The Epigenetic and Metabolic Language of the Circadian Clock

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Abstract The circadian clock controls a large variety of neuronal, endocrine, behavioral and physiological responses in mammals. This control is exerted in large part at the transcriptional level on genes expressed in a cyclic manner. A highly specialized transcriptional machinery based on clock regulatory factors organized in feedback autoregulatory loops governs a significant portion of the genome. These oscillations in gene expression are paralleled by critical events of chromatin remodeling that appear to provide plasticity to circadian regulation. Specifically, the NAD^+-dependent deacetylases SIRT1 and SIRT6 have been linked to circadian control of gene expression. This and additional accumulating evidence shows that the circadian epigenome appears to share intimate links with cellular metabolic processes and has remarkable plasticity, showing reprogramming in response to nutritional challenges. In addition to SIRT1 and SIRT6, a number of chromatin remodelers have been implicated in clock control, including the histone H3K4 tri-methyltransferase MLL1. Deciphering the molecular mechanisms that link metabolism, epigenetic control and circadian responses will provide valuable insights towards innovative strategies of therapeutic intervention.

Introduction

Metabolism, homeostatic balance and behavior follow the 24-h daily cycle (Eckel-Mahan and Sassone-Corsi 2013). Circadian rhythms are virtually present in all life forms on our planet, including mammals, insects, plants, fungi and cyanobacteria. In higher organisms, circadian rhythms have evolved into a complex physiological and molecular system demonstrated by sleep-wake cycles, daily fluctuations in body temperature, blood pressure, cellular regeneration and behavior such as food

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intake and alertness levels (Asher and Sassone-Corsi 2015). Metabolism, nutritional intake and body homeostasis are also under circadian control, displaying rhythms in the levels of circulating hormones and metabolites, as well as enzymes within the biochemical pathways participating in their biosynthesis (Eckel-Mahan and Sassone-Corsi 2013; Gamble et al. 2014). Circadian rhythms are so intimately linked to biological processes that their misregulation may lead to a number of pathologies such as obesity, metabolic syndrome, diabetes, cardiovascular diseases, inflammation, sleep disorders and some cancers (Eckel-Mahan and Sassone-Corsi 2013).

The molecular bases of circadian rhythms have been explored, revealing a remarkable variety of molecular mechanisms that underlie clock function. An important system of circadian control utilizes the core clock molecular machinery that consists of transcription factors and regulators, both activators and repressors, that act in concert to drive circadian expression of an important fraction of the genome. A number of high-throughput transcriptome profiling studies have established that 15–30 % of all transcripts are controlled by the clock, depending on the tissue or cell type (Duffield et al. 2002; Panda et al. 2002; Storch et al. 2002; Ueda et al. 2002). Accumulating evidence has shown that this global program of gene expression is achieved through events of cyclic chromatin remodeling and epigenetic control.

Chromatin Remodeling, Cyclic Transcription and the Clock

The molecular organization of the circadian system relies on a network of cellular oscillators present in virtually every cell of the organism. An intricate network of transcriptional-translational feedback loops constitutes the molecular clock (Eckel-Mahan and Sassone-Corsi 2013; Zhang and Kay 2010). The basic helix-loop-helix (b-HLH)-PAS proteins CLOCK and BMAL1 are core elements of this system and function as transcriptional activators to drive the expression of many clock-controlled genes (CCGs). CLOCK and BMAL1 heterodimers bind E-boxes in CCG promoters and activate their expression. Among the CCGs there are genes encoding other core clock protein repressors Period (PER1-3) and Cryptochromes (CRY1-2). PER and CRY proteins heterodimerize in the cytoplasm and translocate to the nucleus to inhibit CLOCK:BMAL1-mediated transcription. The stability of PER:CRY complexes is regulated by posttranscriptional modifications (Lee et al. 2009) and ubiquitination events (Busino et al. 2007; Hirano et al. 2013; Siepka et al. 2007; Yoo et al. 2013). The time-controlled clearance of the repressors primes for a the next cycle of CLOCK:BMAL1-driven gene activation. This system then leads to the cyclic activation of other regulatory pathways generating interconnected transcriptional feedback loops. These provide remarkable plasticity to the circadian system, eliciting multiple daily oscillations in the transcriptome (Masri and Sassone-Corsi 2010).

