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CATP is a critical component of the *Neurospora* circadian clock by regulating the nucleosome occupancy rhythm at the *frequency* locus

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Rhythmic *frq* transcription is essential for the function of the *Neurospora* circadian clock. Here we show that there is a circadian histone occupancy rhythm at the *frq* promoter that is regulated by FREQUENCY (FRQ). Using a combination of forward genetics and genome sequencing, we identify Clock ATPase (CATP) as an essential clock component. Our results demonstrate that CATP associates with the *frq* locus and other WCC target genes and promotes histone removal at these loci to allow circadian gene transcription. These results indicate that the rhythmic control of histone occupancy at clock genes is critical for circadian clock function.

Keywords: circadian clock; nucleosome; chromatin structure; *Neurospora*

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INTRODUCTION

Eukaryotic circadian oscillators are auto-regulatory circadian feedback loops that are based on regulation of transcription and translation [1,2]. The mechanism of the circadian oscillator of the filamentous fungus *Neurospora crassa* is remarkably similar to those of higher eukaryotes [3–5]. In the *Neurospora* clock, the heterodimeric white collar complex (WCC) formed by two PAS-domain transcription factors WHITE COLLAR-1 (WC-1) and WC-2 binds to the promoter of the *frequency* (*frq*) gene and activates its transcription [6–13]. FRQ and its protein partner FRQ-interacting RNA helicase (FRH) form a complex called FFC that inhibits the expression of *frq* both transcriptionally and post-transcriptionally [8,9,14–18]. The inhibition of *frq* transcription by FFC is mediated by FRQ-dependent WC phosphorylation, which represses WC DNA binding activity and promotes its cytoplasmic localization [8,9,18–22]. The rhythmic activation and repression of *frq* transcription allow the endogenous rhythmic expression of *frq*.

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DNA bound by core histones forms nucleosomes, which are the fundamental units of eukaryotic chromatin. Post-translational modifications of histones are the basis of epigenetic regulation that can have important roles in controlling gene transcription. Rhythmic histone acetylation, and histone methylation of clock gene loci and of many clock-controlled genes have been shown [23,24]. In addition to histone modifications, nucleosome occupancy also impacts gene expression [25,26]. In *Neurospora*, two ATP-dependent chromatin-remodelling factors, CLOCK-SWITCH (CSW-1) and chromodomain helicase DNA-binding-1 (CHD1), have been shown to be involved in the clock function by regulating *frq* transcription [10,27]. These proteins presumably function by regulating the chromatin status of the *frq* locus, but how they act is not clear. In addition, a histone H3K4 methyl-transferase is required for normal circadian rhythms [28]. Still, how nucleosome occupancy is controlled by circadian clocks and whether it has a role in the circadian control of gene expression are not clear.

In this study, we show that there is a circadian nucleosome occupancy rhythm at the *frq* promoter that corresponds to the activation and repression of *frq* transcription. By combining forward genetic- and genome-sequencing approaches, we identified Clock ATPase (CATP), a highly conserved eukaryotic protein, as an essential circadian clock component in *Neurospora*. We showed that CATP controls *frq* transcription and WCC binding to the *frq* promoter by regulating the nucleosome occupancy and chromatin status of the *frq* locus and other WCC target genes.

RESULTS AND DISCUSSION

Rhythmic nucleosome occupancy at the *frq* locus

To investigate the involvement of chromatin structure in the regulation of *frq* transcription, we examined the nucleosome occupancy at the c-box, a WCC-binding site in the *frq* promoter, using a chromatin immunoprecipitation (ChIP) assay with a histone H3 antibody. As shown in Fig 1A, the histone H3 occupancy was rhythmic in constant darkness (DD): low around DD14 and high around DD22. This result indicates that nucleosome occupancy rhythm is anti-phase to the known rhythm of WCC binding at the *frq* c-box [8,10]. Nucleosome occupancy is low when the WCC binding is high and the *frq* transcription is

