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A chromatin-dependent mechanism regulates gene expression at the core of the *Arabidopsis* circadian clock

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The mechanisms of circadian clock function in *Arabidopsis* rely on the complex relationships among core clock components. The current model of the *Arabidopsis* oscillator comprises a myriad of repressors but the mechanisms responsible for activation remain largely unknown. In our recent studies, we have demonstrated that the rhythms in H3 acetylation (H3ac) and H3K4 trimethylation (H3K4me3) are a key mechanism at the positive arm of the oscillator. H3K4me3 rhythmic accumulation is delayed compared with that of H3ac, which opens the possibility for separate roles for each mark. Indeed, the use of inhibitors that block H3K4me3 accumulation was concomitant with increased clock repressor binding, suggesting that H3K4me3 might control the timing from activation to repression. Plants mis-expressing the histone methyltransferase SET DOMAIN GROUP 2 (SDG2/ATXR3) displayed altered H3K4me3 accumulation, oscillator gene expression and clock repressor binding, suggesting that SDG2/ATXR3 is a key component contributing to proper circadian expression.

Circadian rhythms are daily oscillations in gene expression and biological activities that have been observed in almost all organisms, from cyanobacteria to mammals.[1] The circadian clock is the internal mechanism that generates these rhythms allowing organisms to anticipate the environmental changes resulting from the day/night cycles. In *Arabidopsis thaliana*, the circadian clock regulates multiple biological functions, such as the photoperiodic-dependent flowering time, stem and hypocotyl elongation, leaf movement, stomata movement and gene expression of roughly one third of the genes.[2] The fundamental architecture of the circadian clock comprises a complex network of feedback loops.[3] The first transcriptional feedback loop identified in *Arabidopsis*[4] included CCA1 (CIRCADIAN CLOCK ASSOCIATED 1) and LHY (LATE ELONGATED HYPOCOTYL),[5,6] together with TOC1/PRR1 (TIMING OF CAB EXPRESSION 1/PSEUDO-RESPONSE REGULATOR 1), a CCT-domain containing transcription factor expressed in the evening.[7,8] CCA1 and LHY are able to bind to the *TOC1* promoter through the evening element (EE) and repress its expression.[4] TOC1 initially was proposed to activate *CCA1* and *LHY* expression[4] but more recent reports have demonstrated that TOC1 also inhibits the expression of *CCA1* and *LHY*.[9,10] TOC1 not only represses *CCA1* and *LHY* but also the components of the pseudo-response regulator (PRR) family (PRR5, PRR7, PRR9) and other evening-expressed genes.[9] The current model thus contains a myriad of repressors, opening the question about positive factors that might function as activators at the core of the clock.

In eukaryotic cells, DNA wraps around a histone octamer to form nucleosomes, which are structured into higher-order structures to form a chromosome.[11] Histones are subject to multiple post-translational modifications including acetylation, methylation, phosphorylation, ubiquitination and ADP-ribosylation. The complex combination of these modifications regulates gene transcription.[12] Overall, histone acetyltransferases (HAT) acetylate histone lysine residues and favor transcription while histone deacetylases (HDAC) deacetylate histones and induce transcriptional repression.[13-15] Histones can also be mono-, di- or trimethylated on lysines and mono-, symmetrically or asymmetrically dimethylated on arginines.[16] Histone methylation acts as a signal for binding of chromatin remodeling factors, which can activate or repress transcriptional activity. The histone-modifying enzymes that catalyze the transfer of methyl groups are histone methyltransferases (HMT).[16] The two major groups of HMT include lysine-specific (HKMT) and arginine-specific (PRMT) methyltransferases. In *Arabidopsis*, different methyltransferases have been described, including two ATX-RELATED (ATXR3/SDG2 and ATXR7/SDG25) and five *ARABIDOPSIS* TRITHORAX (ATX1–ATX5).[17] Recently, SET DOMAIN GROUP 2 (ATXR3/SDG2) has been identified

