The Time of Metabolism: NAD⁺, SIRT1, and the Circadian Clock

M.M. BELLET, R. OROZCO-SOLIS, S. SAHAR, K. ECKEL-MAHAN, AND P. SASSONE-CORSI

Center for Epigenetics and Metabolism, School of Medicine, University of California, Irvine, Irvine, California 92697

Correspondence: psc@uci.edu

The mammalian cell contains a molecular clock that contributes, within each organism, to circadian rhythms and variety of physiological and metabolic processes. The clock machinery is constituted by intertwined transcriptional–translational feedback loops that, through the action of specific transcription factors, modulate the expression of clock-controlled genes. These oscillations in gene expression necessarily implicate events of chromatin remodeling on a relatively large, global scale, considering that as many 10% of cellular transcripts oscillate in a circadian manner. CLOCK, a transcription factor crucial for circadian function, has intrinsic histone acetyltransferase activity and operates within a large nuclear complex with other chromatin remodelers. CLOCK directs the cyclic acetylation of the histone H3 and of its own partner BMAL1. A search for the histone deacetylase (HDAC) that counterbalanced CLOCK activity revealed that SIRT1, a nicotinamide adenine dinucleotide (NAD⁺)-dependent HDAC, functions in a circadian manner. Importantly, SIRT1 is a regulator of several metabolic processes and was found to interact with CLOCK and to be recruited to circadian promoters in a cyclic manner. 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 among energy balance, chromatin remodeling, and circadian physiology.

Figuring out how metabolism is modulated by environmental and nutritional cues is a task of great conceptual and pharmacological interest. In this respect, an element that occupies a critical position is time. A remarkable array of fundamental physiological functions are circadian in nature, that is, they follow an approximately 24-h cycle. Remarkably, this is the case in almost all organisms, from prokaryotes and plants to humans (Cermakian and Sassone-Corsi 2000; Reppert and Weaver 2001; Bell-Pedersen et al. 2005). The circadian clocks are intrinsic time-tracking systems with which organisms can anticipate environmental changes and adapt themselves to the appropriate time of day (Reppert and Weaver 2001; Young and Kay 2001). In mammals, circadian rhythms are generated in pacemaker neurons within the suprachiasmatic nuclei (SCN) of the hypothalamus. Conceptually, the pacemaker receives input signals, such as light from the retina, and processes them into outputs. These can be the expression of clock-controlled genes (CCGs) or metabolites, which—as we propose here—may feed back on the pacemaker (Fig. 1). Disruption of these rhythms can have a profound influence on human health and has been linked to metabolic disorders, insomnia, depression, coronary heart diseases, various neurodegenerative disorders, and cancer (Sahar and Sassone-Corsi 2009; Bechtold et al. 2010). Recent years have witnessed a spectacular increase in research on biological rhythms, specifically the molecular mechanisms of the circadian clock. These studies have established some intriguing links between the clock and cellular metabolism,

endocrine control, and transcriptional and epigenetic regulation.

THE MOLECULAR ORGANIZATION OF THE CIRCADIAN CLOCK

Transcriptional–translational feedback loops constitute the organizational building blocks of the circadian clock. These are common to a number of organisms, including mammals (Young and Kay 2001; Bell-Pedersen et al. 2005), and the following core circadian clock genes have been identified in mammals: *Clock*, *Bmal1*, *casein kinase 1 epsilon (CK1ε)*, *cryptochromes 1 and 2 (Cry1, Cry2)*, *period 1, 2, and 3 (Per1, Per2, Per3)*, and *Rev-erb-α*. 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 that heterodimerize and induce the expression of *Per* and *Cry* genes via binding to E-box elements (CACGTG) present in their promoters (Reppert and Weaver 2001; Bell-Pedersen et al. 2005). Heterodimers of PER and CRY proteins translocate to the nucleus and inhibit CLOCK–BMAL1-mediated transcription through direct protein–protein interactions (Young and Kay 2001; Bellet and Sassone-Corsi 2010). Importantly, E-boxes are very frequent in the mammalian genome, hinting at the wide potential of possible regulation by the circadian machinery. Indeed, the CLOCK–BMAL1 heterodimer regulates the

