Nucleosome dynamics regulate *Neurospora* circadian clock

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Organisms utilize the circadian clock system to adapt to the predictable changes from day/night cycles. The circadian oscillator is based on transcription feedback loops in which alternate cycles of transcription activation and repression generate rhythmic accumulation of clock gene products and their downstream effectors of physiology. There is growing evidence that dynamic histone accumulation and modification at *cis*-regulatory sites underlie circadian gene transcription. A new study in this issue of *EMBO reports* by Liu and colleagues [1] identified a new factor, named CATP (Clock ATPase), which is necessary for circadian rhythms of histone occupancy at the key oscillator gene *frequency* (*frq*) in the fungus *Neurospora*.

Neurospora and mammals have a similar architecture of the core feedback loop of circadian oscillators. In mammals, the bHLH-PAS (basic helix-loop-helix-Per-ARNT-Sim) transcription factors CLOCK and BMAL1 bind to the promoter region named E-box of *Period* (*Per1*, *Per2* and *Per3*) and *Cryptochrome* (*Cry1* and *Cry2*) genes to drive their transcription. Then, the PER and CRY proteins work as repressors that interact with CLOCK-BMAL1 to inhibit the transcription of *Per* and *Cry* genes, forming the core feedback loop. Furthermore, histone modifications and H3 occupancy at clock gene promoters show circadian oscillation, and many epigenetic modifiers, such as Sirt1, HDAC3, EZH2, MLL1, MLL3 and JARID1a, are reported to stabilize this oscillation [2].

Neurospora crassa has been used to reveal the molecular mechanisms of the circadian clock [3]. It produces asexual conidia in daily paces (conidiation rhythm), and this has been used as readout for circadian rhythms. The PAS-domain-containing transcription factors White Collar-1 (WC-1) and White Collar-2 (WC-2) form the White Collar Complex (WCC) that works similarly to CLOCK and BMAL1 in the mammalian clock (Fig 1). WCC binds to the C-box (Clock-box) in the promoter of the clock gene *frq* to activate its transcription. The presence of C-box elements is not restricted

to *frq*, and is also found in the promoter regions of other clock-controlled genes. The FRQ protein forms a complex with FRQ-interacting RNA helicase (FRH), named FRQ-FRH complex (FFC), which functions similarly to PERs and CRYs in mammals. FFC inhibits transcription of *frq*, contributing to daily activation and repression cycles of *frq* expression. The FRQ protein is phosphorylated by kinases, such as casein kinase I (CKI), casein kinase II (CKII) and calcium/calmodulin-dependent protein kinase I (CAMK1), and

degraded through the ubiquitin-proteasome pathway, mediated by F-box and WD40 repeat-containing protein 1 (FWD-1). Both protein and phosphorylation levels of FRQ show robust circadian rhythms.

As chromatin state shapes transcriptional regulations, DNA methylation, histone modifications and nucleosome assembly/exchange affect transcription of circadian oscillator components. *Neurospora* strains lacking a DNA methyltransferase (*dim-2*), the *frq*-antisense transcript *qrf*, or chromo-domain

Fig 1 | Role of CATP, FRQ and CSW-1 in the circadian oscillation of nucleosome occupancy at the *frq* gene. (A) CATP promotes the removal of nucleosomes at the C-box element in the *frq* promoter to enhance WCC binding for *frq* transcription. (B) Translated FRQ protein and (C) CSW-1 increase the nucleosome occupancy and reduce WCC binding, and this loop cycles once a day to generate a circadian rhythmic expression of *frq*. CATP, Clock ATPase; C-box, Clock-box; CHD1, chromo-domain helicase DNA binding protein 1; CSW-1, Clockswitch; DIM-2, Defective in methylation-2; FRH, FRQ-interacting RNA helicase; *frq*, frequency; SET1, Su(var)3-9, Enhancer-of-Zeste, Trithorax 1; WCC, White Collar Complex.

helicase DNA binding protein 1 (CHD1) show hypo or hyper DNA cytosine methylation at the *frq* locus and an abnormal function of the circadian clock [4]. Hypomethylation in *dim-2* mutants alters resetting of the circadian clock in response to changing lighting conditions, but does not affect period length or robustness of the clock. Hypermethylation of the *frq* locus in *chd1* loss-of-function mutants profoundly dampens FRQ oscillations to a level intermediate between the peak and the trough levels found in wild-type strains. How CHD1 maintains the appropriate DNA methylation state at the *frq* locus is unknown. The recent discovery of a role for the histone H3K4 methyltransferase SET1 in determining the levels of DNA methylation at the *frq* promoter [5] further contributes to the coordinated regulation of the chromatin state in the circadian clock.

