food anticipatory activity and a rise in duodenal secretion, are controlled in a circadian manner (Stephan, 2002). As mentioned above, circadian rhythms are directly dictated by food availability, which is a powerful external cue that entrains peripheral clocks. It has been reported that, in nocturnal animals, limiting food availability to a brief time during the light phase – referred to as restricted feeding – completely inverts the phase of the expression of genes that encode clock components and CCGs in the liver without affecting the SCN clock function (Damiola et al., 2000; Stokkan et al., 2001). Remarkably, restricted feeding also affects peripheral rhythms in arrhythmic *Clock*-mutant mice and in mice that have specific lesions of the SCN when they are exposed to either light-dark cycles or to constant darkness (Hara et al., 2001; Stephan, 2002; Stokkan et al., 2001). These observations suggest that food availability affects not only peripheral clock functions directly, but also through a brain region that is involved in regulating the feeding-mediated circadian behavior. Some evidence indicates that the dorsomedial nuclei have a significant role in this response (Gooley et al., 2006; Mieda et al., 2006); however, the exact location of this food-entrainable oscillator is still under debate (Davidson, 2006).

In contrast to the response to restricted feeding, which only impinges on peripheral clocks, both the SCN and peripheral oscillators exhibit resetting of circadian rhythms during caloric restriction (Challet et al., 1998; Mendoza et al., 2005; Resuehr and Olcese, 2005), indicating that stimuli that are associated with the nutritional value of a meal and with the amount of calories can affect the central circadian oscillator. In support of this hypothesis, it has been shown that a high-fat diet leads to changes in the circadian period in mice (Kohsaka et al., 2007). Furthermore, a number of food components – such as glucose, ethanol, adenosine and caffeine, thiamine and retinoic acid – can entrain or phase-shift circadian rhythms (Froy, 2007). Finally, several stimuli, including insulin, glucose, the glucocorticoid hormone analogue dexamethasone, forskolin and phorbol ester, can trigger circadian expression in vitro by activating specific signaling cascades (Balsalobre et al., 2000; Hirota et al., 2002). Thus, the convergence of distinct signaling pathways that are activated after feeding is likely to influence the clock machinery, probably in a tissue-specific manner.

Nuclear receptors constitute a class of regulators that occupy a prominent position to function at the interface between circadian control and metabolism. These transcription factors coordinate gene expression in response to hormonal and environmental signals through direct binding to specific hormone-responsive DNA elements and by recruiting several co-regulatory proteins and chromatin-remodeling complexes (Sonoda et al., 2008; Trotter and Archer, 2007). Nuclear receptors have a vital role in various biological processes, including development and metabolic homeostasis, by acting as sensors of fat-soluble hormones and dietary lipids. The activity of many nuclear receptors is modulated by a crucial switch that involves binding to specific signaling molecules for their activation – including secreted molecules, such as steroid and thyroid hormones, vitamin D and retinoic acid. Some nuclear receptors are referred to as orphan receptors because their endogenous ligands are unknown.

About half of the known nuclear receptors display cyclic expression in metabolically active tissues, including liver, adipose tissue and skeletal muscle (Yang et al., 2006), a finding that underscores the close link between these regulators and the circadian clock. For example, the orphan receptor REV-ERBα that, as mentioned above, has an important role in the maintenance of circadian rhythm (Akashi and Takumi, 2005; Preitner et al., 2002), also regulates adipogenesis and its expression increases during the differentiation of pre-adipocytes into mature adipocytes (Fontaine et al., 2003). Moreover, REV-ERBα regulates triglyceride mobilization by controlling the circulating levels of triglyceride-rich lipoproteins and also appears to have an important role in regulating vascular inflammatory processes (Migita et al., 2004). Notably, REV-ERBα transcription is rhythmic and is regulated by the clock machinery, exhibiting a diurnal peak of maximal expression. REV-ERBα-deficient mice are not arrythmic, but they have a shorter period length and increased *Clock* and *Bmal1* expression levels (Preitner et al., 2002).