Figure 1. Schematic model of the circadian clock (pacemaker) input and output signals. Light signaling directly influences neurons in the central clock in the hypothalamic suprachiasmatic nuclei, 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. Some metabolites appear to feed back to the central pacemaker and function as “adjusting signals.” This is the case for NAD^+ (nicotinamide adenine dinucleotide), which is used by SIRT1 as coenzyme (Nakahata et al. 2009). CCG, Clock-controlled genes.

transcription of many CCGs, which in turn influence a wide array of physiological functions external to the oscillatory mechanism. This mediates the output function of the clock, thereby controlling, for example, food intake, hormonal synthesis and release, body temperature, and metabolism. Indeed, many mammalian transcripts undergo circadian fluctuations in their expression levels (Akhtar et al. 2002; Duffield et al. 2002; Panda et al. 2002).

How can the clock have such a profound impact on physiology? Genome-wide array analyses indicate that at least 10% of all expressed genes in any tissue are under circadian regulation (Masri and Sassone-Corsi 2010; Hogenesch and Ueda 2011). This high proportion of circadian transcripts suggests that the clock machinery may direct widespread events of cyclic chromatin remodeling and consequent transcriptional activation/repression. Furthermore, genome-wide studies comparing the central SCN pacemaker and peripheral tissues, such as the liver, revealed that between 5% and 10% of cycling genes were identical in both tissue types (Akhtar et al. 2002; Panda et al. 2002). A recent analysis covering 14 mouse tissues identified approximately 10,000 known genes showing circadian oscillations in at least one tissue (Yan et al. 2008). Not surprisingly, the number of common genes showing circadian oscillation in multiple tissues decreased drastically as the number of tissues included in the comparative analysis increased, with only 41 genes displaying circadian oscillation in at least eight out of 14 tissues (Yan et al. 2008). These findings suggest that the core clockwork, which can be assumed to be common to all tissues, interplays with cell-specific transcriptional systems (Masri and Sassone-Corsi 2010).

Finally, in addition to the transcripts that show the approximately 24-h-based oscillation, hundreds of CCG transcripts exist in the liver cycle at the second and third harmonic of circadian oscillations (periods of 12 and 8 h). Both circadian and ultradian transcripts are severely

dampened in ex vivo cellular cultures, whereas in vivo their expression is shifted if the animals are under restricted feeding (Hughes et al. 2009). Thus, both circadian and ultradian transcripts are responsive to systemic cues in vivo, highlighting the critical position of peripheral clocks. These operate indeed as the natural interfaces between nutritional cues and physiology to ensure plasticity and accurate responsiveness of organisms.

CIRCADIAN METABOLISM AND PERIPHERAL CLOCKS

One of the critical discoveries in the field, made about a decade ago, was the finding of independent clocks in peripheral tissues of various organisms (Whitmore et al. 1998; Giebultowicz et al. 2000; Stokkan et al. 2001; Schibler and Sassone-Corsi 2002). This finding had several implications. First, what signal links the central pacemaker to the peripheral clocks, and how independent are the peripheral tissues? It is generally accepted that there are specific humoral signals involved in synchronizing the circadian system, but a comprehensive understanding is still lacking. Another key question has been, How different is the molecular organization of the peripheral clocks versus the central SCN clock? These, and several other questions of fundamental endocrine and physiological significance, remain open. Some important features have, however, been established: The oscillatory function of peripheral clocks in mammals is orchestrated by the SCN (Schibler and Sassone-Corsi 2002). Differently from the SCN, peripheral tissues appear to require physiological stimuli to sustain their circadian rhythms. Thus, it is likely that peripheral clocks are affected by physiological stimuli that may originate from the SCN and/or may be the result of SCN-mediated messages. Indeed, growth factors, some steroids such as glucocorticoids, and retinoic acid have been shown to induce oscillations of clock genes and clock-controlled genes in cultured fibroblasts or peripheral tissues (Le Minh et al. 2001; Preitner et al. 2003). These observations are likely to have physiological relevance because restricted access to food has an effect on peripheral rhythms without affecting the central pacemaker function of the SCN (Schibler and Sassone-Corsi 2002; Preitner et al. 2003). The discovery of independent clocks in peripheral tissues revealed the intrinsic metabolic nature of the circadian system and its homeostatic control.

