Chromatin Remodeling and Circadian Control: Master Regulator CLOCK Is an Enzyme

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The molecular machinery that governs circadian rhythmicity is based on clock gene products organized in regulatory feedback loops. Recently, we have shown that CLOCK, a master circadian regulator, has histone acetyltransferase activity essential for clock gene expression. The Lys-14 residue of histone H3 is a preferential target of CLOCK-mediated acetylation. As the role of chromatin remodeling in eukaryotic transcription is well recognized, this finding identified unforeseen links between histone acetylation and cellular physiology. Indeed, we have shown that the enzymatic function of CLOCK drives circadian control. We reasoned that CLOCK's acetyltransferase activity could also target nonhistone proteins, a feature displayed by other HATs. Indeed, CLOCK also acetylates a nonhistone substrate: its own partner, BMAL1. This protein undergoes rhythmic acetylation in the mouse liver, with a timing that parallels the down-regulation of circadian transcription of clock-controlled genes. BMAL1 is specifically acetylated on a unique, highly conserved Lys-537 residue. This acetylation facilitates recruitment of the repressor CRY1 to BMAL1, indicating that CLOCK may intervene in negative circadian regulation. Our findings reveal that the enzymatic interplay between two clock core components is crucial for the circadian machinery.

INTRODUCTION

The eukaryotic genome is organized in a nucleoprotein structure, chromatin, that enables a large variety of central processes such as regulation of gene expression, DNA repair, apoptosis, and cell division (Luger 2003; Felsenfeld and Groudine 2003). Changes in chromatin organization have a central role in ensuring that the storage, organization, and readout of the genetic information occurs in a proper spatial and temporal sequence during all biological processes. The basic repeating unit of chromatin, the nucleosome core particle, consists of 147 bp of DNA organized in approximately two superhelical turns of DNA wrapped around an octamer of core histones (two copies each of histone H2A, H2B, H3, and H4). Nucleosomes become organized in higher-order structures when interacting with H1 linker histones and more loosely with nonhistone chromatin-associated proteins (Luger 2003). A number of enzymatic processes lead to remodeling of the architecture of chromatin. These are dynamic changes, essential for the transition of chromatin from a condensed to a decondensed state, and vice versa, each state being permissive to specific cellular functions. These states somewhat correlate with the definition of "euchromatin" versus "heterochromatin," which most times identifies with "active" versus "inactive" states of gene expression, respectively (Strahl and Allis 2000; Felsenfeld and Groudine 2003).

Various cellular mechanisms operate that lead to modifications in chromatin structure: One type involves the active participation of ATP-dependent remodeling factors, such as Swi/Snf and NURF (Peterson and Workman 2000), and others implicate posttranslational covalent modifications of histones by specific enzymes. The amino-terminal tails of the core histones H2A, H2B, H3, and H4 are highly conserved and represent the protein

domains where most of the covalent histone modifications occur. Since histone tails are thought to confer secondary and more flexible contacts with DNA, allowing for dynamic changes in the accessibility of the underlying genome, their modifications have been shown to contribute to chromatin remodeling and thereby to the control of a large array of nuclear processes (Cheung et al. 2000b; Felsenfeld and Groudine 2003). The amino-terminal domains of histones are subjected to a large variety of covalent modifications, such as acetylation (Grunstein 1997; Gregory et al. 2001; Roth et al. 2001), phosphorylation (Mahadevan et al. 1991; Sassone-Corsi et al. 1999), and methylation (Bannister et al. 2002), but also ADP-ribosylation (Huletsky et al. 1985) and ubiquitination (Sun and Allis 2002). In several cases, the combinatorial association of some specific modifications has been reported and coupled to unique nuclear functions (Cheung et al. 2000a; Lo et al. 2000), strongly suggesting that the coordinate action of multiple enzymes converges to promote physiological changes in chromatin organization (Lo et al. 2001; Merienne et al. 2001).

CHROMATIN REMODELING AND GENE EXPRESSION: AN ENZYMATIC AFFAIR

The four core histones have amino-terminal tails whose primary sequences are highly conserved from yeast to mammals. Remarkably, histone tails have a high density of residues that are prone to posttranslational modifications. For the most part, the enzymes that elicit these modifications are also conserved among species. Various position-specific modifications have been associated with distinct chromatin-based outputs (Strahl and Allis 2000; Felsenfeld and Groudine 2003). For example, modifications in the histone H3 amino-terminal tail have been cou-

pled to transcriptional regulation (Lys-9/Lys-14 acetylation, Ser-10 phosphorylation), transcriptional silencing (Lys-9 methylation), histone deposition (Lys-9 acetylation), and chromosome condensation/segregation (Ser-10/Ser-28 phosphorylation). It has been proposed that various combinations of histone modifications may elicit differential regulation of the chromatin condensation state that corresponds to distinct biological responses (Fischle et al. 2003).

Histone phosphorylation is remarkable because it is directly linked to intracellular signaling pathways. Indeed, activation of a specific transduction system by an extracellular stimulus results in the stimulation of kinase cascades and phosphorylation at distinct sites on histones. Various serine-threonine kinases have been implicated for phosphorylating potential serine phosphoacceptor sites on histone H2A (Ser-1 and Ser-18), H2B (Ser14 and Ser-32), H3 (Ser-10 and Ser-28), and H4 (Ser-1). The distance between the serine residues in H2A, H2B, and H3 is conserved among all histones, with a constant spacing of 18 residues. The significance of this intriguing feature is unclear. Phosphorylation of Ser-10 in histone H3 has been studied in detail (Sassone-Corsi et al. 1999; Clayton et al. 2000) and found to be elicited by a number of kinases, all belonging to the AGC branch of cyclic nucleotide-regulated protein kinase, including RSK-2, MSK1, PKA, and IKK α (Nowak and Corces 2004).