*S*pecific cyclic chromatin transitions occur in a genome-wide scale and are associated with circadian waves of transcription (Masri and Sassone-Corsi 2010). Several chromatin remodelers have been found to be involved in circadian control. The protein CLOCK was found to operate as an acetyltransferase on histone H3 at K9 and K14 (Doi et al. 2006), modifications associated with a chromatin state permissive for transcription. CLOCK acts in concert with other histone acetyltransferases (HATs) (Etchegaray et al. 2003), such as CBP (CREB binding protein), p300 and with the CBP-associated factor PCAF (Lee et al. 2010; Curtis et al. 2004; Takahata et al. 2000). A number of histone deacetylases (HDACs) have been found to counterbalance these HATs. For example, the circadian repressor PER recruits SIN3A-HDAC1 (Duong et al. 2011), whereas the protein CRY1 associates with the complex SIN3B-HDAC1/2 (Naruse et al. 2004). The circadian regulator REV-ERBα recruits the NCoR-HDAC3 complex in a rhythmic manner to chromatin via a process that has been linked to the control of lipids metabolism in the liver (Sun et al. 2011). Thus, a variety of circadian repressive complexes appear to exist that may elicit distinct functions at unique times of the circadian cycle. The nicotinamide adenine dinucleotide (NAD^+)-dependent class III of HDACs was found to play a critical role in connecting cellular metabolism to circadian physiology. The founding member, SIRT1, gives the name to this class of enzymes, collectively known as sirtuins. There are seven sirtuins, all involved in various aspects of metabolism, inflammation and aging; their intracellular localization is nuclear, cytoplasmic or mitochondrial. The nuclear proteins SIRT1 and SIRT6 have been shown to contribute to circadian transcription (Nakahata et al. 2008; Masri et al. 2014).

A number of chromatin post-translational modifications have been linked to clock function in addition to acetylation. The first evidence that a histone modification may play a role in circadian transcription was the light-inducible phosphorylation at H3-S10 in SCN neurons (Crosio et al. 2000). The activating histone methylation H3K4me3 has also been linked to clock control and it seems to be essential to permit circadian chromatin transitions that lead to activation of CCG expression (Ripperger and Schibler 2006). MLL1, a H3K4 histone methyltransferase (HMT), was shown to elicit CLOCK:BMAL1 recruitment to chromatin at specific circadian promoters and for the cyclic tri-methylation at H3K4 (Katada and Sassone-Corsi 2010). Also the repressive mark H3K27me3 is clock controlled at the *Per1* promoter through a mechanism that involves the methyltransferase EZH2 (Etchegaray et al. 2006). Additional chromatin remodelers involved in circadian function include the demethylase JARID1a that appears to inhibit HDAC1, thereby enhancing CLOCK:BMAL1-mediated transcription (DiTacchio et al. 2011), and the FAD (Flavin Adenine Dinucleotide)-dependent demethylase LSD1 whose function is controlled by PKCα-mediated circadian phosphorylation (Nam et al. 2014).

Cellular Metabolism and the Circadian Clock Converge

A large number of human studies and animal models provide solid evidence of the reciprocal regulation between the circadian clock and cellular and organismal homeostasis (Eckel-Mahan and Sassone-Corsi 2013; Dallmann et al. 2012; Eckel-Mahan et al. 2012, 2013; Hatori et al. 2012; Kasukawa et al. 2012). The clock regulates metabolism by controlling the expression of a large fraction of the genome. Moreover, the oscillator appears to sense the cellular energy state and consequently adapts its function accordingly.