CIRCADIAN REMODELING OF CHROMATIN

The transcriptional control of a significant proportion of the genome by the clock invokes genome-wide mechanisms of chromatin remodeling. Indeed, the highly specialized, temporally based regulation of transcription that characterizes circadian rhythms elects the cellular clock as a prominent model for the study of dynamic regulations of chromatin remodeling (Crosio et al. 2000; Borrelli et al. 2008; Zocchi and Sassone-Corsi 2010). Finally, because circadian rhythms are tightly coupled to physiological and metabolic control (Rutter et al.

2002), clock-controlled chromatin reorganization is likely to reveal as yet unexplored pathways linking histone modifications to cellular metabolism.

The first evidence linking circadian gene expression to dynamic events of chromatin remodeling was obtained by studying histone modifications in SCN neurons in response to a light stimulus (Crosio et al. 2000). Subsequent studies indicated that activation of CCGs by CLOCK–BMAL1 is associated with circadian changes in histone modifications at their promoters (Etchegaray et al. 2003; Curtis et al. 2004; Naruse et al. 2004), showing that transcription-permissive chromatin states are dynamically established in a circadian-time-specific manner. Specifically, histone H3 is acetylated in chromatin, which encompasses the *Per1*, *Per2*, and *Cry1* promoters when these genes are actively transcribed (Doi et al. 2006; Ripperger and Schibler 2006). In addition, H3 Ser-10 phosphorylation is involved in the transcriptional response to light in the SCN. Thus light-mediated signaling leads to nuclear responses that in turn influence the state of higher chromatin organization (Crosio et al. 2000). Moreover, the coupled modification of Ser10 phosphorylation and Lys14 acetylation on H3 has been shown to be a hallmark of transcriptional activation (Cheung et al. 2000; Lo et al. 2000), a notion that underscores the implication of histone acetyltransferases (HATs) in circadian gene expression (Masri and Sassone-Corsi 2010). Finally, histone methylation may be important for circadian clock function, as indicated by RNA interference (RNAi) experiments in cultured fibroblasts. The Polycomb group protein EZH2 and methylation at H3 Lys27 are associated with *Per* gene promoters, extending the activity of the Polycomb group proteins to the core clockwork mechanism of mammals (Etchegaray et al. 2006).

Another study of the protein CLOCK revealed that, in addition to operating as a transcription factor in combination with BMAL1, it has the enzymatic properties of an acetyltransferase (Doi et al. 2006). The carboxy-terminal region of CLOCK displays a significant sequence homology with the carboxy-terminal domain of ACTR, a domain previously described to have intrinsic HAT activity (Chen et al. 1997). In this region, at least six independent amino acid regions are found to share significant sequence similarity between the two proteins. Importantly, the amino acid residues common to CLOCK and ACTR are evolutionarily conserved in both proteins. It is also noteworthy that CLOCK and ACTR share a number of other structural features outside of the carboxy-terminal glutamine-rich region, including a putative NRID (nuclear receptor interaction domain). Using HAT-in-gel assays, it was demonstrated that CLOCK indeed has intrinsic HAT activity directed toward K9/K14 of histone H3 (Doi et al. 2006) and that it is also able to acetylate nonhistone proteins, specifically its partner BMAL1 (Hirayama et al. 2007). The question of whether CLOCK may be able to acetylate other nonhistone proteins is important, as it may connect its enzymatic, circadian function to a number of cellular pathways. In this respect, the finding that CLOCK acetylates the glucocorticoid receptor the (GR) could be highly relevant (Nader

et al. 2009). CLOCK acetylates the GR at multiple lysines in the hinge region, resulting in inhibition of GR binding to DNA. Because glucocorticoids have been implicated in a variety of circadian responses, and are good candidates as possible mediators of the SCN control on the peripheral clocks, these observations could have remarkable physiological importance. Indeed, glucocorticoid secretion peaks just before the onset of the activity period and it is classically regulated by the hypothalamus–pituitary–gland–adrenal axis, which in turn is regulated by the SCN.