Histone acetylation is believed to have a pivotal role in the modulation of chromatin structure associated with transcriptional activation (Grunstein 1997; Wade and Wolffe 1997; Kuo and Allis 1998; Struhl 1998; Workman and Kingston 1998). In support of this notion, a wide variety of nuclear proteins involved in transcriptional control have been demonstrated to possess intrinsic histone acetyltransferase (HAT) activity (Kouzarides 1999; Sterner and Berger 2000; Roth et al. 2001). In particular, a number of transcriptional coactivators, including GCN5 (Brownell et al. 1999), PCAF (Yang et al. 1996), CBP/p300 (Bannister and Kouzarides 1996; Ogryzko et al. 1996), SRC-1 (Spencer et al. 1997), and ACTR (Chen et al. 1997) are known to acetylate histones, thereby facilitating the *trans*-activation exerted by a number of DNA-binding transcription factors. Furthermore, HAT function has been ascribed also to TAFII250, a component of the TATA-box-binding TFIID complex of the basal transcription machinery (Mizzen et al. 1996), and to ATF-2, a sequence-specific DNA-binding transcription factor (Kawasaki et al. 2000). Thus, HATs constitute a family of proteins with remarkably diverse features.

Amino acid sequence analyses of HAT proteins reveal an important feature: HATs fall into distinct families that share relatively poor sequence similarity. For example, ACTR/SRC-1 is thought to constitute a unique class of HAT (Chen et al. 1997; Spencer et al. 1997), whereas p300/CBP displays only limited homology with the GCN5-related *N*-acetyltransferase superfamily (Martinez-Balbas et al. 1998). The MYST family of HATs is particularly interesting as these proteins show similarity with other acetyltransferases exclusively within the acetyl-coenzyme-A-binding motif (denominated "motif A") (Yamamoto and Horikoshi 1997). Accumulating evi-

dence indicates that histone acetylation exerted by various classes of HATs contributes to plasticity in transcriptional control by increasing the dynamic changes in chromatin structure (Fischle et al. 2003).

Histone methylation has been linked mostly to gene silencing, although some examples of its role in transcriptional activation have been reported. Specifically, methylation of the Lys-4 of H3 has been found to be a positive mark for gene activation, possibly in combination to acetylation at Lys-14 (Berger 2007). Three forms of histone methylation exist, each one of them promoting a different regulatory function. Indeed, mono-, di-, and three-methylated lysines are obtained by the action of different types of histone methyltransferases (HMTs). Some of the enzymes devoted to the removal of the methyl groups, the demethyltransferases, have been implicated in neurological disorders (Shi 2007). Importantly it is the combined association of different posttranslational modifications that appears to bring unique biological functions. Thus, the identification of the enzymes involved in histone modifications, and their regulatory interplays, has far-reaching physiological consequences.

PERIPHERAL VERSUS CENTRAL CLOCKS: WHERE IS THE DIFFERENCE?

The finely controlled transcriptional regulation within the circadian system is absolutely remarkable. Indeed, more than 10% of all mammalian transcripts undergo circadian fluctuations in their expression levels (Akhtar et al. 2002; Duffield et al. 2002; Panda et al. 2002), underscoring that genome-wide mechanisms must operate in order to ensure such global transcriptional regulation. The highly specialized, temporally based regulation of gene expression that characterizes circadian oscillations elects the cellular clock as a prominent model for the study of dynamic regulations of chromatin remodeling (Crosio et al. 2000). Moreover, as circadian function is intimately coupled to physiological and metabolic control (Dunlap 1999; Rutter et al. 2001; Schibler and Naef 2005), clock-controlled chromatin reorganization is likely to reveal yet unexplored pathways linking histone modifications to cellular metabolism. In this context, the finding that peripheral tissues also contain independent clocks is of fundamental importance (Whitmore et al. 2000; Giebultowicz 2001; Stokkan et al. 2001; Schibler and Sassone-Corsi 2002). Peripheral clocks are not fully self-sustained and autonomous and, differently from the suprachiasmatic nucleus (SCN), require specific stimuli in order to sustain their circadian rhythms (Cermakian and Sassone-Corsi 2000; King and Takahashi 2000; Reppert and Weaver 2002). We favor a scenario where peripheral clocks are affected by physiological stimuli that may originate from the SCN and/or may be the result of SCN-mediated messages (Schibler and Sassone-Corsi 2002). This view has been substantiated by a number of studies that have identified growth factors, some steroids such as glucocorticoids, and retinoic acid to induce oscillations of clock genes and clock-controlled genes in cultured fibroblasts or peripheral tis-

sues (Kramer et al. 2001, 2005). These observations underscore the presence of possible differences at the molecular level between the organization of the clock mechanism in the SCN and that in peripheral tissues. These may operate following some unique tissue-specific regulatory pathways since restricted access to food has an effect on peripheral rhythms without affecting the central pacemaker function of the SCN (Schibler and Sassone-Corsi 2002).

PLASTICITY IN CIRCADIAN REGULATION AND CHROMATIN REMODELING

How is the oscillatory expression of clock-controlled genes regulated, so that transcription-permissive chromatin states are dynamically established in a circadian time-specific manner? Interestingly, the activation of clock-controlled genes (CCGs) by CLOCK:BMAL1 has been shown to be coupled to circadian changes in histone acetylation at their promoters (Etchegaray et al. 2003; Curtis et al. 2004; Naruse et al. 2004; Nakahata et al. 2007). Specifically, histone H3 is acetylated in chromatin that encompasses the *Per1*, *Per2*, and *Cry1* promoters when these genes are actively transcribed. The molecular dissection of the CLOCK:BMAL1-mediated *trans*-activation mechanism is likely to provide significant information on how circadian regulation of histone acetylation is achieved. Several lines of genetic evidence also indicate that the carboxy-terminal glutamine-rich region of CLOCK exerts a central function in the circadian *trans*-activation of target genes in flies and mice (Antoch et al. 1997; King et al. 1997; Allada et al. 1998; Gekakis et al. 1998; Jin et al. 1999). In addition, H3 Ser-10 phosphorylation is involved in the transcriptional response to light in the SCN. Indeed, light-mediated signaling leads to nuclear responses that in turn influence the state of higher chromatin organization (Crosio et al. 2000). As the coupled modification of Ser-10 phosphorylation and Lys-14 acetylation on H3 has been shown to be a hallmark of transcriptional activation (Cheung et al. 2000a; Lo et al. 2000), it appears that the combined implication of HATs and other enzymatic pathways is crucial for circadian gene expression (Curtis et al. 2004; Ripperger and Schibler 2006). 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 Lys-27 are found to be associated to *Per* gene promoters, extending the activity of the polycomb group proteins to the core clockwork mechanism of mammals (Etchegaray et al. 2006).