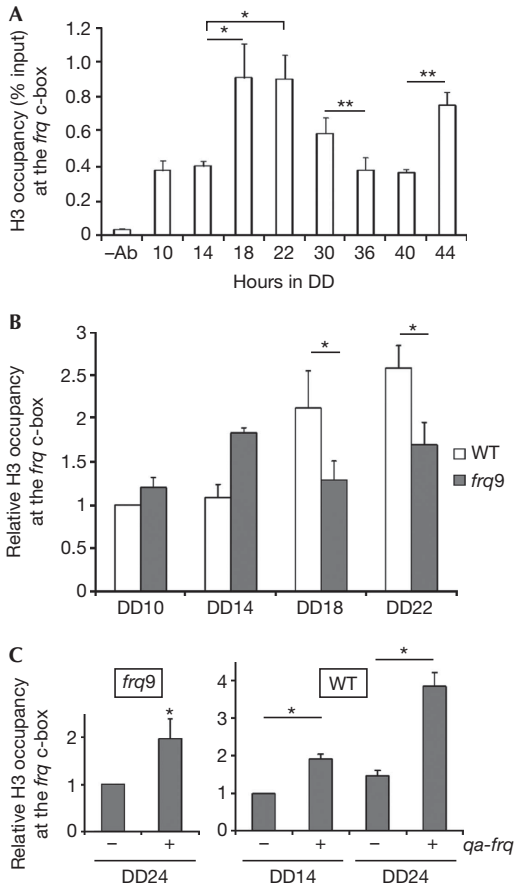

Fig 1 | Nucleosome occupancy at the *frq* c-box shows a circadian rhythm. (**A**) Histone H3 ChIP assay with the WT strain in DD. (**B**) H3 ChIP assay in the WT and *frq*^{9} strains. (**C**) H3 ChIP assays of the *frq*^{9} and WT strains with or without QA-induced FRQ. Mean with standard errors ($n = 3$). *$P < 0.05$, **$P < 0.01$ (paired student's *t*-test). ChIP, chromatin immunoprecipitation; DD, constant darkness; QA, quinic acid; WT, wild type.

activated, and is high when the WCC binding is low and the *frq* transcription is repressed.

To further confirm this result, we performed the histone H3 ChIP assays with the *frq*^{9} strain, in which a premature stop codon results in a truncated FRQ protein and the loss of circadian rhythm [29]. The H3 occupancy rhythm was lost in the *frq*^{9} strain; no histone occupancy increase was observed at DD18 or 22 (Fig 1B). Because FRQ protein levels peak at these time points when *frq* transcription is repressed, this result suggests that FRQ promotes nucleosome formation at the *frq* locus. To test this hypothesis, we induced FRQ expression in *frq*^{9} and wild-type strains that harbour a quinic acid-inducible *frq* construct. As expected, ectopic expression of FRQ resulted in a significant increase in H3 occupancy at the *frq* c-box in both strains (Fig 1C). These results indicate that FRQ is required for high nucleosome occupancy on its own promoter in the repressive phase. Nucleosome occupancy is important in regulating transcription: gene expression is inhibited when nucleosome occupancy is high [25,26]. Therefore, the rhythmic nucleosome occupancy at the *frq* locus likely has a critical role in the circadian negative feedback process.

Identification of a new *Neurospora* clock mutant

In our analyses of different progenies from a routine cross between *Neurospora* strains, we identified a clock mutant strain, *mut10* that arose from a spontaneous mutation. As shown by race tube assay, *mut10* has lost the normal circadian conidiation rhythm: this strain showed a low-amplitude long-period rhythm for the first 2 days and then became arrhythmic (Fig 2A). In addition, the circadian conidiation rhythm was also abolished in the *bd mut10* double mutant (supplementary Fig S1 online). To determine the molecular phenotype of the mutant, we examined the FRQ expression profile at different time points in DD. As shown previously [30], FRQ oscillated in both its level and its phosphorylation profile in a wild-type strain. In contrast, the *mut10* strain only exhibited a very low-amplitude oscillation after the light-to-dark transition, and there was little change in FRQ phosphorylation after DD12 (Fig 2B; supplementary Fig S2A online). Furthermore, we introduced a bioluminescence reporter construct (*frq-luc*) [31], in which luciferase expression is controlled by the *frq* promoter, into the mutant. As shown in Fig 2C, a robust circadian rhythm of luciferase activity was observed in the wild-type strain, but no rhythmic expression of luciferase was observed in the *mut10* strain, indicating that the normal clock function is abolished in the *mut10* strain.

CATP is an essential clock component

To identify the mutation responsible for the clock phenotype in the *mut10* strain, we crossed it with a Mauriceville strain and performed race tube assays to examine the resulting progenies. We then performed cleaved amplified polymorphic sequence analyses utilizing the previously identified cleaved amplified polymorphic sequence markers in the genome [32]. The mutation was mapped to an ~1-Mbp region in the right arm of linkage group III. To identify the mutation, whole-genome sequencing of the *mut10* strain was performed. Comparison of the genome sequence of a wild-type strain with that of *mut10* within the genetically mapped region revealed a single nucleotide change within the open reading frame of NCU06484 (Fig 3A). This mutation results in a nonsense mutation that changes Glu880 to a premature stop codon. The predicted protein product of NCU06484 is highly conserved in eukaryotic genomes from fungi to human and contains two ATPase domains and a non-canonical bromodomain (Fig 3A). Because of its role in the clock and its ATPase domains, this gene was named *catp*, for Clock ATPase.