Histone H3 and H4 acetylation, nucleosome depletion and histone exchange at the transcriptional start site changes the nucleosome–DNA interaction rendering the DNA accessible to transcription factors and RNA polymerase. Experimentally, such changes are detected by changes in immunoprecipitated chromatin using antibodies against transcription factors, histones and histone variants, and by measuring susceptibility of the chromatin to limited digestion by DNases. Occupancy of the *frq* promoter by WCC and DNase hypersensitivity of this region show circadian oscillation [4]. Increased hypersensitivity to DNase or open chromatin is likely to favour WCC recruitment during transcriptional activation, while compact chromatin facilitates eviction of WCC during transcriptional repression. When WC-2 levels at the *frq* promoter and *frq* transcription begin to rise, an ATP-dependent chromatin remodelling enzyme, CSW-1 (Clockswitch), is also recruited to the *frq* promoter [6]. CSW-1 facilitates chromatin compaction and reduction in WC-2 levels at the *frq* promoter, thus promoting repression. Strains lacking CSW-1 show a relatively open chromatin structure at the *frq* promoter. Consequently, WC-2 is not effectively evicted from the site and *frq* transcription is not fully repressed. If CSW-1 is required to fully compact the chromatin, there must be additional factors to open the chromatin for the next round of activation.

Cha *et al* found such a factor that promotes open chromatin structure at the *frq* promoter. First, by chromatin immunoprecipitation (ChIP) assays, they tested whether histone H3 occupancy at the *frq* C-box show circadian

rhythms. Histone H3 occupancy reflects compactness of the chromatin and show daily changes with a peak at night-time when WCC levels at the *frq* promoter are low. A mutant strain expressing truncated FRQ and with an abnormal circadian rhythm, the *frq⁹* strain, showed lower occupancy of histone H3 at the C-box across a day. Expression of wild-type FRQ resulted in higher H3 occupancy, implying that FRQ promotes nucleosome occupancy at *frq* (Fig 1). These results indicate that chromatin remodelling is regulated by the circadian clock.

So which gene(s)/protein(s) are the responsible remodelling factor(s)? Cha *et al* identified a spontaneous mutant strain named *mut10*, with aberrant conidiation rhythms. In the *mut10* strain, the FRQ protein rhythm, its phosphorylation rhythm and the *frq* promoter activity rhythm are dampened. The responsible gene for this mutant was genetically mapped, and the authors found that it is highly conserved in eukaryotes. The protein product has two ATPase domains and a non-canonical bromodomain, and was therefore named CLOCK ATPase (CATP). The *mut10* strain has a premature stop codon in CATP, and therefore expresses a truncated CATP protein. A *catp* knockout (*catp^{KO}*) strain shows similar circadian phenotypes as the *mut10* strain. Whilst CATP does not affect global transcription, the expression of some WCC target genes is specifically reduced in *catp^{KO}*. The *catp^{KO}* strain shows a lower expression of FRQ, higher H3 occupancy at the *frq* C-box across a day and reduced WCC occupancy at the *frq* C-box, compared with the wild-type strain. A marker for completion of transcription initiation, ser5 phosphorylated RNA polymerase II, and histone H3 acetylation, a marker for transcriptionally active chromatin, were also reduced on the *frq* promoter in *catp^{KO}*. In summary, these results suggest that CATP promotes a relatively open chromatin structure facilitating WCC recruitment and activation of *frq* transcription.

How does CATP function in facilitating an open chromatin structure? At least two scenarios arise: CATP is part of a larger complex that facilitates histone H3 removal or it directly acts on histone H3. The yeast homologue of CATP is known to bind to histone H3 *in vitro* [7], suggesting that CATP might directly affect histone levels. The authors also showed that the *mut10* circadian phenotype could be rescued by exogenously expressing CATP. However, mutant forms with point mutations in the ATPase or bromodomain of CATP could not rescue the conidiation

rhythms of the *catp^{KO}* strain, or the *frq* C-box at histone H3 occupancy, demonstrating that the ATPase and bromodomain of CATP are required for circadian oscillations. Altogether these observations suggest a model in which CATP binds to the histone through its non-canonical bromodomain and uses the ATPase activity to evict or degrade histones, thus promoting a more open chromatin structure.

The suggested function of CATP in creating an open chromatin structure complements the role of CSW-1 in chromatin compaction. Alternating actions of CATP and CSW-1 on the *frq* promoter can generate a circadian rhythm in chromatin structure and constitute a mechanism underlying circadian transcription (Fig 1). Specific recruitment of these factors to oscillator components might be mediated by their association with WCC or FFC complexes. In mammals, chromatin regulation of circadian rhythm has largely focused on recruitment of histone-modifying enzymes to the promoter region by association with some of the circadian transcription factors [2]. The suggested functions of CATP, CSW-1, CHD1, SET1 and *frq*-antisense *qrf* now offer a broad picture in which histone-modifying enzymes, histone exchange/eviction factors, DNA methylation and antisense transcription fine-tune the pace, robustness and adaptability of the circadian clock to ambient conditions. Given the conserved mechanism of circadian transcription between fungi and mammals, the recent discovery of *per* antisense RNA (*asPer*; [8]), and a growing list of chromatin regulators in clock function [2], the future will present a better understanding of the epigenetic regulation of circadian rhythms.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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