The finding that the central element of the core clock mechanism, the transcription factor CLOCK, has intrinsic HAT activity (Doi et al. 2006) has modified our view because this protein should not be simply considered a transcription factor but an enzyme (Fig. 1). This finding also further underscored the importance of the E box as a pivotal element in circadian control, because chromatin conformational changes may occur specifically and efficiently at predetermined locations identified by the presence of E boxes.

Figure 1. Schematic model of the role that chromatin remodeling has within the physiological pathways of circadian rhythmicity. Metabolic, nutritional, and environmental circadian cues are likely to modulate chromatin remodeling, likely via control of the HAT function of CLOCK by means of intracellular signaling. Furthermore, by regulating chromatin transitions, CLOCK exerts its function on a variety of output CCGs, thereby influencing cellular physiology and metabolism. Importantly, the expression of at least 10% of all mammalian transcripts oscillate in a circadian manner.

THE PROTEIN CLOCK IS AN ENZYME

The carboxy-terminal glutamine-rich region of CLOCK, a region implicated in *trans*-activation function (Allada et al. 1998; Gekakis et al. 1998), has some important features. Except for a polyglutamine stretch, no characteristic structural motifs were previously described in this region. A closer look revealed that the carboxy-terminal region of CLOCK displays a significant sequence homology with the carboxy-terminal domain of ACTR, a domain shown 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. These include the highly conserved basic helix-loop-helix (bHLH)-PAS domain at the amino termini, a NRID (nuclear receptor interaction domain), as well as serine-rich regions within the middle portion of both proteins. Although CLOCK is a significantly smaller protein as compared to ACTR, these common features result in a

strikingly similar organization overall (Doi et al. 2006). Analysis of the primary sequence shows that BMAL1, the heterodimeric partner of CLOCK, which is also a bHLH-PAS domain-containing protein (Hirayama and Sassone-Corsi 2005), displays no similarity to the HAT region of ACTR.

An additional feature makes CLOCK quite unique as a HAT. Acetyl-coenzyme-A (CoA)-binding motifs are hallmarks of HAT proteins (Sterner and Berger 2000). Detailed sequence comparison between the acetyl-CoA-binding motifs of various HATs revealed that CLOCK contains a motif within the carboxy-terminal glutamine-rich region. This amino acid sequence stretch shares significant similarity to the so-called "motif A" in the HAT family denominated MYST (for its founding members MOZ, Ybf2/Sas3, Sas2, and Tip60). In particular, CLOCK shows high sequence similarity to yeast Esa1 and other MYST members, including yeast Sas3, fly MOF, and human Tip60. Importantly, residues that have been demonstrated to be involved in acetyl-CoA interaction by crystal structure analysis of the Esa1 protein (Yan et al. 2000) are shared with CLOCK. It is significant that these residues are all fully conserved in CLOCK proteins of various species. One intriguing feature of the motif A in CLOCK, when compared to MYST family members, is the insertion of five amino acids, also fully conserved among species. As compared to Esa1, the additional five amino acids would lengthen the loop comprised between β 9 and α 3, a region that is demonstrated to be exposed at the protein surface (Yan et al. 2000). Intriguingly, the putative acetyl-CoA-binding motif in *Drosophila* CLOCK lacks the five extra amino acids present in the vertebrate counterparts, which increases its similarity to Esa1 and other MYST family members. Finally, a number of acetyl-CoA-binding motifs from various *N*-acetyltransferases also show similarities. In conclusion, the structural features of CLOCK appear to identify it as a novel type of DNA-binding HAT. Indeed, the general organization is similar to a SRC/ACTR-type of HAT, but the acetyl-CoA-binding motif is more related to the MYST class. Thus, the unique combination of protein domains in CLOCK seems to form a somewhat hybrid HAT.

CIRCADIAN PHYSIOLOGY NEEDS A HAT

Our biochemical analyses have established that CLOCK is indeed a bona fide HAT with high specific enzymatic activity for H3 and H4, as it does not acetylate H2A and H2B. Site-directed mutagenesis indicated that H3 Lys14 is where most of the acetylation occurs (Doi et al. 2006). Most importantly, using an experimental system based on mouse embryonic fibroblast (MEF) cells derived from homozygous *Clock* mutant (*c/c*) mice (Vitaterna et al. 1994), we showed that the HAT function of CLOCK is essential for the circadian control of CCGs (Doi et al. 2006). As *Clock* is essential for circadian rhythm (Antoch et al. 1997; King et al. 1997), MEF *c/c* cells show no cyclic expression of clock genes (Pando et al. 2002). Importantly, ectopic expression of CLOCK is able to rescue the circadian expression of endogenous target genes. MEF *c/c* cells stably transfected with a CLOCK expression plasmid were

subjected to a serum shock, a stimulus commonly used to trigger circadian gene transcription in a variety of cell lines (Balsalobre et al. 1998; Pando et al. 2002). Although the MEF *c/c* cells had no functional circadian clock, CLOCK ectopic expression restored circadian oscillation of the endogenous *Per1* gene and of the *Dbp* gene, an E-box-regulated circadian CCG output gene. On the contrary, ectopic expression of a HAT-deficient CLOCK failed to restore the circadian *trans*-activation of *mPer1* and *Dbp*, demonstrating that the HAT activity of CLOCK is necessary for circadian gene expression (Doi et al. 2006). These findings underscore the importance of chromatin remodeling in circadian regulation and reveal the molecular pathways by which such essential control is achieved (Nakahata et al. 2007).