Several levels of interplay exist between cellular metabolism and chromatin remodeling (Masri and Sassone-Corsi 2010; Feng and Lazar 2012; Katada et al. 2012). Acetylation of histones or non-histone nuclear proteins depends on the supply of acetyl-CoA in the nuclear compartment. The main carbon source in mammals is glucose, which generates acetyl-CoA because of the enzyme adenosine triphosphate (ATP)-citrate lyase (ACLY). ACLY protein levels are cyclic in the liver (Mauvoisin et al. 2014), and ACLY activity controls global histone acetylation depending on glucose availability (Wellen et al. 2009). Thus, circadian changes in histone acetylation are controlled not only by specific HATs but also by interconnected metabolic pathways and enzymes supplying nuclear acetyl-CoA. A similar regulation involves S-adenosyl methionine (SAM), the metabolite used by methyltransferases to deliver methyl groups. Changing SAM levels directly influence H3K4me3 levels in mouse pluripotent stem cells (Shyh-Chang et al. 2013). Also, treatment with 3-deazaadenosine (DAA), an inhibitor of SAH (S-adenosylhomocysteine) hydrolysis that hinders transmethylation, elongates the circadian period (Fustin et al. 2013). Further research is necessary to decipher the impact of one carbon metabolism in the circadian transcriptome.

Nicotinamide adenine dinucleotide (NAD^+) is a pivotal metabolite for the circadian epigenome. NAD^+ shows robust diurnal rhythms in synchronized cells and mice (Bellet et al. 2013; Nakahata et al. 2009; Ramsey et al. 2009), and operates as a cofactor for class III of HDACs, the sirtuins (see next section).

The core machinery may be directly influenced by changing metabolic states. Specifically, the DNA-binding function of NPAS2:BMAL1 and CLOCK:BMAL1 heterodimers was shown to be influenced by the redox states of NAD(H) or NADP (H) (Rutter et al. 2001). This finding implied that CLOCK:BMAL1 transcriptional activity should be sensitive to the levels of cellular redox. While a causal evidence for this regulation has not been explored, circadian oscillations in intracellular redox potentials are evolutionary conserved (Eckel-Mahan and Sassone-Corsi 2013; Asher and Sassone-Corsi 2015). Thus, while the ability of NPAS2 or CLOCK to sense the intracellular redox state in vivo remains to be proven, independent evidence provides interesting information. Indeed, crystallographic analyses of the CRY1-PER2 complex indicate that a disulfide bond between two cysteine residues in CRY1 weakens its interaction with PER2, whereas a reduced state of CRY1 stabilizes the complex and facilitates transcriptional repression (Schmalen et al. 2014). In this scenario, CRY2 would retain specific FAD (Flavin

Adenine Dinucleotide) binding activity, and FAD competes for CRY2 binding pocket with the ubiquitin ligase complex SCF^{FBXL3}, which has been shown to control period length by regulating CRYs stability (Xing et al. 2013). Interestingly, this finding provides a possible approach to pharmacologically adjust circadian period length by using small molecules resembling FAD (Hirota et al. 2012).

Posttranslational modifications of clock proteins have been shown to modify their regulatory capacity. For example, CLOCK, BMAL1 and PER2 can be O-linked *N*-acetylglucosamine (GlcNAc)-modified by the enzyme O-GlcNAc transferase (OGT), which results in a change in their activities (Kaasik et al. 2013; Li et al. 2013). Importantly, liver-specific ablation of OGT leads to dampened oscillation of *Bmal1* and gluconeogenic genes. Thus, glucose levels dictate the availability of GlcNAc, OGT serving as a signal transducer between cellular metabolism and circadian components. Along the same lines, phosphorylation of CRY1 by the nutrient sensor kinase AMPK (AMP-activated protein kinase) connects cellular energy levels with the circadian clock by adjusting it to the changing intracellular ratio of AMP/ATP (Jordan and Lamia 2013; Gomes et al. 2013).