The premature stop codon in the *mut10* mutant occurs immediately after the first ATPase domain. Thus *mut10* should express a truncated CATP protein without the second ATPase domain and the bromodomain. We generated an antibody against amino terminus of CATP and examined CATP expression in wild-type, *mut10*, and a *catp*^{KO} strain was obtained from the *Neurospora crassa* knockout library [33]. As shown in Fig 3B, the CATP antibody recognized four high-molecular-weight bands in the wild-type strain but not in the *catp*^{KO} strain, indicating that they are CATP protein products. In the *mut10* mutant, the wild-type CATP bands were absent and two main bands of lower molecular weight were observed, indicating that the premature stop codon resulted in truncated CATP products.

As shown in Fig 3C, the race tube phenotype of the *catp*^{KO} strain is very similar to that of the *mut10* strain with the circadian

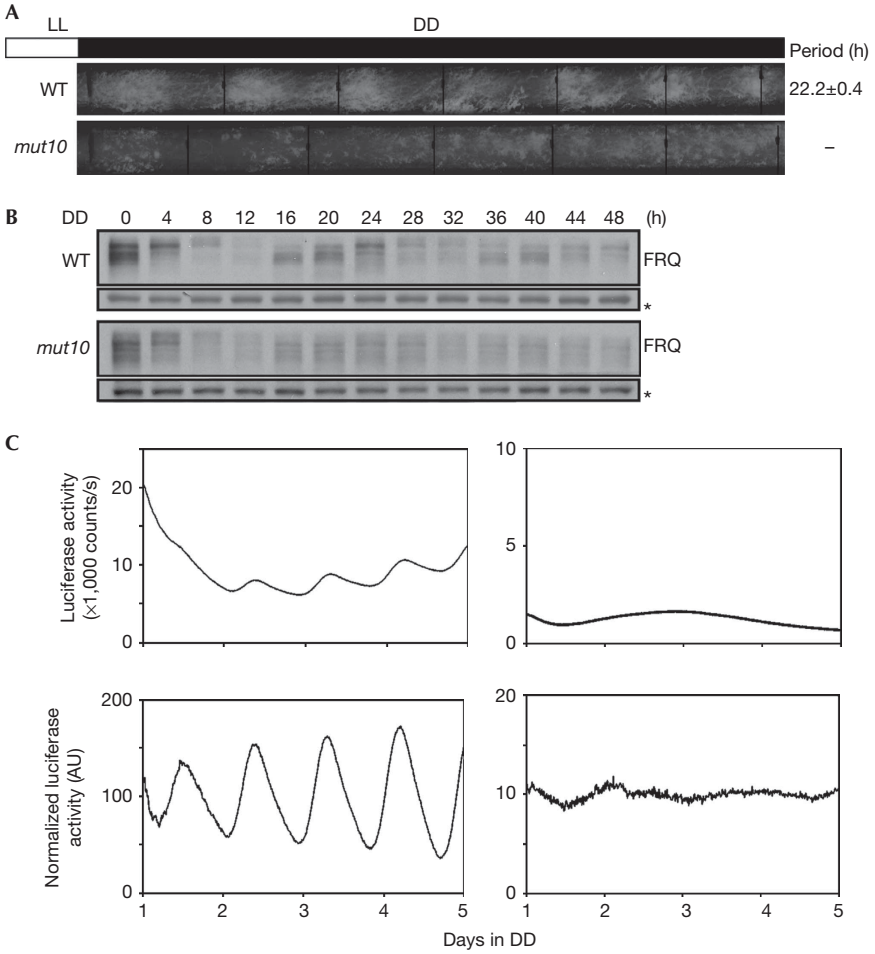

Fig 2 | Loss of normal circadian rhythms in the *mut10* strain. (**A**) Race tube analysis showing the conidiation rhythms in DD. Period of the WT strain is shown as average ± s.e.m. ($n = 6$). (**B**) Western blot analyses showing FRQ expression in the *mut10* strain in DD. Asterisks indicate nonspecific bands. (**C**) Luciferase reporter assay showing the *frq* promoter activity in the WT (left) and *mut10* (right) strains in DD. Raw data (above) were normalized to subtract the baseline calculated by LumiCycle Analysis software. WT, wild type.

conidiation rhythm abolished after 2 days in DD. Furthermore, the circadian conidiation and slower growth phenotype of *mut10* was rescued by introduction of a construct in which wild-type *capt* was expressed under the control of the quinic acid-inducible *qa-2* promoter (Fig 3C). We tried to obtain the *catp*^{KO} *ras-1*^{bd} double mutant and were not successful, suggesting that the *catp*^{KO} strain is sterile probably because of meiotic silencing. Together, these results indicate that CATP is a critical component in the *Neurospora* circadian clock.