BMAL1 IS ACETYLATED BY CLOCK

The finding that CLOCK is a HAT (Doi et al. 2006) suggested that the acetyltransferase enzymatic activity could also target other nonhistone proteins. This feature is displayed by other HATs (Glozak et al. 2005; Zhang and Dent 2005) and is demonstrated to have profound physiological significance. In a search for putative targets, we have found that CLOCK mediates the acetylation of its own heterodimerization partner, BMAL1 (Hirayama et al. 2007).

In a survey aimed at identifying proteins that may be acetylated rhythmically *in vivo*, we analyzed various clock proteins, such as BMAL1, CLOCK, and PER1 in the mouse liver at different zeitgeber times. Although, as expected, these proteins oscillate in abundance and phosphorylation levels (Lee et al. 2001; Matsuo et al. 2003), acetylation of BMAL1 displays a robust circadian oscillation with a peak at ZT15 (Hirayama et al. 2007). Significantly, the other clock proteins are not acetylated. Ongoing studies in our laboratory on a variety of nuclear proteins and transcription factors indicate that BMAL1 is one of the few substrates for CLOCK, underscoring the specificity of the assay. Importantly, CLOCK is directly responsible for BMAL1 acetylation in cultured mammalian cells (Hirayama et al. 2007).

From a mechanistic point of view, it is notable that heterodimerization between the two proteins seems to be required for BMAL1 acetylation. Indeed, an amino-terminally truncated CLOCK (CLOCKAN) mutant that is unable to interact with BMAL1 because it lacks the PAS domains, although it still possesses HAT activity (Doi et al. 2006), did not induce BMAL1 acetylation. On the other hand, a CLOCK protein with three single-amino-acid mutations in the HAT domain (CLOCK-mutA) that we had previously shown to have reduced HAT activity (Doi et al. 2006) displayed a drastically reduced acetylation of BMAL1, demonstrating that the intrinsic HAT activity of CLOCK is required for BMAL1 acetylation.

HIGH SPECIFICITY: CLOCK ACETYLATES A SINGLE LYSINE IN BMAL1

In addition to acetylation, BMAL1 is posttranslationally modified by phosphorylation (Kondratov et al. 2003; Hirayama and Sassone-Corsi 2005) and SUMOylation

(Cardone et al. 2005). SUMOylation, as acetylation, occurs on lysine residues and thus we investigated whether an interplay converging on BMAL1 may exist between these two enzymatic pathways. We had previously identified four candidate lysines, K223, K229, K259, and K272, as subjected to SUMOylation in mouse BMAL1. Our analysis then demonstrated that K259 is the major *in vivo* SUMOylation site in BMAL1 (Cardone et al. 2005). We have found that none of these lysines are acetylated by CLOCK (Fig. 2), demonstrating that the target lysine residues for the two modifications are different. Thus, we embarked in the search for the lysines that are specifically acetylated by CLOCK. We first generated two carboxy-terminally truncated BMAL1 proteins (amino acids 1–282 and 1–469) (Fig. 2A). Both of these mutant proteins interact with CLOCK but are not acetylated, indicating that the target lysines for CLOCK-dependent acetylation must be located in the carboxyl terminus of mouse BMAL1 (amino acids 470–631; Fig. 2B). Somewhat conveniently, this region contains only four potential target lysines at positions 475, 494, 537, and 538, rendering our analysis quite accessible. Each lysine residue was mutated to arginine and tested for

CLOCK-mediated acetylation. Strikingly, all mutant proteins are acetylated at levels comparable to wild-type BMAL1, with the exception of K537R (Fig. 2C). Specificity was confirmed also in an *in vitro* acetylation assay using bacterially purified GST-BMAL1 and a mutant GST-BMAL1-K537R in the presence of ^3H -labeled acetyl-CoA. Thus, K537 is the major acetylation site for CLOCK (Hirayama et al. 2007), and it is remarkable that this lysine is highly conserved among all vertebrate BMAL1s (Fig. 2A).

BMAL1 ACETYLATION IS ESSENTIAL FOR CIRCADIAN RHYTHMICITY

The unique acetylation profile and the remarkable specificity of CLOCK in targeting one single lysine in BMAL1 prompted us to establish the physiological relevance of BMAL1 acetylation. To investigate the requirement for circadian rhythmicity of BMAL1 acetylation, we performed rescue experiments using MEFs generated from *Bmal1*-null mice. Lack of BMAL1 expression in these MEFs results in a dysfunctional circadian clock and arrhythmic gene expression (Konratov et al. 2003; Cardone et al. 2005). We used retroviruses expressing either wild-type BMAL1, an acetylation-deficient BMAL1 (K537R), or green fluorescent protein (GFP), and infected *Bmal1*^{−/−} MEFs (Fig. 3). To synchronize the infected MEFs, we used dexamethasone (DEX), and circadian oscillation was monitored by a real-time bioluminescence assay based on an *mPer2* promoter-driven luciferase reporter vector (Nagoshi et al. 2004; Sato et al. 2006). Although wild-type BMAL1 rescued circadian *mPer2* expression, the BMAL1 (K537R) mutant was unable to do so. This experiment demonstrates that the acetylation of BMAL1 is essential for circadian gene regulation (Fig. 3).