The Central Role of Sirtuins

The intracellular availability in time and space of specific metabolites constitutes an intriguing level of control for their protein sensors (Katada et al. 2012). In this respect, the circadian oscillation in NAD^+ concentration represents a revealing paradigm. The NAD^+ biosynthetic salvage pathway controls the conversion of nicotinamide (NAM) to β-nicotinamide mononucleotide (NMN); this step is catalyzed by a rate-limiting step enzyme, the nicotinamide phosphoribosyltransferase (NAMPT, also known as visfatin). The circadian machinery controls the transcription of the *Nampt* gene through direct binding of CLOCK:BMAL1 to E-boxes in the promoter (Nakahata et al. 2009; Ramsey et al. 2009). NMN is converted to NAD^+ by the enzymes nicotinamide mononucleotide adenylyltransferase 1-3 (NMNAT1-3) (Fig. 1). Thus, a transcriptional-enzymatic feedback loop controls NAD^+ biosynthesis and availability that in turn could result in circadian function of a variety of NAD^+-dependent enzymes. Moreover, there is a differential regulation of NAD^+ levels and NAD^+-consuming enzymes in various cell compartments (Gomes et al. 2013; Yang et al. 2007). In this respect the sirtuins deserve special attention. Indeed, of the seven mammalian sirtuins, three (SIRT1, SIRT3 and SIRT6) have been functionally linked to circadian control and found to modulate cyclic outputs in response to metabolic cues.

SIRT3 is a mitochondrial enzyme that displays robust changes in its deacetylase activity in response to NAD^+ levels (Hebert et al. 2013; Peek et al. 2013; Masri et al. 2013). SIRT3 controls mitochondrial function, including fatty acid oxidation and intermediary metabolism, by directly targeting rate-limiting enzymes for mitochondrial biochemical processes (Peek et al. 2013). As mitochondrial fatty acid

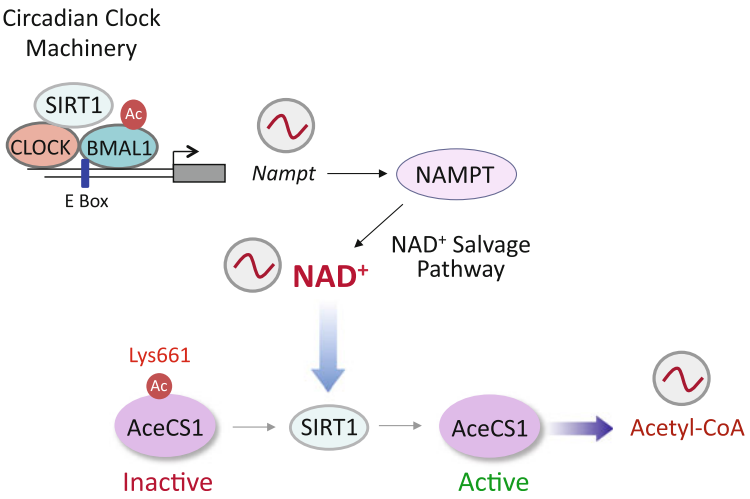

Fig. 1 Metabolism and the circadian clock converge. A paradigm example is represented by the role of SIRT1 and other sirtuins in clock regulation. The circadian machinery controls a large fraction of the genome through the transcriptional regulation of CCGs. One of the CCGs is the gene encoding the protein NAMPT, the rate-limiting enzyme in the NAD^+-salvage pathway. Cyclic transcriptional control of the *Nampt* gene results in the cyclic synthesis of NAD^+, which in turn is consumed rhythmically by enzymes such as SIRT1, whose deacetylase activity is consequently cyclic. One of the non-histone targets is the enzyme AceCS1, which contributes to the synthesis of Acetyl-CoA. AceCS1 is acetylated at one residue, Lys661, and its cyclic deacetylation by SIRT1 activates the enzyme, resulting in cyclic synthesis of Acetyl-CoA and thereby oscillating availability of acetyl groups required for global acetylation

oxidation and protein acetylation show circadian rhythmicity (Masri et al. 2013), the link with NAD^+ availability through SIRT3 is of particular interest. Also, mitochondria from *Bmal1*$^{-/-}$ mice display reduced oxidative ability and decreased mitochondrial NAD^+ levels (Peek et al. 2013). These findings, together with the implication of SIRT1 in circadian control, raise the possibility that the sirtuins-NAD^+ link with the clock may represent a critical molecular pathway to govern the process of aging.

