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Suppression of WHITE COLLAR-independent *frequency* Transcription by Histone H3 Lysine 36 Methyltransferase SET-2 Is Necessary for Clock Function in *Neurospora*[*]

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The circadian system in *Neurospora* is based on the transcriptional/translational feedback loops and rhythmic *frequency* (*frq*) transcription requires the WHITE COLLAR (WC) complex. Our previous paper has shown that *frq* could be transcribed in a WC-independent pathway in a strain lacking the histone H3K36 methyltransferase, SET-2 (su(var)3–9-enhancer-of-zeste-trithorax-2) (1), but the mechanism was unclear. Here we disclose that loss of histone H3K36 methylation, due to either deletion of SET-2 or *H3K36R* mutation, results in arrhythmic *frq* transcription and loss of overt rhythmicity. Histone acetylation at *frq* locus increases in *set-2*^{KO} mutant. Consistent with these results, loss of H3K36 methylation readers, histone deacetylase RPD-3 (reduced potassium dependence 3) or EAF-3 (essential SAS-related acetyltransferase-associated factor 3), also leads to hyperacetylation of histone at *frq* locus and WC-independent *frq* expression, suggesting that proper chromatin modification at *frq* locus is required for circadian clock operation. Furthermore, a mutant strain with three amino acid substitutions (histone H3 lysine 9, 14, and 18 to glutamine) was generated to mimic the strain with hyperacetylation state of histone H3. *H3K9QK14QK18Q* mutant exhibits the same defective clock phenotype as *rpd-3*^{KO} mutant. Our results support a scenario in which H3K36 methylation is required to establish a permissive chromatin state for circadian *frq* transcription by maintaining proper acetylation status at *frq* locus.

Despite evolutionary distance, circadian clock oscillators are conserved among the organisms to perform their cellular and behavioral activities. The eukaryotic circadian clock oscillator contains positive and negative elements that form auto-regulatory negative feedback loops (2–8).

In the *Neurospora* circadian negative feedback loop, the GATA-family transcription factors WHITE COLLAR-1 (WC-1)[3] and WHITE COLLAR-2 (WC-2) serve as positive elements. Typically, WC-1 and WC-2 form a heterodimer through their Per-Arnt-Sim (PAS) domain and rhythmically bind the promoter of the clock gene *frequency* (*frq*) to activate its transcription. FREQUENCY (FRQ) and its partner FRQ-interacting RNA helicase (FRH) act as negative elements. FRQ is the core factor in *Neurospora* oscillator, which determines the normal clock rhythm (9–16). The translated FRQ interacts with FRH and forms the FFC (FRQ-FRH complex) to inhibit its own transcription by suppressing the activity of the WC complex. FRQ is progressively phosphorylated and degraded through the ubiquitin-proteasome pathway. Degradation of FRQ derepresses the WC complex, initiating a new circle of *frq* transcription (14, 17–19). The circadian oscillator creates a robust rhythmic system with a period of ~22 h in *Neurospora crassa* (17, 20). Rhythmic activation and repression of *frq* transcription result in cyclic changes of *frq* mRNA abundance. In *Neurospora*, histone modifiers and chromatin remodelers are required for rhythmic transcription of *frq* gene and clock-controlled genes (1, 9, 22–24). However, the mechanism of transcriptional regulation of *frq* by different histone modifications is not fully understood.

Although *frq* transcription is WC-dependent, our previous studies have shown that *frq* can be transcribed in a WC-independent manner in the absence of transcriptional corepressors, RCO-1 or RCM-1 (1, 25). Unlike the WC-dependent *frq* transcription, WC-independent *frq* transcription is constitutive, which disrupts the rhythmical WC-dependent *frq* transcription through interference the negative feedback loop. Moreover, our results indicate that histone modifications are required to suppress the WC-independent *frq* transcription. In *rco-1*^{KO} mutant, the histone modifications at *frq* locus are changed, including increased H3K4me3 and H3K9ac, and decreased H3K36me3.