Our interest then turned to the molecular mechanism by which BMAL1 acetylation influences circadian rhythmicity: What we found revealed a regulatory pathway highly significant for circadian physiology. Our analyses

Figure 2. Highly specific acetylation of BMAL1. (A) Schematic representation of mBMAL1 protein showing the positions of bHLH, PAS A (A), PAS B (B), and CRY-binding site (CRY binding) (Hirayama and Sassone-Corsi 2005). Numbers indicate the amino acid residues in the mouse protein. The extent of two deletion mutants (amino acids 1–282 and 1–469) is shown as two gray bars on the top. The sequence alignment of BMAL1 with the target lysine for acetylation and its surrounding amino acids from various species is shown. (Bold) Target lysines. (B,C) Identification of the target lysine for acetylation. Cells were cotransfected with expression vectors for Myc-CLOCK and Flag-Myc-BMAL1 wild type or for each BMAL1 mutant described. Flag-Myc-BMAL1 proteins were immunoprecipitated, and acetylation was determined by western blotting using anti-pan-acetyl-Lys. Expression of precipitated BMAL1 was determined by western blotting using anti-BMAL1 (B, right panel) or anti-Myc (C, bottom panel) antibodies.

Figure 3. Acetylation of BMAL1 is essential to rescue the circadian rhythmicity in BMAL1-deficient cells. Retrovirus-infected *Bmal1*^{−/−} MEFs were used to rescue circadian rhythmicity by expressing either a wild-type BMAL1 protein (light gray dots) or the BMAL1(K537R) mutant (gray dots) and compared with the lack of circadian oscillation in the noninfected *Bmal1*^{−/−} MEFs (black dots). The *mPer2* promoter luciferase reporter plasmid was transfected into the retrovirus-infected cells, and *mPer2* promoter activity was monitored by a real-time bioluminescence assay. Expression levels were plotted as arbitrary units.

show that the BMAL1(K537R) mutant is recruited to the *mPer2* promoter with an efficiency equivalent to that of BMAL1. In addition, the K537R mutation has no effect on BMAL1 protein stability, capacity of association with CLOCK, and subcellular localization of the protein. Thus, we reasoned that BMAL1 acetylation could be involved in modulating CRY-mediated repression. This possibility would rationalize the results obtained in the rescue circadian experiments (Fig. 3) and is supported by the notion that impairment of CRY-mediated repression of the CLOCK:BMAL1 complex leads to loss of circadian rhythmicity (Sato et al. 2006). Indeed, the BMAL1(K537R) mutant showed a drastically reduced sensitivity to CRY-mediated repression compared to wild-type BMAL1 (Fig. 4). In other terms, our findings indicate that acetylation of BMAL1 by CLOCK may be an essential regulatory switch as it facilitates CRY-dependent repression. On the basis of association studies, our working model hypothesizes that acetylation of BMAL1 at K537 induces a conformational switch that generates a better “docking site” for CRY.

CONCLUSIONS AND PERSPECTIVES

The notion that at least 10% of all cellular transcripts oscillate in a circadian manner underscores the importance of chromatin remodeling in the control of the circadian gene expression (Nakahata et al. 2007). The finding that CLOCK is a HAT pointed to unforeseen links between histone acetylation and cellular physiology. Indeed, because of these multiple implications, a number of exciting avenues of research are now evident. One of

them relates to the search and finding of nonhistone targets for CLOCK. Our findings demonstrate that CLOCK exerts its enzymatic activity on another core component of the clock machinery *in vivo* in a circadian manner. As we demonstrated that BMAL1 enhances the intrinsic HAT activity of CLOCK (Doi et al. 2006), it is tempting to speculate that BMAL1 regulates its own acetylation by reciprocally controlling CLOCK enzymatic activity. Acetylation of proteins is recognized as an essential regulatory mechanism having both stimulatory and inhibitory effects on transcription (Workman and Kingston 1998; Sterner and Berger 2000). We have demonstrated that acetylation may operate at yet another level of control, because BMAL1 acetylation serves to increase the repressive function of another regulator, CRY. Our results also indicate that CLOCK enzymatic activity has a dual regulatory function. Indeed, we have shown that it contributes to the negative limb of the circadian feedback loop (Hirayama et al. 2007), whereas CLOCK-mediated acetylation of histones participates in the transcriptional stimulation of CCGs, acting within the positive limb of the loop (Doi et al. 2006). Thus, CLOCK enzymatic function contributes in multiple ways to the time-dependent regulation of transcription.

Future studies aimed at deciphering the structural determinants of HAT function, its regulation by BMAL1, and the possible intervention of additional regulators are on the way. Like other HATs, CLOCK is likely to associate with a number of nuclear proteins in a chromatin complex. The study of this complex will reveal additional control elements for circadian transcription, as well as putative histone deacetyltransferases (HDACs) involved

Figure 4. The enzymatic function of CLOCK governs the circadian machinery. Shown is a schematic model of CLOCK-mediated histone and nonhistone acetylation and its role within the physiological pathways of circadian rhythmicity. The HAT function of CLOCK regulates promoters of CCGs and clock genes (such as *Per1*) by inducing locally open organization of the chromatin. For example, acetylation on Lys-14 of histone H3 is thought to elicit chromatin remodeling by inducing a transcription-permissive state. Acetylation at Lys-14 could also be favored by additional concerted modifications of the histone tails. For example, phosphorylation at Ser-10, an event directly coupled to activation of intracellular signaling pathways, such as light-induced signals in SCN neurons (Crosio et al. 2000), induces a more efficient acetylation at Lys-14. We envisage a scenario where circadian control of chromatin remodeling by CLOCK may be influenced by the dynamic assembly of a multiprotein regulatory complex. In addition, CLOCK is responsible for BMAL1 acetylation at K537, an event that increases the repression potential by CRY of the CLOCK:BMAL1 complex. Thus, the acetyltransferase enzymatic activity of CLOCK has a dual function, by regulating the circadian machinery by targeting both histone and nonhistone proteins. Indeed, although acetylation of histone H3 results in the positive regulation of CCGs, acetylation of BMAL1 is involved in the negative repression by CRY.

in enzymatically counteracting the acetylation. Thus, many questions remain to be addressed. The exciting part is now to design experiments and gather the results that will elucidate the intricacies of how circadian control is linked to metabolism, cell cycle, and physiology. Uncovering the intimate links between chromatin remodeling and the circadian clock constitutes a conceptually novel challenge that will provide more excitement for future studies.