The implication of nuclear sirtuins in clock function is multiple. SIRT1 is both nuclear and cytoplasmic whereas SIRT6 is exclusively nuclear and mostly chromatin bound, localized at transcriptionally active genomic loci. SIRT1 and SIRT6 operate through distinct mechanisms to coordinate the clock machinery in a differential manner and thereby delineate the circadian transcriptional output (Masri et al. 2014). Because of these different mechanisms of action, in the liver these two sirtuins coordinate circadian expression of distinct groups of genes. SIRT6 exerts its function by coordinating CLOCK:BMAL1 recruitment to specific chromatin sites (Masri et al. 2014). SIRT1, which is mostly nucleoplasmic and is recruited to chromatin only 'on demand', deacetylates histones and non-histone proteins. Among the non-histone targets of SIRT1 there are the clock proteins BMAL1 and PER2 (Asher et al. 2008; Hirayama et al. 2007). SIRT1 is also able

to deacetylate MLL1, thereby controlling its methyltransferase activity. Thus, there is control in H3K4 tri-methylation through the cyclic oscillation of NAD^+ levels (Aguilar-Arnal et al. 2015).

SIRT1-mediated deacetylation also affects circadian levels of other metabolites besides NAD^+. Specifically, intracellular acetyl-CoA levels are controlled by the clock through SIRT1-controlled deacetylation of the enzyme acetyl-CoA Synthetase 1 (AceCS1) (Sahar et al. 2014). This acetylation switch controls AceCS1 activity, leading to cyclic synthesis of acetyl-CoA (Fig. 1), that then is likely to influence the acetylation levels of histones and non-histone proteins (Sahar et al. 2014). In contrast, SIRT6 deacetylase activity seems to be efficient in removing long chain fatty acids from lysine residues (Jiang et al. 2013). In this respect it is noteworthy that not only on NAD^+, but also on fatty acids, control the activity of SIRT6 (Feldman et al. 2013). Thus, SIRT6 appears to occupy a key position in the control of fatty acids metabolism by the clock. Indeed, CLOCK:BMAL1-driven activation of genes involved in fatty acid biosynthesis is modulated by SIRT6 (Masri et al. 2014).

High-throughput analysis of the transcriptome and metabolome along the circadian cycle has revealed notable differences in the metabolic functions of SIRT1 and SIRT6. Using mice with liver-specific deletion of either SIRT1 or SIRT6, a specific role for SIRT6 was shown in dictating the synthesis and breakdown of fatty acid pathways, as well as their storage into triglycerides. SIRT6 operates at least in part through the control of alternative circadian transcriptional pathways, specifically because of the chromatin recruitment of the sterol regulatory element-binding protein 1 (SREBP1) (Masri et al. 2014). Thus, it is through genomic partitioning that the two deacetylases contribute to a parallel segregation of cellular metabolism (Masri et al. 2014).

Finally, these findings suggest a role for genome topology in circadian control (Aguilar-Arnal et al. 2013). Our studies have identified the presence of circadian interactomes where co-regulated genes are physically associated in the circadian epigenome. Nuclear sirtuins may constitute a paradigm for other chromatin remodelers that could contribute in the cyclic control of the nuclear landscape. Also, specific changes in the nuclear localization of NAD^+ may provide the possibility of restricting the distribution of this metabolite to "niches" of activity (Katada et al. 2012).

Conclusion

The ability of the circadian clock machinery to sense the metabolic state of the cell in a time-specific manner places it in a strategic position. Indeed, fascinating findings reviewed in this article demonstrate the direct implication of the clock in the maintenance of cellular homeostasis. The clock machinery appears to integrate environmental and metabolic signals to directly translate them in plasticity in gene expression so to favor the adaptation of the organism to specific conditions. As the

circadian transcriptional landscape is highly complex, including dynamic changes in nuclear organization (Katada et al. 2012; Aguilar-Arnal et al. 2013), it becomes critical to decipher how the nuclear landscape integrates metabolic cues and shapes the transcriptional output. It is through the analysis of the specific coordination that key chromatin remodelers have with clock transcription factors that we will gain insights into how the intracellular metabolic state communicates with the clock machinery. As disruption of clock function has been linked to a variety of pathological conditions, revealing the clock mechanisms will lead to innovative strategies towards the pharmacological treatment of metabolic syndromes, obesity, diabetes, inflammation and even cancer.

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