The HAT domain of CLOCK contains a stretch of amino acids that shares significant similarity to the so-called “motif A” in the HAT family, denominated MYST (for its founding members MOZ, Ybf/2Sas3, Sas2, and Tip60) (Doi et al. 2006). This is important because highly specific interactions have been described between nuclear receptors (NRs) and proteins with a MYST-type of “motif A,” such as the androgen receptor and the HBO1 protein. Thus, it is conceivable that, in addition to the GR, CLOCK may acetylate other NRs.

SIRT1, A DEACETYLASE WITH CIRCADIAN ACTIVITY

Circadian rhythms and changes in cellular energy state seem to be tightly linked, as suggested by the abnormal metabolic phenotypes displayed by mice mutant for clock genes (Eckel-Mahan and Sassone-Corsi 2009). A genome-wide RNAi approach has revealed that reduced circadian amplitude can be caused by individual knock-down of approximately 1000 genes (Zhang et al. 2009). Importantly, pathway analysis indicates that these genes encode components of the insulin and hedgehog signaling, cell cycle, and folate metabolism. Thus, the clock machinery is interconnected with a number of cellular functions. In this respect, the search for the HDAC (histone deacetylase), that would physiologically counterbalance the enzymatic activity elicited by CLOCK, revealed an intriguing twist.

HDAC-mediated deacetylation of histones correlates with gene silencing (Berger 2007), and several HDACs have been implicated in the reversible acetylation of non-histone proteins, including p53, Hsp90, MyoD, and E2F1 (Glozak et al. 2005). Four classes of mammalian HDACs exist, based on their structure and regulation (Yang and Seto 2008). Class III is composed of seven mammalian enzyme homologs of yeast Sir2 (silencing information regulator) and are known as SIRT1 to SIRT7. These proteins are structurally distinct from the other HDACs and have the property of dynamically sensing energy cellular metabolism using NAD⁺ (nicotinamide adenine dinucleotide) as a coenzyme (Bordone and Guarente 2005). In this reaction, nicotinamide is liberated from NAD⁺ and the acetyl group of the substrate is transferred to cleaved NAD⁺, generating the metabolite *O*-acetyl-ADP ribose (Sauve et al. 2006). Owing to the NAD⁺ dependency, SIRT1s are thought to constitute a functional link between metabolic activity and genome stability and, possibly, aging (Bordone and Guarente 2005).

SIRT1, the mammalian ortholog of Sir2, is a nuclear protein that occupies a privileged position in the cell and governs critical metabolic and physiological processes. SIRT1 helps cells to be more resistant to oxidative or radiation-induced stress, promotes mobilization of fat from white adipose tissues, and mediates the metabolism of energy sources in metabolically active tissues (Bordone and Guarente 2005). Thus, the finding that SIRT1 acts as a “rheostat” to modulate CLOCK-mediated acetylase activity and circadian function established an intriguing molecular link (Nakahata et al. 2008). SIRT1 was found to associate with CLOCK and to be recruited at circadian promoters, whereas *Sirt1* genetic ablation or pharmacological inhibition of SIRT1 activity leads to significant disturbances in the circadian cycle. Importantly, whereas the protein levels of SIRT1 do not oscillate, as analyzed in several tissues and under various experimental conditions (Fig. 2A) (Nakahata et al. 2008), its enzymatic activity oscillates in a circadian manner (Fig. 2B), with a peak that corresponds to the lowest level of H3 acetylation at various CCGs (Nakahata et al. 2008, 2009). This may also be due to a role of SIRT1 in controlling the stability of some clock proteins, such as PER2 (Asher et al. 2008). Thus, it is possible that SIRT1 contributes to transduce signals originated by cellular metabolites to the circadian machinery (Nakahata et al. 2008). Some further evidence in this direction was obtained when it was found that the levels of NAD^+ oscillate in a circadian manner in serum-entrained mouse embryonic fibroblasts (MEFs) and in mice livers (see below; Nakahata et al. 2009).