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REFERENCES

- Akhtar R.A., Reddy A.B., Maywood E.S., Clayton J.D., King V.M., Smith A.G., Gant T.W., Hastings M.H., and Kyriacou C.P. 2002. Circadian cycling of the mouse liver transcriptome, as revealed by cDNA microarray, is driven by the suprachiasmatic nucleus. *Curr. Biol.* **12**: 540.
- Allada R., White N.E., So W.V., Hall J.C., and Rosbash M. 1998. A mutant *Drosophila* homolog of mammalian *Clock* disrupts circadian rhythms and transcription of *period* and *timeless*. *Cell* **93**: 791.
- Antoch M.P., Song E.J., Chang A.M., Vitaterna M.H., Zhao Y., Wilsbacher L.D., Sangoram A.M., King D.P., Pinto L.H., and Takahashi J.S. 1997. Functional identification of the mouse circadian *Clock* gene by transgenic BAC rescue. *Cell* **89**: 655.
- Balsalobre A., Damiola F., and Schibler U. 1998. A serum shock induces circadian gene expression in mammalian tissue culture cells. *Cell* **93**: 929.
- Bannister A.J. and Kouzarides T. 1996. The CBP co-activator is a histone acetyltransferase. *Nature* **384**: 641.
- Bannister A.J., Schneider R., and Kouzarides T. 2002. Histone methylation: Dynamic or static? *Cell* **109**: 801.
- Berger S.L. 2007. The complex language of chromatin regulation during transcription. *Nature* **447**: 407.
- Brownell J.E., Mizzen C.A., and Allis C.D. 1999. An SDS-PAGE-based enzyme activity assay for the detection and identification of histone acetyltransferases. *Methods Mol. Biol.* **119**: 343.
- Cardone L., Hirayama J., Giordano F., Tamaru T., Palvimo J.J., and Sassone-Corsi P. 2005. Circadian clock control by SUMOylation of BMAL1. *Science* **309**: 1390.
- Cermakian N. and Sassone-Corsi P. 2000. Multilevel regulation of the circadian clock. *Nat. Rev. Mol. Cell Biol.* **1**: 59.
- Chen H., Lin R.J., Schiltz R.L., Chakravarti D., Nash A., Nagy L., Privalsky M.L., Nakatani Y., and Evans R.M. 1997. Nuclear receptor coactivator ACTR is a novel histone acetyltransferase and forms a multimeric activation complex with P/CAF and CBP/p300. *Cell* **90**: 569.
- Cheung P., Allis C.D., and Sassone-Corsi P. 2000a. Signaling to chromatin through histone modifications. *Cell* **103**: 263.
- Cheung P., Tanner K.G., Cheung W.L., Sassone-Corsi P., Denu J.M., and Allis C.D. 2000b. Synergistic coupling of histone H3 phosphorylation and acetylation in response to epidermal growth factor stimulation. *Mol. Cell* **5**: 905.
- Clayton A.L., Rose S., Barratt M.J., and Mahadevan L.C. 2000. Phosphoacetylation of histone H3 on *c-fos*- and *c-jun*-associated nucleosomes upon gene activation. *EMBO J.* **19**: 3714.
- Crosio C., Cermakian N., Allis C.D., and Sassone-Corsi P. 2000. Light induces chromatin modification in cells of the mammalian circadian clock. *Nat. Neurosci.* **3**: 1241.
- Curtis A.M., Seo S.B., Westgate E.J., Rudic R.D., Smyth E.M., Chakravarti D., Fitzgerald G.A., and McNamara P. 2004. Histone acetyltransferase-dependent chromatin remodeling and the vascular clock. *J. Biol. Chem.* **279**: 7091.
- Doi M., Hirayama J., and Sassone-Corsi P. 2006. Circadian regulator CLOCK is a histone acetyltransferase. *Cell* **125**: 497.
- Duffield G.E., Best J.D., Meurers B.H., Bittner A., Loros J.J., and Dunlap J.C. 2002. Circadian programs of transcriptional activation, signaling, and protein turnover revealed by microarray analysis of mammalian cells. *Curr. Biol.* **12**: 551.
- Dunlap J.C. 1999. Molecular bases for circadian clocks. *Cell* **96**: 271.
- Etchegaray J.P., Lee C., Wade P.A., and Reppert S.M. 2003. Rhythmic histone acetylation underlies transcription in the mammalian circadian clock. *Nature* **421**: 177.
- Etchegaray J.P., Yang X., DeBruyne J.P., Peters A.H., Weaver D.R., Jenuwein T., and Reppert S.M. 2006. The polycomb group protein EZH2 is required for mammalian circadian clock function. *J. Biol. Chem.* **281**: 21209.
- Felsenfeld G. and Groudine M. 2003. Controlling the double helix. *Nature* **421**: 448.
- Fischle W., Wang Y., and Allis C.D. 2003. Binary switches and modification cassettes in histone biology and beyond. *Nature* **425**: 475.
- Gekakis N., Staknis D., Nguyen H.B., Davis F.C., Wilsbacher L.D., King D.P., Takahashi J.S., and Weitz C.J. 1998. Role of the CLOCK protein in the mammalian circadian mechanism. *Science* **280**: 1564.
- Giebultowicz J.M. 2001. Peripheral clocks and their role in circadian timing: Insights from insects. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* **356**: 1791.
- Glozak M.A., Sengupta N., Zhang X., and Seto E. 2005. Acetylation and deacetylation of non-histone proteins. *Gene* **363**: 15.
- Gregory P.D., Wagner K., and Horz W. 2001. Histone acetylation and chromatin remodeling. *Exp. Cell Res.* **265**: 195.
- Grunstein M. 1997. Histone acetylation in chromatin structure and transcription. *Nature* **389**: 349.
- Hirayama J. and Sassone-Corsi P. 2005. Structural and functional features of transcription factors controlling the circadian clock. *Curr. Opin. Genet. Dev.* **15**: 548.
- Hirayama J., Sahar S., Grimaldi B., Tamaru T., Takamatsu K., Nakahata Y., and Sassone-Corsi P. 2007. CLOCK-mediated acetylation of BMAL1 controls circadian function. *Nature* (in press).
- Huletsky A., Niedergang C., Frechette A., Aubin R., Gaudreau A., and Poirier G.G. 1985. Sequential ADP-ribosylation pattern of nucleosomal histones. ADP-ribosylation of nucleosomal histones. *Eur. J. Biochem.* **146**: 277.
- Jin X., Shearman L.P., Weaver D.R., Zylka M.J., de Vries G.J., and Reppert S.M. 1999. A molecular mechanism regulating rhythmic output from the suprachiasmatic circadian clock. *Cell* **96**: 57.
- Kawasaki H., Schiltz L., Chiu R., Itakura K., Taira K., Nakatani Y., and Yokoyama K.K. 2000. ATF-2 has intrinsic histone acetyltransferase activity which is modulated by phosphorylation. *Nature* **405**: 195.
- King D.P. and Takahashi J.S. 2000. Molecular genetics of circadian rhythms in mammals. *Annu. Rev. Neurosci.* **23**: 713.
- King D.P., Zhao Y., Sangoram A.M., Wilsbacher L.D., Tanaka M., Antoch M.P., Steeves T.D., Vitaterna M.H., Kornhauser J.M., Lowrey P.L., Turek F.W., and Takahashi J.S. 1997. Positional cloning of the mouse circadian clock gene. *Cell* **89**: 641.
- Kondratov R.V., Chernov M.V., Kondratova A.A., Gorbacheva V.Y., Gudkov A.V., and Antoch M.P. 2003. BMAL1-dependent circadian oscillation of nuclear CLOCK: Posttranslational events induced by dimerization of transcriptional activators of the mammalian clock system. *Genes Dev.* **17**: 1921.
- Kouzarides T. 1999. Histone acetylases and deacetylases in cell proliferation. *Curr. Opin. Genet. Dev.* **9**: 40.
- Kramer A., Yang F.C., Karves S., and Weitz C.J. 2005. A screen for secreted factors of the suprachiasmatic nucleus. *Methods Enzymol.* **393**: 645.
- Kramer A., Yang F.C., Snodgrass P., Li X., Scammell T.E., Davis F.C., and Weitz C.J. 2001. Regulation of daily locomotor activity and sleep by hypothalamic EGF receptor signaling. *Science* **294**: 2511.
- Kuo M.H. and Allis C.D. 1998. Roles of histone acetyltransferases and deacetylases in gene regulation. *Bioessays* **20**: 615.