The peroxisome proliferator-activated receptors (PPARs) also constitute a link between metabolism and circadian control. PPARα regulates lipid metabolism and binds to the *Bmal1* promoter to modulate its expression. Moreover, its own expression is regulated by CLOCK–BMAL1 through E-boxes present in its promoter region (Canaple et al., 2006; Oishi et al., 2005). Consequently, genes that are induced or inhibited by PPARs are also rhythmically expressed. PGC1α is an inducible transcriptional coactivator of the genes encoding PPARs, and is involved in integrating metabolic and clock signals. It has a prominent role in regulating oxidative phosphorylation by controlling mitochondrial biogenesis. PGC-1α expression oscillates in a circadian manner and contributes to *Bmal1* transcription through the co-activation of the ROR family of orphan nuclear receptors. Accordingly, PGC-1α-null-mice show defects in circadian expression of clock and metabolic genes (Liu et al., 2007). Finally, PER2 has been shown to physically interact with several nuclear receptors (Schmutz et al., 2010) and to directly regulate PPARγ function (Grimaldi et al., 2010). These findings

indicate that several physiologically relevant connections between nuclear receptors and clock functions exist that remain to be uncovered.

Linking the circadian clock to metabolic disorders

Metabolic tissues have the remarkable property of being able to respond to oscillatory changes in circulating levels of glucose, fatty acids, triglycerides and several hormones (Eckel-Mahan and Sassone-Corsi, 2009). Many proteins that are expressed rhythmically in mammalian peripheral tissues have central roles in different metabolic processes. Examples include key rate-limiting enzymes involved in the metabolism of cholesterol [such as cholesterol 7α hydroxylase (CYP7A1)] and lipids (such as acetyl-CoA carboxylase, malate dehydrogenase and fatty acid synthase), glucose [such as phosphoenolpyruvate carboxykinase (PEPCK), glycogen phosphorylase and glycogen synthase], amino acid regulation (such as serine dehydratase) and detoxification pathways (such as cytrochrome P450 enzymes) (Froy, 2007; Kohsaka and Bass, 2007). It is intuitive that altering the cyclic pattern of clock control would result in aberrant levels of crucial metabolic enzymes, possibly leading to metabolic disorders. The regulation of CYP7A1, the rate-limiting enzyme in the synthesis of bile acid from cholesterol, represents a good example. The *Cyp7a1* gene is activated by the nuclear receptor liver X receptor (LXR) when plasma cholesterol levels are high and by the transcription factor sterol response element binding protein (SREBP) when they are low. The cyclic expression of *Cyp7a1* is mainly controlled by REV-ERBα, which drives the cyclic activation of both SREBP and LXR (Brewer et al., 2005; Le Martelot et al., 2009).

The serum levels of many hormones oscillate over a 24-hour period. For example, the concentrations of insulin (La Fleur, 2003) and glucagon (Ruiter et al., 2003) both display a daily rhythm that drives the oscillation of glucose plasma levels, which peaks at the beginning of the activity period. Whereas insulin secretion seems to be indirectly regulated by SCN-mediated control of feeding activity, glucagon and glucose levels oscillate independently of food intake. Hormones secreted from the adrenal glands, such as corticosterone and adrenaline, also show circadian patterns of expression (De Boer and Van der Gugten, 1987). Interestingly, glucocorticoids are likely to be candidates as mediators of the SCN-mediated control of peripheral clocks (Le Minh et al., 2001; Reddy et al., 2007). Glucocorticoid secretion, which peaks just before the onset of the activity period, is governed by the hypothalamus–pituitary gland–adrenal axis that is, in turn, regulated by the SCN. The function of glucocorticoids is largely exerted through binding to the glucocorticoid receptor and its subsequent interactions with glucocorticoid response elements (GREs) in the genome. A recent report suggested that many clock genes are direct GR target genes, by virtue of the presence of a GRE in their sequence (So et al., 2009). Glucocorticoids have also been found to modulate circadian resetting mediated by restricted feeding through inhibiting the uncoupling of central and peripheral clocks (Le Minh et al., 2001). Intriguingly, the glucocorticoid receptor has been recently found to be a target of CLOCK-mediated acetylation (Nader et al., 2009). Finally, other hormones that are important in regulating food intake and fat mass – such as adiponectin, leptin and ghrelin – are also known to oscillate (Ando et al., 2005; Bodosi et al., 2004).