CATP positively regulates *frq* expression

We compared FRQ expression in DD in the wild-type strain to that in the *catp*^{KO} strain (Fig 3D; supplementary Fig S2B online). Unlike the robust oscillation of FRQ levels in the wild-type strain, the *catp*^{KO} strain had a very low-amplitude FRQ fluctuation after the light-to-dark transition with no obvious changes in FRQ phosphorylation profiles. In addition, the overall FRQ levels were lower in the mutant. We next examined *frq* mRNA levels in DD by quantitative reverse transcriptase polymerase chain reaction analysis (Fig 3E). The *catp*^{KO} strain exhibited lower *frq* mRNA levels in DD than in the wild-type strain, as the FRQ protein. These lower levels of *frq* mRNA in the *catp*^{KO} strain are consistent with the results of expression of luciferase from the *frq* promoter in the *mut10* strain (Fig 2C). These results indicate that CATP positively regulates *frq* transcription.

We also checked the light-induced gene expression in the *catp*^{KO} strain and found that the light-induction of *frq*, *al-1* and *vvd* expression was near normal (supplementary Fig S3 online), suggesting that the role of CATP is specific for circadian control of gene expression.

CATP is essential for the nucleosome occupancy rhythm

Yta7, the yeast homologue of CATP, is thought to function at the boundary of silent and active chromatin states and interacts with histones as well as histone chaperones [34,35]. To understand the role of CATP, we examined nucleosome occupancy of the *frq* c-box in the *catp*^{KO} strain. In contrast to the rhythmic H3 occupancy in the wild-type strain, the H3 occupancy level in the *catp*^{KO} strain was arrhythmic and constantly high in DD (Fig 4A). The biggest difference in nucleosome occupancy between the two