- Lee C., Etchegaray J.P., Cagampang F.R., Loudon A.S., and Reppert S.M. 2001. Posttranslational mechanisms regulate the mammalian circadian clock. *Cell* **107**: 855.
- Lo W.S., Duggan L., Emre N.C., Belotserkovskaya R., Lane W.S., Shiekhattar R., and Berger S.L. 2001. Snf1—A histone kinase that works in concert with the histone acetyltransferase Gcn5 to regulate transcription. *Science* **293**: 1142.
- Lo W.S., Trievel R.C., Rojas J.R., Duggan L., Hsu J.Y., Allis C.D., Marmorstein R., and Berger S.L. 2000. Phosphorylation of serine 10 in histone H3 is functionally linked in vitro and in vivo to Gcn5-mediated acetylation at lysine 14. *Mol. Cell* **5**: 917.
- Luger K. 2003. Structure and dynamic behavior of nucleosomes. *Curr. Opin. Genet. Dev.* **13**: 127.
- Mahadevan L.C., Willis A.C., and Barratt M.J. 1991. Rapid histone H3 phosphorylation in response to growth factors, phorbol esters, okadaic acid, and protein synthesis inhibitors. *Cell* **65**: 775.
- Martinez-Balbas M.A., Bannister A.J., Martin K., Haus-Seuffert P., Meisterernst M., and Kouzarides T. 1998. The acetyltransferase activity of CBP stimulates transcription. *EMBO J.* **17**: 2886.
- Matsuo T., Yamaguchi S., Mitsui S., Emi A., Shimoda F., and Okamura H. 2003. Control mechanism of the circadian clock for timing of cell division in vivo. *Science* **302**: 255.
- Merienne K., Pannetier S., Harel-Bellan A., and Sassone-Corsi P. 2001. Mitogen-regulated RSK2-CBP interaction controls their kinase and acetylase activities. *Mol. Cell. Biol.* **21**: 7089.
- Mizzen C.A., Yang X.J., Kokubo T., Brownell J.E., Bannister A.J., Owen-Hughes T., Workman J., Wang L., Berger S.L., Kouzarides T., Nakatani Y., and Allis C.D. 1996. The TAF(II)250 subunit of TFIID has histone acetyltransferase activity. *Cell* **87**: 1261.
- Nagoshi E., Saini C., Bauer C., Laroche T., Naef F., and Schibler U. 2004. Circadian gene expression in individual fibroblasts: Cell-autonomous and self-sustained oscillators pass time to daughter cells. *Cell* **119**: 693.
- Nakahata Y., Grimaldi B., Sahar S., Hirayama J., and Sassone-Corsi P. 2007. Signaling to the circadian clock: Plasticity by chromatin remodeling. *Curr. Opin. Cell Biol.* **19**: 230.
- Naruse Y., Oh-hashii K., Iijima N., Naruse M., Yoshioka H., and Tanaka M. 2004. Circadian and light-induced transcription of clock gene *Per1* depends on histone acetylation and deacetylation. *Mol. Cell. Biol.* **24**: 6278.
- Nowak S.J. and Corces V.G. 2004. Phosphorylation of histone H3: A balancing act between chromosome condensation and transcriptional activation. *Trends Genet.* **20**: 214.
- Ogryzko V.V., Schiltz R.L., Russanova V., Howard B.H., and Nakatani Y. 1996. The transcriptional coactivators p300 and CBP are histone acetyltransferases. *Cell* **87**: 953.
- Panda S., Antoch M.P., Miller B.H., Su A.I., Schook A.B., Straume M., Schultz P.G., Kay S.A., Takahashi J.S., and Hogenesch J.B. 2002. Coordinated transcription of key pathways in the mouse by the circadian clock. *Cell* **109**: 307.
- Pando M.P., Morse D., Cermakian N., and Sassone-Corsi P. 2002. Phenotypic rescue of a peripheral clock genetic defect via SCN hierarchical dominance. *Cell* **110**: 107.
- Peterson C.L. and Workman J.L. 2000. Promoter targeting and chromatin remodeling by the SWI/SNF complex. *Curr. Opin. Genet. Dev.* **10**: 187.
- Reppert S.M. and Weaver D.R. 2002. Coordination of circadian timing in mammals. *Nature* **418**: 935.