On the basis of the findings discussed above, it is expected that disrupting the circadian clocks results in aberrant metabolic functions. Indeed, mice that lack the expression of circadian clock genes or express variant clock proteins, have abnormal metabolic phenotypes. For example, homozygous *Clock* mutant mice are hyperphagic and obese, and develop a phenotype that resembles metabolic syndrome, including features such as hyperlipidemia, hyperglycemia and hepatic steatosis (Turek et al., 2005). *Bmal1*-deficient mice have impaired insulin responsiveness, altered gluconeogenesis and suppressed diurnal variations in glucose and triglyceride levels (Marcheva et al., 2010; Rudic et al., 2004). Interestingly, fluctuation in body weight is associated with the length of the light phase (Bray and Young, 2007). Clinical and epidemiological studies in humans have shown a link between the lack of adequate sleep and metabolic disorders, and have identified sleep deprivation as an independent risk factor for obesity and hypertension (Gangwisch et al., 2005). These studies associate circadian disruption with cardiovascular and metabolic complications (Laposky et al., 2008). Furthermore, night-shift workers show higher triglyceride levels, lower HDL cholesterol levels and are more likely to be obese compared with day workers, with their body mass index directly proportional to the duration of night-shift-work (Duez and Staels, 2008).

SIRT1: a circadian enzymatic rheostat

The close relationship between metabolic control and circadian rhythm is, for the most part, based on the transcriptional regulation of a large number of CCGs by the clock machinery. When SIRT1 was identified in the search for a HDAC that counterbalanced the HAT function of CLOCK in controlling the circadian remodeling of chromatin at CCGs promoters, a molecular link was established (Asher et al., 2008; Nakahata et al., 2008). The enzymatic function of SIRT1 requires NAD^+ as a coenzyme (Imai et al., 2000; Vaziri et al., 2001), which suggests that cellular energy levels are intimately connected to the circadian cycle.

The levels of SIRT1 expression do not change significantly throughout the circadian cycle (Nakahata et al., 2008; Nakahata et al., 2009; Ramsey et al., 2009), similar to the almost constant levels of CLOCK expression in most mammalian tissues (Lee et al., 2001; Ripperger and Schibler, 2006; Yagita et al., 2001; Yamamoto et al., 2004). Given that CLOCK and SIRT1 physically interact, and that their association does not seem to undergo major daily changes (at least in the liver), it is therefore particularly interesting that SIRT1 activity is regulated in a circadian manner (Nakahata et al., 2008). This indicates that the CLOCK–SIRT1 complex most probably determines changes in the acetylation of its targets based, at least in part, on the oscillation of SIRT1 activity.

SIRT1 associates with CLOCK–BMAL1 within a chromatin complex that is recruited to CCG promoters in a circadian manner (Fig. 4). SIRT1 appears to modulate circadian gene expression by repressing transcription through its HDAC activity in a time-dependent manner, presumably by enhancing localized chromatin condensation. In fact, in *Sirt1*-null MEFs, the level of acetylated H3 Lys9 and Lys14 residues at CCGs promoters is increased in a constitutive and non-oscillating manner (Nakahata et al., 2008). Pharmacological and genetic approaches have demonstrated that lack of SIRT1 leads to a significant increase in the amplitude of transcription of circadian genes, both in cultured cells and in livers from entrained mice (Nakahata et al., 2008).