Sirtuins, and SIRT1 in particular, establish a variety of functional interactions with other proteins implicated in cellular metabolism and signaling (Bordone and Guarente

2005). At the level of chromatin, SIRT1 enzymatic activity targets preferentially histone H3 at Lys9 and Lys14, and histone H4 at Lys16 (Imai et al. 2000). In addition, a number of nonhistone proteins are regulated by SIRT1-mediated deacetylation, including p53, FOXO3, PGC-1 α , and LXR (Sauve et al. 2006), stressing the pivotal function that this regulator plays in cellular control and responses. We have found that SIRT1 readily deacetylates BMAL1, contributing to its cyclic acetylation levels (Hirayama et al. 2007; Nakahata et al. 2008, 2009). We have generated an antibody that recognizes BMAL1 only when acetylated at the unique Lys537 residue. This anti-Ac-BMAL1 antibody demonstrates that acetylation is induced by CLOCK and that it is highly specific, as demonstrated using a K537R mutant (Fig. 3A,B). Using this antibody we could show that BMAL1 deacetylation occurs in the presence of both SIRT1 and NAD^+ and is inhibited by nicotinamide (Fig. 3C). In a series of additional experiments, it was demonstrated that acetylation is circadian and it controls the efficient repression by CRY proteins (Hirayama et al. 2007). These findings have been recently corroborated by quantitative analyses of the BMAL1-CRY interaction by fluorescence polarization and isothermal titration calorimetry (Czarna et al. 2011).

Recent results have established an interdependence between SIRT1 and the nutrient-responsive adenosine monophosphate-activated protein kinase (AMPK), which contributes to the metabolic adaptation to fasting and exercise in skeletal muscle (Canto et al. 2010). Importantly, AMPK was found to play a role in the control of circadian rhythms by phosphorylating and inducing the destabilization of CRY1 in a cyclic manner (Lamia et al. 2009). Stimulation of AMPK signaling alters circadian

Figure 2. The levels of SIRT1 do not cycle, but its deacetylase activity does. (A) To assess whether SIRT1 protein levels oscillate in a circadian manner, nuclear extracts from mouse liver were prepared following various methods. Western blot (WB) analyses using specific antibodies demonstrated that SIRT1 levels displayed marginal or no oscillation. Nuclear extracts (NE) were prepared following, using a nuclear lysis buffer containing 450 mM NaCl (high salt) or 150 mM NaCl (low salt), following the method by Andrews and Fallar (1991); alternatively, nuclear extracts were prepared using the method of Gorski et al. (1986). Nuclei were purified over a sucrose cushion and the resulting nuclear pellet was extracted. Nuclei were lysed with ammonium sulfate and chromatin was centrifuged out. Nuclear proteins were precipitated by addition of 0.3 g/mL solid ammonium sulfate. Nuclear extracts were also prepared using the commercially available kit NE-PER Nuclear and Cytoplasmic Extraction Reagents (PIERCE Biotechnology). In all of these conditions, the SIRT1 protein did not show oscillation (see also Nakahata et al. 2008). (B) Mouse liver total extracts were used to immunoprecipitate SIRT1. SIRT1 levels were noncyclic in total extract at different times of the circadian cycle (*lower panel*). The same immunoprecipitates were subjected to deacetylase enzymatic assays as described (Nakahata et al. 2009). The histone deacetylase (HDAC) activity of SIRT1 followed a circadian profile.

Figure 3. A specific antibody that recognizes BMAL1 only when it is acetylated at K537. (A) We have raised a polyclonal antibody against an 11 amino acid peptide in which K537 is uniquely acetylated. The antibody has been successfully tested in western analysis, immunoprecipitations, and immunohistochemistry. (B) Results of a transfection in 293 cells in which CLOCK, BMAL1, or a BMAL1(K537R) mutant is ectopically expressed. Western analysis shows that the antibody specifically recognizes BMAL1 when it is acetylated and that CLOCK elicits this event (Hirayama et al. 2007). (C) SIRT1 deacetylates BMAL1(K537) in vitro. Acetylated BMAL1 was prepared from HDAC inhibitor-treated JEG3 cultured cells transfected with Flag-Myc-BMAL1 and Myc-CLOCK. Recombinant SIRT1 and deacetylation buffer were used from the SIRT1 Fluorimetric Activity Assay/Drug Discovery Kit (AK-555; BIOMOL International). Immunoprecipitated (IP) AcBMAL1 and recombinant SIRT1 were incubated in deacetylation buffer with 5 mM NAD⁺ or 10 mM nicotinamide (NAM) for 90 min at 37°C. SIRT1 is able to deacetylate BMAL1 only in the presence of NAD⁺.

rhythms, and mice in which the AMPK pathway is genetically disrupted show alterations in peripheral clocks. Thus, it appears that phosphorylation by AMPK enables CRY1 to transduce nutrient signals to circadian clocks in mammalian peripheral organs. How SIRT1, and possibly NAD⁺ oscillations, may be involved in this control system is as yet unclear.