- Ripperger J.A. and Schibler U. 2006. Rhythmic CLOCK-BMAL1 binding to multiple E-box motifs drives circadian Dbp transcription and chromatin transitions. *Nat. Genet.* **38**: 369.
- Roth S.Y., Denu J.M., and Allis C.D. 2001. Histone acetyltransferases. *Annu. Rev. Biochem.* **70**: 81.
- Rutter J., Reick M., Wu L.C., and McKnight S.L. 2001. Regulation of clock and NPAS2 DNA binding by the redox state of NAD cofactors. *Science* **293**: 510.
- Sassone-Corsi P., Mizzen C.A., Cheung P., Crosio C., Monaco L., Jacquot S., Hanauer A., and Allis C.D. 1999. Requirement of Rsk-2 for epidermal growth factor-activated phosphorylation of histone H3. *Science* **285**: 886.
- Sato T.K., Yamada R.G., Ukai H., Baggs J.E., Miraglia L.J., Kobayashi T.J., Welsh D.K., Kay S.A., Ueda H.R., and Hogenesch J.B. 2006. Feedback repression is required for mammalian circadian clock function. *Nat. Genet.* **38**: 312.
- Schibler U. and Naef F. 2005. Cellular oscillators: Rhythmic gene expression and metabolism. *Curr. Opin. Cell Biol.* **17**: 223.
- Schibler U. and Sassone-Corsi P. 2002. A web of circadian pace-makers. *Cell* **111**: 919.
- Shi Y. 2007. Histone lysine demethylases: Emerging roles in development, physiology and disease. *Nat. Rev. Genet.* **8**: 829.
- Spencer T.E., Jenster G., Burcin M.M., Allis C.D., Zhou J., Mizzen C.A., McKenna N.J., Onate S.A., Tsai S.Y., Tsai M.J., and O'Malley B.W. 1997. Steroid receptor coactivator-1 is a histone acetyltransferase. *Nature* **389**: 194.
- Sterner D.E. and Berger S.L. 2000. Acetylation of histones and transcription-related factors. *Microbiol. Mol. Biol. Rev.* **64**: 435.
- Stokkan K.A., Yamazaki S., Tei H., Sakaki Y., and Menaker M. 2001. Entrainment of the circadian clock in the liver by feeding. *Science* **291**: 490.
- Strahl B.D. and Allis C.D. 2000. The language of covalent histone modifications. *Nature* **403**: 41.
- Struhl K. 1998. Histone acetylation and transcriptional regulatory mechanisms. *Genes Dev.* **12**: 599.
- Sun Z.W. and Allis C.D. 2002. Ubiquitination of histone H2B regulates H3 methylation and gene silencing in yeast. *Nature* **418**: 104.
- Vitaterna M.H., King D.P., Chang A.M., Kornhauser J.M., Lowrey P.L., McDonald J.D., Dove W.F., Pinto L.H., Turek F.W., and Takahashi J.S. 1994. Mutagenesis and mapping of a mouse gene, Clock, essential for circadian behavior. *Science* **264**: 719.
- Wade P.A. and Wolffe A.P. 1997. Histone acetyltransferases in control. *Curr. Biol.* **7**: R82.
- Whitmore D., Foulkes N.S., and Sassone-Corsi P. 2000. Light acts directly on organs and cells in culture to set the vertebrate circadian clock. *Nature* **404**: 87.
- Workman J.L. and Kingston R.E. 1998. Alteration of nucleosome structure as a mechanism of transcriptional regulation. *Annu. Rev. Biochem.* **67**: 545.
- Yamamoto T. and Horikoshi M. 1997. Novel substrate specificity of the histone acetyltransferase activity of HIV-1-Tat interactive protein Tip60. *J. Biol. Chem.* **272**: 30595.
- Yan Y., Barlev N.A., Haley R.H., Berger S.L., and Marmorstein R. 2000. Crystal structure of yeast Esa1 suggests a unified mechanism for catalysis and substrate binding by histone acetyltransferases. *Mol. Cell* **6**: 1195.
- Yang X.J., Ogryzko V.V., Nishikawa J., Howard B.H., and Nakatani Y. 1996. A p300/CBP-associated factor that competes with the adenoviral oncoprotein E1A. *Nature* **382**: 319.
- Zhang K. and Dent S.Y. 2005. Histone modifying enzymes and cancer: Going beyond histones. *J. Cell. Biochem.* **96**: 1137.

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