Importantly, the deacetylase activity of SIRT1 also targets non-histone circadian regulators. At least two clock regulators, BMAL1 and PER2, have been shown to be SIRT1 targets (Asher et al.,

2008; Nakahata et al., 2008). For both proteins, loss of SIRT1 results in a significant increase in acetylation levels. Mutations in BMAL1, whose CLOCK-mediated acetylation is crucial for circadian function (Hirayama et al., 2007) lead to a premature-aging phenotype in mice – which is of interest given that SIRT1, which directly deacetylates BMAL1, was shown to protect against age-related diseases in mouse models for neurological disorders (Kondratov et al., 2006; Kim et al., 2007). These findings suggest that SIRT1 functions as a modulator of the circadian machinery. We like to think that SIRT1 acts as a 'rheostat' within the clock system by controlling the balance of acetylation and chromatin remodeling by the circadian clock. Indeed, as its enzymatic activity is directly dependent on the redox state of the cell, SIRT1 might constitute a direct connection between metabolic activity, chromatin modifications and clock machinery (Fig. 4). Finally, as SIRT1 deacetylates clock proteins in a circadian manner, it can be assumed that it acts on other targets in the same way, but this remains an open question at the moment.

NAD$^+$ – a circadian metabolite

Given that SIRT1 utilizes NAD$^+$ as a coenzyme, this metabolite might have a direct role in circadian rhythm. As a coenzyme of oxidoreductases, the major function of NAD$^+$ in cell metabolism is to transfer electrons in redox reactions. Whereas NADP$^+$ is mainly used as a reducing agent in anabolic reactions, high NAD$^+$ levels favors oxidative reactions, by which nutrients are degraded to release energy in the form of ATP that can be directly used by the cell. As the cycling of NAD$^+$ in redox reactions does not change its overall levels, its consumption is mainly the result of other reactions, mostly post-translational modifications performed by NAD$^+$-dependent deacetylases (sirtuins) and mono- or poly-ribosyltransferases (Berger et al., 2004). As mentioned above, SIRT1 activity is regulated by the NAD$^+$:NADH ratio present in the cells (Lin et al., 2004). NAD$^+$ availability is essential for SIRT1 to catalyze a reaction by which NAD$^+$ is converted into nicotinamide and O-acetyl-ADP-ribose, and which removes an acetyl group from the substrate. Thus, SIRT1 has a central role in the molecular pathway that controls the intracellular levels of NAD$^+$, the NAD$^+$-salvage pathway, which is conserved from yeast to mammalian cells. Here, the enzyme nicotinamide (NAM) phospho-ribosyltransferase (NAMPT) occupies a central position as it catalyses the rate-limiting step (Revollo et al., 2004) (Fig. 5). Remarkably, the expression of the *Nampt* gene is circadian as its promoter contains E-boxes to which CLOCK, BMAL1 and SIRT1 are recruited in a circadian manner. This clock-controlled regulation leads to the circadian oscillation of NAMPT enzymatic activity and, ultimately, that of NAD$^+$ (Nakahata et al., 2009; Ramsey et al., 2009). This control system is a unique example in which the transcriptional feedback loop of the circadian clock is connected to an enzymatic cycle, the NAD$^+$-salvage pathway. SIRT1 occupies a central position as it participates in both loops (Fig. 5). Another interesting finding that arises from these studies is that SIRT1 is directly responsible for the oscillatory levels of its own coenzyme.