SET-2 is the histone H3K36 methyltransferase in *Neurospora*, which is required for growth and development (26). The homolog of SET-2 in yeast Set2, it directly regulates gene transcription through interacting with phosphorylated RNA po-

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[3] The abbreviations used are: WC, WHITE COLLAR; *frq*, *frequency*; ORF, open reading frame; UTR, untranslated region; C-Box, clock box; PLRE, proximal light-regulated element; TSS, transcription start site; qPCR, quantitative PCR.

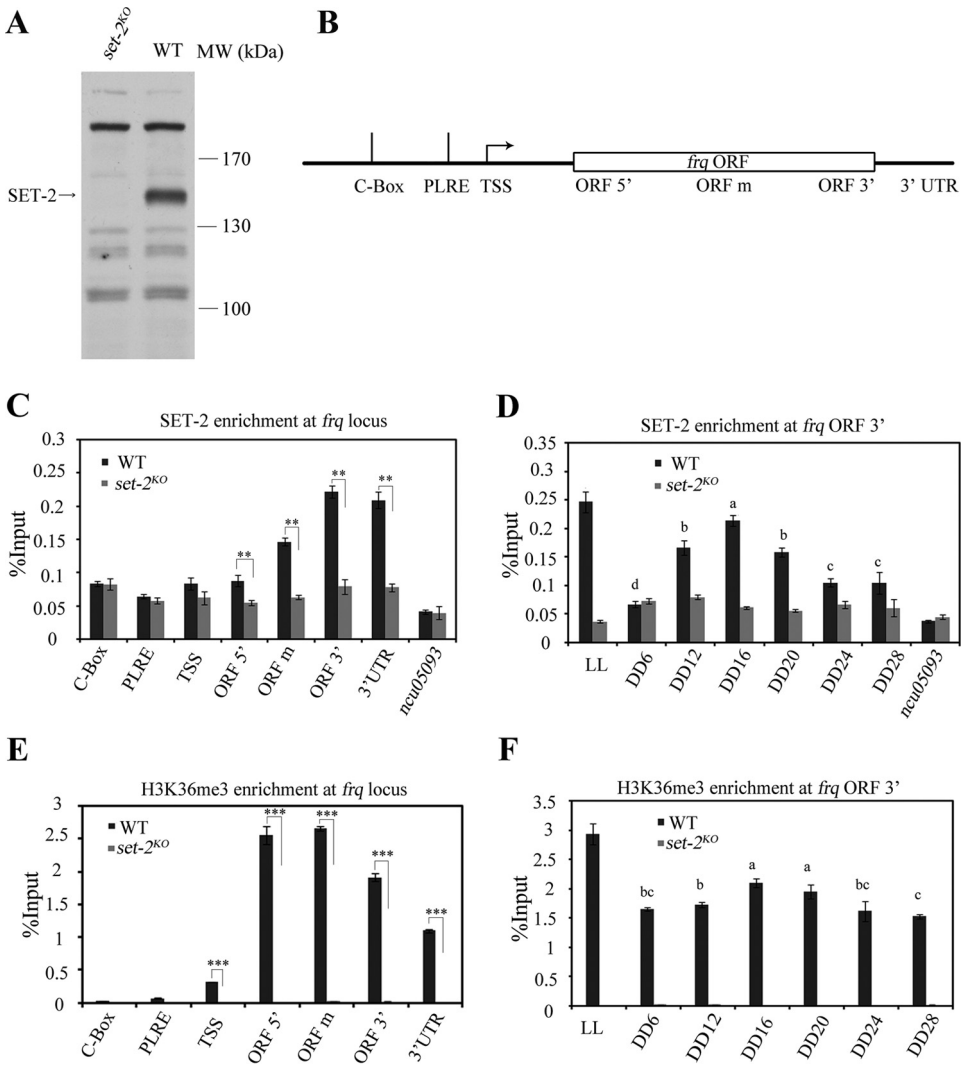

FIGURE 1. **SET-2 is recruited to *frq* gene and regulates its transcription by catalyzing H3K36me3.** *A*, Western blot analysis showing a specific band present in the wild-type strain but not the *set-2*^{KO} strain. The *arrow* points out the SET-2 protein band detected by our SET-2 antibody. *WT*, wild-type; *MW*, molecular weight. *B*, schematic depiction of the *frq* locus. *C*, ChIP analysis showing the enrichment of SET-2 at the *frq* locus in DD16. ChIP experiment performed on chromatin isolated at DD16 when *frq* expression is maximal by using the SET-2 antibody. The amount of DNA associated with SET-2 was determined by qPCR. *D*, rhythmic association of SET-2 at the 3′-end of *frq* gene in different time points. Samples were grown for the indicated hours in constant darkness (*DD*) prior to harvesting and processing for ChIP. *LL*, constant light. *E*, ChIP analysis shows that the H3K36me3 levels are increased at the 3′-end of *frq* in the wild-type strain. *F*, rhythmic changes of H3K36 trimethylation at the 3′-end of *frq* gene in different time points. The *set-2*^{KO} strain was used as a negative control in the ChIP assay, no SET-2 binding at *ncu05093* promoter in *C* and *D* also acts as a negative control. Error bars show the mean ± S.D. ($n = 3$). Significance in *C* and *E* was assessed by using a two-tailed *t* test. *, $p < 0.05$, **, $p < 0.01$; ***, $p < 0.001$. The statistical analysis was also performed on wild-type from DD6 to DD24 in *D* and *F*, bars with different letters in *D* and *F* are statistically significant, $p < 0.05$, one-way Anova and Tukey's HSD test.