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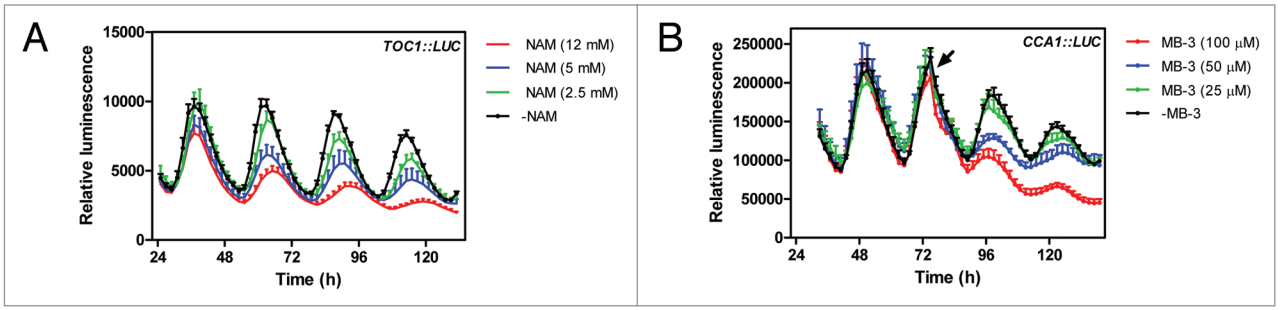

Figure 1. Effects of blocking histone acetylation and K4 trimethylation on circadian gene expression. (**A**) *TOC1::LUC* luminescence in WT plants entrained under LD cycles and subsequently released to constant light (LL) conditions. Luminescence was examined in the presence of 12 mM, 5 mM or 2.5 mM of NAM. As control, plants were treated only with the solvent. (**B**) *CCA1::LUC* luminescence in WT plants entrained under LD cycles and subsequently released to constant light (LL) conditions. Luminescence was examined in the presence of 100 μM, 50 μM or 25 μM of MB-3. As control, plants were treated only with the solvent. The arrow indicates the circadian time of inhibitor administration.

as the major histone methyltransferase responsible for H3K4me3 in *Arabidopsis thaliana*.[18,19]

Several studies have shown the connection between chromatin remodeling and the plant circadian clock. For instance, histone H3 acetylation at the *TOC1* promoter was shown to closely correlate with *TOC1* circadian expression.[20] Moreover, recent reports have extended the analysis demonstrating that H3 acetylation and H3K4me3 associate with the rhythmic transcription of *CCA1*, *LHY* and *TOC1*[21,22] while H3K36me2 shows a negative correlation with their expression. Other studies have also implicated *JUMONJI DOMAIN CONTAINING 5/30* (*JMD5/JMD30)*, a putative histone demethylase, in circadian clock regulation.[23,24] Taken together, these reports suggest that chromatin remodeling might be a key mechanism in the regulation of the plant circadian clock.

Our previous work revealed that circadian oscillations of H3ac at the *TOC1* promoter regulate *TOC1* rhythmic expression.[20,25] In a recent report,[26] we have extended these studies to other oscillator genes to demonstrate that the rhythms of H3K4me3, H3K9ac and H3K56ac are a regulatory mechanism common to the morning (CCA1, *LHY, PRR9* and *PRR7*) and evening (*TOC1* and *LUX*) expressed oscillator genes. Our studies show that H3K4me3 and H3K56ac mostly accumulated around the 5' end of the oscillator genes, with a peak around the time of their maximal expression. Recent publications reporting H3K4me3 and H3ac location on *TOC1*, *CCA1* and *LHY* loci support our results.[21,22] The distribution of histone marks have been shown to be important for their effect on transcription. For instance, methylation of K36 by Set2 usually occurs within the ORF of actively transcribed genes. However, misaccumulation of this mark within the promoter correlates with repression.[27]