Changes in NAD$^+$ levels are most likely to have also other effects in addition to those on SIRT1. The DNA-binding activity of CLOCK (and its homologue NPAS2) itself appears to be regulated by cellular redox state. It has been reported that motorial activity or food availability directly and rapidly affects the CLOCK–BMAL1- (and NPAS2–BMAL1)-driven transcription, which is increased in the presence of high NADH and NADPH levels (Rutter et al., 2001). Moreover, the activity of other NAD$^+$-consuming enzymes might be crucially affected by oscillatory levels of NAD$^+$. In this context, one class of NAD$^+$-dependent enzymes, the poly-ADP-ribose polymerases (PARPs) – which are markedly activated in response to stimuli that induce a DNA-damage response (Schreiber et al., 2006) – is of special interest because of the possible connections between circadian metabolism, aging and cancer. PARP-1 binds to DNA single-strand breaks in order to exert its main function of surveying genome integrity. It is also involved in other important cellular events, including apoptosis, cell-cycle regulation and modulation of gene expression. Intriguingly, a functional interplay between SIRT1 and PARP1 has been established (Kolthur-Seetharam et al., 2006).

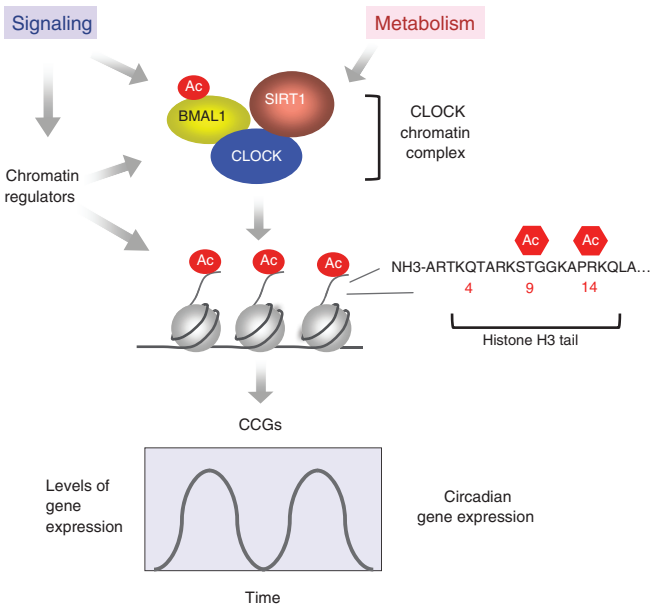

Fig. 4. The CLOCK chromatin complex. CLOCK is associated in a nuclear complex with BMAL1 and SIRT1, an HDAC that is directly regulated by cellular metabolism. This complex is recruited to circadian gene promoters in a cyclic manner and is thought to be responsible for the circadian acetylation of histone H3 at K9 and K14. A balance between HAT and HDAC functions controls the opening and closing of chromatin loci at the level of circadian gene promoters through the modification of histone acetylation levels, thus leading to cyclic gene expression. It has been predicted that other chromatin remodelers and regulators participate in the CLOCK chromatin complex, possibly in a time-dependent and tissue-specific manner.

Finally, other sensors of redox state in the cell might have a role in the circadian system. The activity of AMP-activated protein kinase (AMPK) oscillates in a circadian manner; AMPK phosphorylates CRY1, leading to its destabilization and to de-repression of circadian transcription (Lamia et al., 2009). AMPK is activated through phosphorylation by other kinases under conditions of increased intracellular AMP:ATP ratio. This typically occurs during metabolic stresses that interfere with cellular energy homeostasis by either inhibiting ATP production (low blood glucose, hypoxia) or by accelerating its consumption (muscle contraction). AMPK activation switches on catabolic pathways that produce ATP by enhancing oxidative metabolism and mitochondrial biogenesis, whereas anabolic and ATP-consuming processes – including cell growth and proliferation – are switched off. Recent studies have shown that the metabolic actions of SIRT1

and AMPK often converge. Indeed, several functional connections exist between the two pathways, for example SIRT1 acts upstream of AMPK signaling by regulating the activity of its kinase LKB1 through deacetylation. Conversely, AMPK itself enhances SIRT1 activity by increasing cellular NAD^+ levels (Canto et al., 2009).