NAD⁺, AN OSCILLATING METABOLITE

The involvement of SIRT1 in circadian regulation demonstrated a direct link between cyclic rhythms and energy metabolism in the cell (Asher et al. 2008; Nakahata et al. 2008). Yet, analyses along the circadian cycle in various cell types demonstrated that the expression levels of the SIRT1 gene and protein are noncyclic (Fig. 2) (Nakahata et al. 2008; Ramsey et al. 2009). On the contrary, the HDAC activity of the enzyme is circadian, indicating that some other sort of regulation—unrelated to protein levels—had to intervene to regulate SIRT1 function (Fig. 2). Thus the question we had was, How can two enzymes that are mostly expressed in a nonoscillatory manner lead to circadian acetylation of K9/K14 in histone H3 and K537 in BMAL1? (Fig. 4). This apparent discrepancy was solved by subsequent studies that revealed that NAD⁺ levels oscillate in a circadian fashion in all cell types analyzed and that it is through the cyclic availability of its own coenzyme that SIRT1 HDAC activity is circadian (Nakahata et al. 2009; Ramsey et al. 2009). The circadian regulation of NAD⁺ synthesis is itself conceptually remarkable because it links the transcriptional feedback loop of the circadian clock to an enzymatic pathway.

A major enzymatic loop that operates in most cells is the NAD⁺ salvage pathway. Enzymes such as SIRT1 and poly(ADP-ribose) polymerase-1 (PARP-1) heavily

use NAD⁺ as coenzyme, risking depletion of the intracellular stores, which can lead to cell death. Thus, levels of NAD⁺ need to be controlled even in the absence of de novo biosynthesis through nutritional pathways. The NAD⁺ salvage pathway allows nicotinamide (NAM), the

Figure 4. What leads to cyclic acetylation if CLOCK and SIRT1 levels do not oscillate? The levels of K9/K14 H3 acetylation at the *Dbp* promoter monitored by chromatin immunoprecipitation (Nakahata et al. 2008) and of BMAL1 as monitored using the anti-AcBMAL1 antibody (see Fig. 3) oscillate in a circadian manner. These events are elicited by a complex interplay of circadian regulators, in which CLOCK and SIRT1 appear to have a central role. Ultimately, circadian gene expression (*lower panel*) may not depend exclusively on proteins but also on the circadian oscillation of metabolites, such as NAD⁺ (Nakahata et al. 2009; see also Fig. 5).

by-product of enzymes that use NAD^+ as coenzyme, to be reconverted into NAD^+ via the use of a group of nicotinamide mononucleotide adenylyltransferases (NMNATs) and the nicotinamide phosphoribosyltransferase enzyme NAMPT. Importantly, NAMPT is the rate-limiting step enzyme within the NAD^+ salvage pathway (Nakahata et al. 2009; Ramsey et al. 2009). Thus, changes in NAMPT activity will directly dictate the levels of intracellular NAD^+ .

The rhythmicity of NAD^+ levels parallels the antiphasic oscillation in NAM, both oscillations being abolished in cells with a mutation in the circadian machinery (Nakahata et al. 2009). Thus, the clock directs the oscillation of critical metabolites. Because SIRT1 associates with and modulates CLOCK–BMAL1 (Nakahata et al. 2008), this suggested the presence of an enzymatic feedback loop, in which CLOCK–BMAL1 would control its own activity by directing the oscillatory synthesis of NAD^+ . This was demonstrated to indeed be the case. The regulatory region of the *Nampt* gene contains two E-box promoter elements, known to bind CLOCK–BMAL1. Additional experiments demonstrated that the expression of the *Nampt* gene is indeed controlled by CLOCK–BMAL1 in a complex that contains SIRT1. Thus, SIRT1 is present in both the transcriptional regulatory loop of the *Nampt* gene and the NAD^+ enzymatic salvage pathway. This two-ways control results in the circadian