The accumulation of H3K56ac and H3K4me3 around the peak of mRNA expression suggested that these marks might be associated with clock gene activation. Indeed, the rhythmic expression of the oscillator genes damped when acetylation and H3K4me3 were blocked with different inhibitors. Following the results of a previous study showing that nicotinamide (NAM) affected H3K4me3 and clock gene expression in mammals,[28] we treated seedlings with NAM and found that oscillator gene expression was reduced by treatment with the inhibitor in a dose-dependent manner (**Fig. 1**). Treatment with NAM also associated with a significant reduction in H3K4me3. Although NAM was previously shown to inhibit histone deacetylase,[29] our assay revealed that H3K56ac accumulation decreased when plants were treated with NAM. When we blocked histone acetylation by inhibition with MB-3[30] or with C646[31] we also observed a dose-dependent reduction of oscillator gene expression (**Fig. 1**). Remarkably, the combined treatment with both NAM and C646 damped low the oscillation of clock gene expression suggesting that acetylation and H3K4me3 are key histone modifications for the activation of oscillator gene expression. Our conclusions suggesting a chromatin remodeling mechanism within the positive arm of the oscillator are consistent with the results demonstrating that the key clock component of the mammalian oscillator, CLOCK, has HAT activity that is crucial for the circadian clock.[32] Therefore, it seems that the plant and mammal circadian systems share a common chromatin-dependent mechanism required for the activation of oscillator genes.

We also found that the oscillations in H3K4me3 were followed by rhythms in H3K4me2, which were antiphasic to H3ac and partially overlapping with H3K4me3. Detailed comparisons revealed clear coexistence of H3K4me2 and H3K4me3 around dusk for the oscillator genes expressed in the morning and around dawn for the genes expressed in the evening. Therefore, our results show the sequential enrichment H3ac→H3K4me3→H3K4me2 that can be temporally associated with the rhythmic expression of the oscillator genes, from peak to trough.

The detailed study of both H3ac and H3K4me3 accumulation in a time course experiment also revealed other interesting features. For instance, the peak of H3K4me3 was delayed in comparison to that of H3K56ac. A similar delay of H3K4me3 accumulation compared with H3K9ac was found at the promoter of the mouse albumin D element-binding protein (DBP).[33] Acetylation seems to precisely coincide with the peak of maximal gene expression, suggesting a main role as an activating mark. The extended accumulation of H3K4me3 and its overlap with both acetylation and H3K4me2 suggest that H3K4me3 might somehow function as a transition mark at the boundaries between activation and repression. Notably, analysis of clock repressor

binding showed that blocking H3K4me3 by pharmacological inhibition increases the binding and enhances gene repression. These results might be relevant for explaining the observed cross-talk between histone acetylation and methylation. We proposed that H3K4me3 might control the timing of repression by preventing an advanced repression phase, and thus ensuring the proper timing for activation by histone acetylation.

The last piece of the study focused on characterizing the histone methyltransferase SDG2/ATXR3 as the possible molecular component responsible for H3K4me3 accumulation at the promoters of the oscillator genes. Our analysis showed a decreased methylation and reduced gene expression in mutant lines while the opposite was observed in plants expressing additional copies of SDG2/ATXR3. Furthermore, clock repressor binding was also affected in these plants, which is fully consistent with the function of H3K4me3 as a transition mark controlling the timing of repression. The effects on repressor binding were reverted by treatment with NAM, suggesting that H3K4me3 accumulation is indeed able to directly modulate the clock repressor binding. Recent findings have shown that the methyltransferase MLL1 mediates H3K4 methylation to regulate CLOCK target genes.[34] Further work would be required to check if the mechanism controlling clock repressor binding by H3K4me3 is conserved in other circadian systems.

Despite the advances in our understanding of the connections between chromatin remodeling and plant circadian function, many challenges are still in front of us. Discovering the chromatin related components and clock effectors that lie at the interface of pre- and post-transcriptional regulation is one of the plausible future objectives.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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