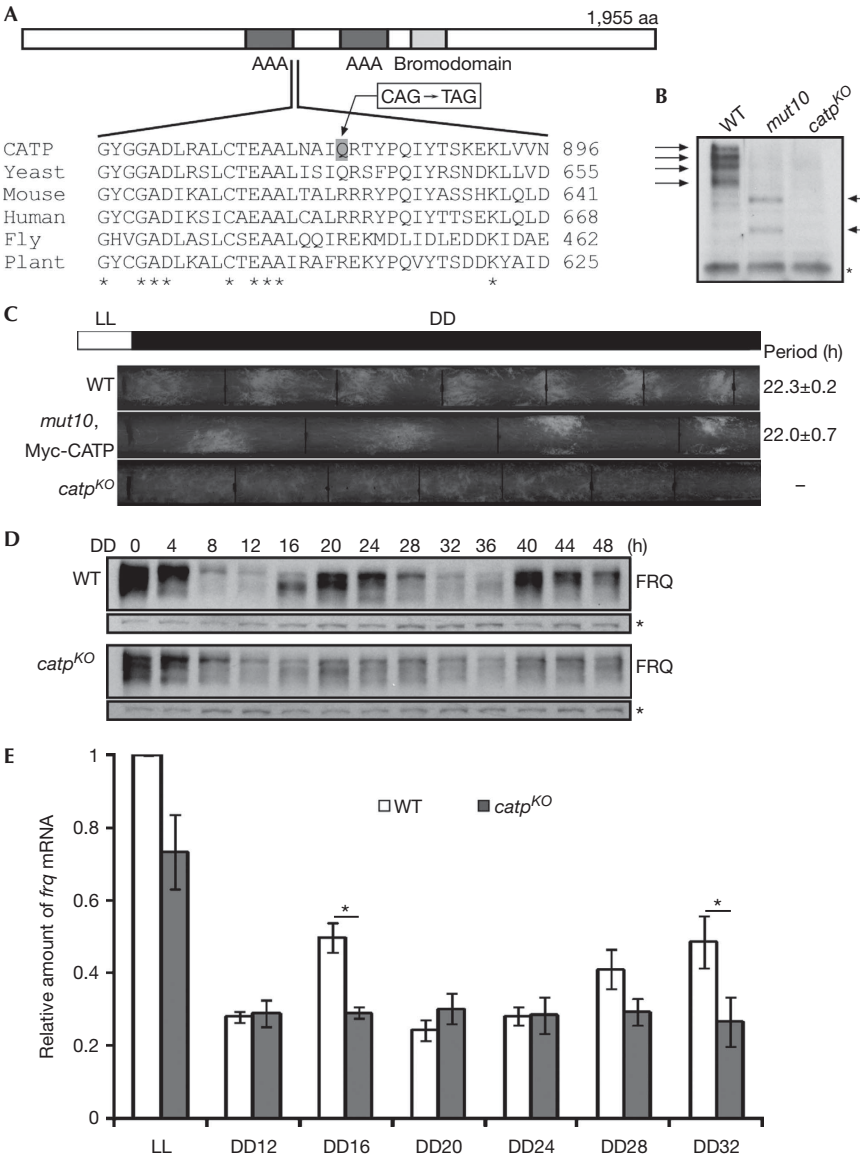

Fig 3 | CATP is a critical component of the *Neurospora* circadian clock. (**A**) Domain structure of CATP and amino-acid sequence alignment of CATP homologues. (**B**) Western blot analysis showing CATP expression (indicated by arrows) in different strains. (**C**) Race tube analysis showing the circadian conidiation phenotype of different strains. Periods are shown as average ± s.e.m. ($n = 6$). (**D**) Western blot analysis showing FRQ expression in DD. (**E**) qRT–PCR results showing that rhythmic expression of *frq* is abolished in the *catp*^{KO} strain. Mean with standard errors ($n = 3$). *$P < 0.05$ (paired student's *t*-test). CATP, Clock ATPase; DD, constant darkness; KO, knockout; qRT–PCR, quantitative reverse transcriptase polymerase chain reaction; WT, wild type.

strains was observed at DD14, a time point when *frq* transcription peaks in the wild-type strain.

A ChIP assay using a WC-2 antibody showed that WCC-binding rhythm at the *frq* c-box was abolished and remained low throughout DD in the *catp*^{KO} strain (Fig 4B). In addition, ChIP assays using an antibody specific for the phosphorylated Ser5 of the carboxy-terminal domain of the largest subunit of RNA polymerase II (pol II) showed that levels of the phosphorylated pol II were significantly reduced in the *frq* open reading frame in the *catp*^{KO} strain compared with those in the wild-type strain (Fig 4C). Because pol II Ser5 phosphorylation is a marker for the completion of transcriptional initiation [36], this result indicates that CATP promotes transcription initiation of *frq*. It was previously shown that histone H3 acetylation, a marker for active chromatin, fluctuates at the *frq* locus [10]. As shown in Fig 4D, the rhythmic histone H3 Lys14 acetylation was abolished and the level of H3 Lys14 acetylation was reduced in the *catp*^{KO} strain. Together, these results indicate that CATP regulates the rhythmic *frq* transcription by controlling nucleosome occupancy and chromatin structure to allow rhythmic activation and repression of *frq* transcription.