expression of the *Nampt* gene, a circadian function of the NAD^+ salvage pathway and thereby in a circadian synthesis of NAD^+ . Importantly, the use of FK866, a highly specific NAMPT pharmacological inhibitor, abolished NAD^+ circadian oscillations and thereby SIRT1 cyclic activity (Nakahata et al. 2009). This finding is of interest because FK866 is used to control cell death in human cancer tissues. Thus, in addition to revealing a critical enzymatic circadian cycle, these results suggest that a direct molecular coupling exists among circadian clock, energy metabolism, and cell survival.

Thus, the circadian clock is directly implicated in controlling the intracellular levels of critical metabolites, generating an interlocking of the transcriptional feedback clock loop with the enzymatic feedback loop of the NAD^+ salvage pathway (Fig. 5). This view is confirmed by recent results using mice deficient in CD38, a NAD^+ hydrolase, which display NAD^+ levels elevated during most of the circadian cycle. CD38-null mice show altered circadian rhythmicity, CCG expression, and aberrant metabolism (Sahar et al. 2011). The oscillation of NAD^+ could have important consequences for cellular physiology, including changes in chromatin remodeling and downstream molecular pathways. Thus, a direct link between circadian control and metabolic regulation exists, revealing that SIRT1 is implicated in controlling the

Figure 5. The metabolite NAD^+ oscillates in a circadian manner. The circadian clock machinery controls the cyclic synthesis of NAD^+ through control of the NAD^+ salvage pathway (Eckel-Mahan and Sassone-Corsi 2009; Nakahata et al. 2009; Ramsey et al. 2009). The gene encoding the enzyme NAMPT, 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. Thus, the feedback transcriptional loop of circadian regulation is intimately linked to an enzymatic feedback loop. Through this regulation, SIRT1 controls the cellular levels of its own coenzyme NAD^+ . (NAD^+ , nicotinamide adenine dinucleotide; NAM, nicotinamide; NMN, nicotinamide mononucleotide; ~, oscillation of CCGs [*Nampt*] and metabolites [NAD^+].) Other connections with critical metabolic pathways are also indicated.

cellular levels of its own coenzyme. It would be critical to understand how other sirtuins may contribute to circadian rhythms.

CONCLUSION

Evidence accumulating in the past few years depicts a scenario of increasing functional and molecular connections among cellular metabolism, circadian clock, and epigenetic control. The conceptual significance of these findings relates to how cells interpret the environment, by modifying their genomic response and possibly establishing a “memory” of recurrent nutritional and environmental cues. In this context, it is the chromatin landscape that most likely functions as molecular substrate of where to “write” or “erase” specific marks by posttranslational modifications of DNA and/or histones (Borrelli et al. 2008). Thus, we hypothesize that the chromatin remodelers (“writers” and “erasers” of posttranslational modifications) specifically implicated in circadian control of the genome play a central role in translating changes in cellular metabolism into epigenetic regulation. As described in this chapter, CLOCK and SIRT1 appear to occupy a central position as circadian regulators of protein acetylation (Masri and Sassone-Corsi 2010). The recent finding that the histone H3-K4 methyltransferase MLL1 controls the chromatin recruitment to circadian promoters of the CLOCK–BMAL1 complex extended the reasoning to histone methylation (Katada and Sassone-Corsi 2010). Indeed, MLL1 associates with CLOCK–BMAL1 only at specific circadian times, dictating their circadian function on CCGs. It would be highly interesting to unravel whether changes in the intracellular levels of *S*-adenosyl methionine, a major source of methyl groups for methyltransferases, could determine whether MLL1 circadian function can be linked to the changing levels of a metabolite. In this sense, variable availability of *S*-adenosyl methionine, could function as NAD⁺, linking the metabolite to gene expression, through the modulation of an epigenetic regulator. In our view, the circadian transcriptome and proteome are oscillating in concert, and in a coherent manner with the metabolome, in a system where the circadian machinery occupies a central place, providing the tempo for synchronicity.

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M.M. Bellet, R. Orozco-Solis, S. Sahar, et al.

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