Conclusions

How extensive is the interplay between cellular metabolism and circadian cycles? It is currently difficult to fully address this question, but accumulating evidence shows there is a mutual relationship – the clock controls some crucial metabolic pathways and metabolism feeds back to the clock machinery (Eckel-Mahan and Sassone-Corsi, 2009). In this sense, the classic scheme of the circadian pacemaker in peripheral clocks could be conceptually modified to include the potential role of metabolic outputs, such as NAD^+, in feeding back to the pacemaker and acting as adjusting signals (Fig. 6). How complex and varied this 'adjusting' activity is it is not yet understood, but we predict that it could be more extensive than currently known. One additional level of complexity is tissue specificity. It is highly probable that different sets of metabolites oscillate with varied amplitude or period in different tissues. Here, it would be highly informative to compare the scenario of the circadian regulation in different peripheral tissues with the clock function in the SCN.

Further circadian metabolomic studies should at the very least provide a more complete picture of which metabolites oscillate and when. However, a crucial challenge will be to identify and decipher the specific molecular pathways that determine each particular oscillation. We anticipate that these studies will provide valuable leads to develop successful therapeutic intervention of metabolic disorders.

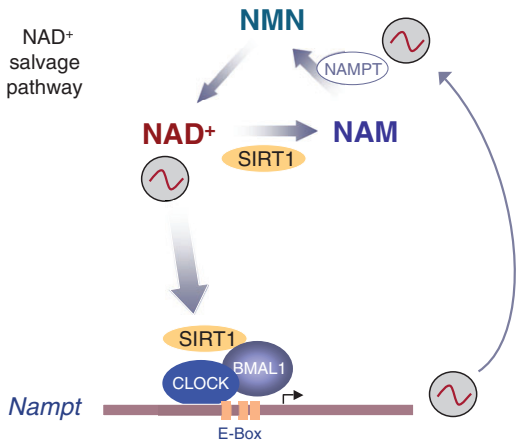

Fig. 5. The metabolite NAD^+ oscillates in a circadian manner. Recent studies (Nakahata et al., 2009; Ramsey et al., 2009) have shown that the circadian clock machinery controls the cyclic synthesis of NAD^+ through control of the NAD^+ salvage pathway. The gene encoding the enzyme NAMPT, whose transcription is the rate-limiting step in the NAD^+ salvage pathway, contains E-boxes and is controlled by CLOCK–BMAL1. A crucial step in the NAD^+ salvage pathway is controlled by SIRT1, which also contributes to the regulation of the *Nampt* promoter by associating with CLOCK–BMAL1 in the CLOCK chromatin complex (see Fig. 4). Thus, the feedback transcriptional loop of circadian regulation is closely linked to an enzymatic feedback loop. Through this regulation, SIRT1 controls the cellular levels of its own coenzyme NAD^+. NAD^+, nicotinamide adenin dinucleotide; NAM, nicotinamide; NMN, nicotinamide mononucleotide; ~, oscillation of CCGs (*Nampt*) and metabolites (NAD^+).

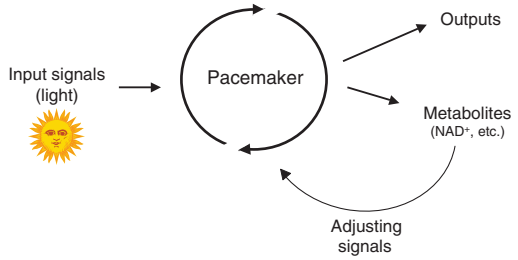

Fig. 6. Model of the circadian clock (pacemaker) and its most important input signal, light. Light signaling directly influences neurons in the central clock in the hypothalamic SCN, thereby modulating the self-sustained clock circadian regulation. The outputs of the circadian system include a large array of physiological, metabolic and neuronal functions. Disruption of clock function can cause dramatic pathophysiological effects, including neurodegeneration and cancer. Some metabolites appear to feed back to the central pacemaker and function as adjusting signals. An example for this is NAD^+, which is used by SIRT1 as coenzyme (Nakahata et al., 2009).

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