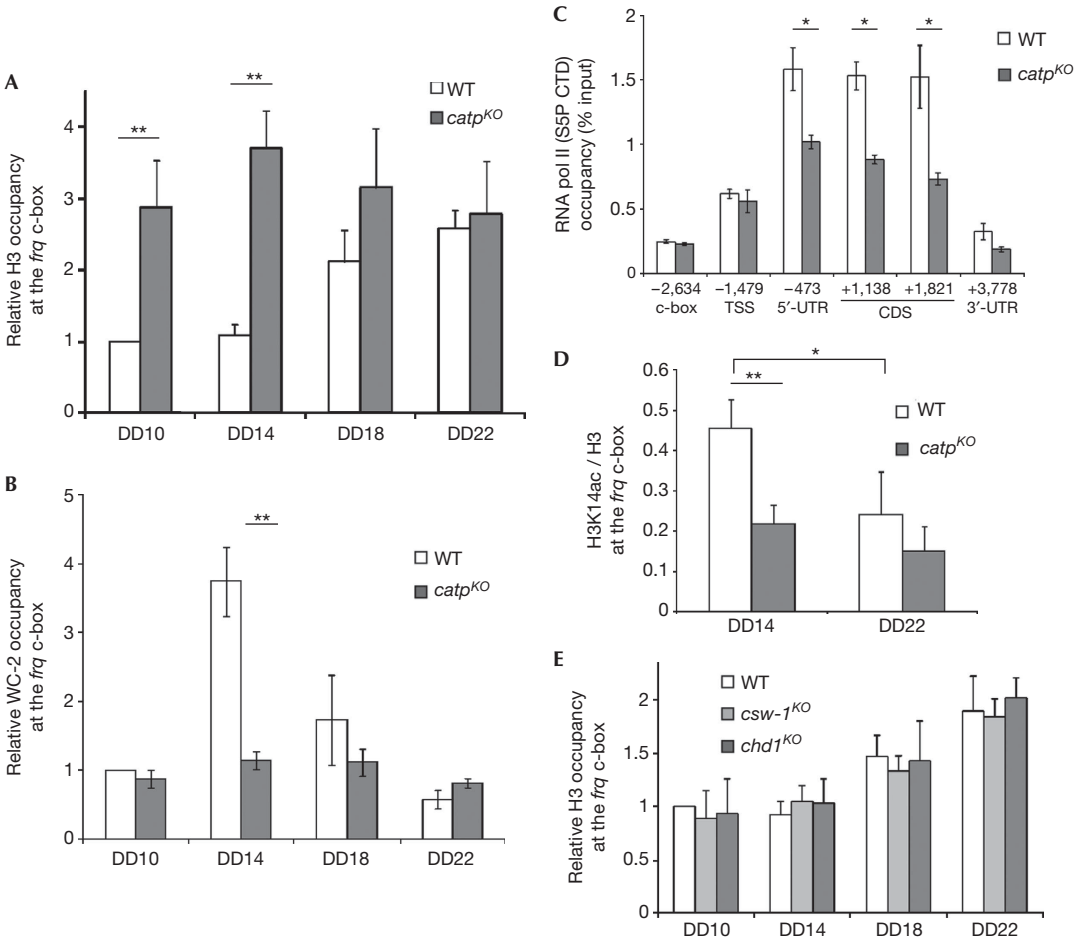

Fig 4 | CATP regulates chromatin structure at the *frq* locus. (**A**) H3 ChIP assay showing histone occupancy in DD. (**B**) WC-2 ChIP assays with the WT and the $catp^{KO}$ strains. (**C**) ChIP assays of the indicated strains using an S5P CTD antibody at DD14. Primer sets corresponding to different *frq* regions with indicated distances from the start codon (+1) were used to analyse pol II occupancies. (**D**) ChIP assays showing H3K14 acetylation at the *frq* c-box. IP/input values of H3K14ac ChIP assays were divided by those of H3 ChIP assays to calculate the ratio of acetylated H3K14. (**E**) H3 ChIP assays with indicated strains. Mean with standard errors ($n = 3$). *$P < 0.05$, **$P < 0.01$ (paired student's *t*-test). CATP, Clock ATPase; ChIP, chromatin immunoprecipitation; DD, constant darkness; IP, immunoprecipitation; KO, knockout; TSS, transcription start site; UTR, untranslated region; WT, wild type.

To examine whether CATP functions in the same pathway as CSW-1 and CHD1 to regulate *frq* transcription, we examined the histone occupancy at the *frq* promoter in the *csw-1* and *chd1* knockout strains. As shown in Fig 4E, the H3 occupancy levels were similar in the wild-type strain and these two mutant strains, indicating that the role of CATP in regulating histone occupancy at the *frq* promoter is independent of these two chromatin-remodelling enzymes.

CATP associates with chromatin at the *frq* locus

ChIP assays using both the Myc-tagged CATP and the antibody against endogenous CATP showed that it binds to the *frq* promoter and open reading frame regions (Fig 5A; supplementary Fig S4A online). These results indicate that CATP associates with chromatin at the *frq* locus, probably by interacting with the histones [34,37]. In addition, these results indicate that the role of CATP in regulating *frq* transcription is specific and is not because of a nonspecific effect of CATP on general transcription.

We then examined whether CATP also acts on other WCC target genes. *hsf-2*, *csp-1* and *adv-1* are directly regulated by WCC [38]. In contrast, *frh* expression is not influenced by WCC [15]. Myc-tagged CATP showed nonspecific binding to the *tubulin* gene (Fig 5A), so it was used as the internal negative control for the ChIP assays (supplementary Figs S4A and S5B online). As shown in Fig 5B, there was significant CATP binding in the promoter regions of *hsf-2* and *csp-1* but not at the *adv-1* and *frh* loci. Consistent with this result, histone H3 ChIP showed that the nucleosome occupancy at the *hsf-2* and *csp-1* loci, but not at *adv-1* and *frh* loci, were significantly higher in the $catp^{KO}$ strain than in the wild-type strain (Fig 5C; supplementary Fig S4B online). Furthermore, quantitative reverse transcriptase polymerase chain reaction analyses showed that the mRNA levels of *hsf-2* and *csp-1* but not if *adv-1* and *frh*, were downregulated in the $catp^{KO}$ strain (Fig 5D; supplementary Fig S4C online). Together, these results indicate that, in addition to *frq*, CATP affects the chromatin structures to regulate transcription of some WC target genes.

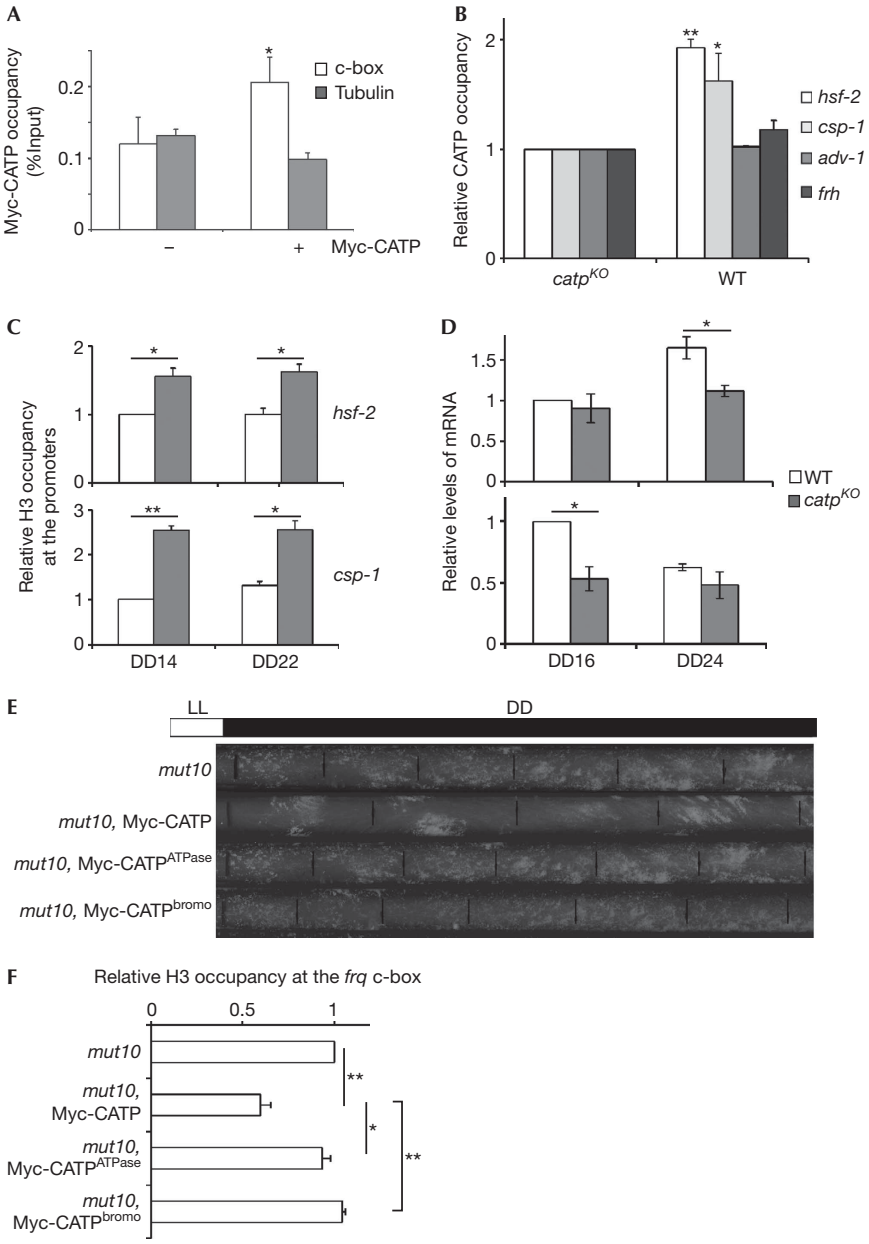

Fig 5 | CATP associates with chromatin and requires its conserved domains for its function. (**A**) c-Myc ChIP assays with the Myc-tagged CATP strain at DD18. (**B**) ChIP assays using anti-CATP antibody to analyse CATP binding to the indicated loci. (**C**) H3 ChIP assays for *hsf-2* and *csp-1* loci in a WT strain at the indicated time points. (**D**) qRT–PCR analyses of *hsf-2* and *csp-1*. (**E**) Race tube assays showing the circadian conidiation rhythms of CATP mutants. (**F**) H3 ChIP assays of the indicated strains in DD14. Mean with standard errors ($n = 3$). $^{*}P < 0.05$, $^{**}P < 0.01$ (paired student's *t*-test). CATP, Clock ATPase; ChIP, chromatin immunoprecipitation; DD, constant darkness; KO, knockout; qRT–PCR, quantitative reverse transcriptase PCR; WT, wild type.

Conserved domains of CATP required for its function

The ATPase domains and the non-canonical bromodomain are conserved in all CATP eukaryotic homologues. We generated Myc-tagged CATP constructs that contain point mutations in the ATPase domain or bromodomain (CATP^{ATPase} and CATP^{bromo}). Although the wild-type CATP construct could rescue the circadian conidiation phenotype of the *mut10* strain, the mutant CATP constructs could not (Fig 5E). H3 ChIP assays showed that the nucleosome occupancy at the *frq* c-box was significantly reduced in the wild-type CATP strain but remained at high levels in the CATP^{ATPase} and CATP^{bromo} strains (Fig 5F). These results demonstrate that the ATPase domain and the bromodomain of CATP are essential for its function in the clock.

Concluding remarks

In this study, we identified CATP as the critical clock component that regulates the nucleosome occupancy at the *frq* locus. Our

results indicate that the nucleosome occupancy rhythm is important for clock function. They indicate that CATP promotes the removal of nucleosomes at the *frq* locus to enhance WCC binding. In contrast, FRQ promotes an increase of nucleosome occupancy at the c-box. Although the expression of CATP is not circadian (supplementary Fig S5 online), the counter balance of these two effects, therefore, results in rhythmic nucleosome occupancy at the *frq* promoter, which allows rhythmic binding of the WCC and rhythmic activation and repression of *frq* transcription. The regulation of nucleosome occupancy at the *frq* locus is, thus, part of the circadian negative feedback process that permits rhythmic transcription.

CATP specifically associates with the *frq* locus, suggesting that CATP regulates nucleosome occupancy directly on chromatin. In addition, CATP also associates with the WCC target genes, *hsf-2* and *csp-1* [38], and modulates the histone occupancy at these loci. Thus, CATP also participates in the circadian output pathway, but it remains to be demonstrated whether CATP acts directly or indirectly on nucleosomes.

Two chromatin remodelers, CSW-1 and CHD1, were previously shown to affect clock function by regulating the chromatin structure at the *frq* locus [10,27]. CHD1 appears to affect the *frq* chromatin structure near the 3′ UTR region, and DNA methylation at the *frq* locus is altered in the *chd1* mutant. On the other hand, micrococcal nuclease I assays showed that there is a rhythm of nuclease sensitivity near the c-box region of the *frq* promoter and CSW-1 appeared to promote the nuclease resistance in this region [10]. However, as the nuclease accessibility rhythms were present in both the wild-type and the *csw-1*^{KO}strains, an additional chromatin-remodelling factor is likely involved in regulating nucleosome occupancy at the *frq* promoter [10]. Interestingly, using ChIP assays, we found that the H3 occupancy at the *frq* c-box region is not altered in either *csw-1* or *chd1* mutants, suggesting that CATP acts differently from CSW-1 and CHD1. The difference between our results and the earlier findings might be because of the use of different assays. Nonetheless, the combination of these results indicates that CATP, CSW-1 and CHD1 act together to maintain the normal chromatin structure at the *frq* locus. In addition, we showed that histone H3K14 acetylation is also reduced in the *catp* mutant (Fig 4D), suggesting that changes in nucleosome occupancy and histone modifications act together to regulate the chromatin state of the *frq* locus.

The mammalian homologue of CATP, ANCCA, is required for the chromatin association of transcriptional activators [39,40]. Mutations in the yeast homologue of CATP, yta7, further result in increased nucleosome occupancy at some genetic loci [41]. These results indicate that the role of CATP homologues in regulating nucleosome occupancy to permit transcription factor binding is conserved. How CATP and its homologues regulate nucleosome occupancy is not clear. It is possible that its non-canonical bromodomain interacts with histones and the ATPase domains generate energy to promote removal or degradation of nucleosomes. Consistent with this model, both Yta7 and ANCCA associate with histones [35,40]. The conservation of circadian clock mechanisms from *Neurospora* to animals suggests that the circadian control of histone occupancy by CATP homologues might also be a critical process of circadian process in other organisms.

METHODS

Luciferase reporter assay. p*frq-luc-I* (a generous gift from Dr Jay Dunlap) was transformed into wild-type and *mut10* strains at the *his-3* locus. The luciferase assay was performed and analysed as previously described [31,42].

ChIP assays. ChIP assays were performed as previously described [22]. Bound DNA was eluted using Chelex 100 resin (Bio-Rad) and subjected to quantitative reverse transcriptase polymerase chain reaction. Occupancies were normalized by the ratio of ChIP to input (histone H3 ChIP assays) or the relative binding to *β-tubulin* gene (internal negative control for WC-2 and CATP ChIP assays).

Construction of point mutations of CATP. To generate mutant constructs of CATP, site-directed mutagenesis was carried out as previously described [43]. The mutated residues ^{700}GTGKT^{704} in *CATP*^{ATPase} were changed to ATAEA; in *CATP*^{bromo},^{1226}DPNF^{1229} was mutated to AAAA. The constructs were transformed into a *mut10*, *his-3* strain at the *his-3* locus.

Supplementary information is available at EMBO *reports* online (http://www.emboreports.org).

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Author contributions: J.C. and Y.L. designed the experiments and wrote the paper; J.C. and M.Z. performed the experiments.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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