lymerase II CTD. During transcription elongation, Set2-mediated H3K36me3 is recognized by the histone deacetylase complex Rpd3S. Rpd3S complex removes the acetyl modification from the histones and maintains the gene body in a hypoacetylation status (27–30). Meanwhile, Set2-mediated H3K36me3 affects the interaction between histone H3 and its chaperones and suppresses histone exchange in nucleosomes of transcribed genes. Histone modifications directly affect the chromatin structures, which play important roles in regulating gene transcription. Soluble histones are predominantly acetylated and increased histone exchange lead to the accumulation of histone acetylation on chromatin. Thus, loss of H3K36me3 results in increased histone acetylation at the open reading frame (ORF) of genes and cryptic transcription from promoters within ORFs (31, 32). Our previous study indicates that RCO-1 functions to regulate H3K36 methylation levels at the *frq* locus to suppress WC-independent *frq* transcription. Consistent with the decreased H3K36me3 and increased H3K9ac at *frq* locus in *rco-1*^{KO} mutant, deletion of *set-2* gene results in WC-independent *frq* transcription and impaired circadian rhythm (1), suggesting that crosstalk of different histone modifications correlates with the rhythmic transcription of *frq* gene. However, the mechanism that SET-2 represses the WC-independent *frq* transcription remains unknown.

Here we show that SET-2 is rhythmically recruited to the *frq* gene body and leads to rhythmic H3K36 methylation at *frq* locus. Loss of SET-2 results in elevated histone acetylation at *frq* ORF, which makes the chromatin more accessible for WC-independent *frq* transcription and disrupts the circadian clock rhythm. Thus, SET-2 is implicated in establish-

ing a permissive chromatin state for circadian transcription of *frq* gene.

Experimental Procedures

Strains and Culture Conditions—The 87-3 (*bd, a*) was used as the wild-type strain in this study. The *bd ku70^{RIP}* strain got previously (14) was used as the host strain for generating the *rpd-3* or *eaf-3* knock-out mutants and *H3K36R* or *H3K9QK14QK18Q* knock-in mutants. The *wc-1^{RIP}*, *wc-2^{RIP}*, and *set-2^{KO}* strains made before (1, 11, 33, 34) were also included in this study. The newly created strains were *rpd-3^{KO} wc-1^{RIP}*, *eaf-3^{KO} wc-2^{RIP}*, *H3K9QK14QK18Q wc-1^{RIP}* double mutants. Liquid culture conditions were the same as recommended (35).

Race Tube Assay—Race tube medium contained 1×Vogel's salts, 0.1% glucose, 0.17% arginine, 50 ng/ml biotin, and 1.5% agar. Conidia of different strains were inoculated at one end of each race tube and were grown under constant light (LL) for 1 day to synchronize the clock. Race tubes were then transferred to constant darkness (DD) and the position of the advancing mycelia front was marked at 24-h intervals on the tube. When growth was completed, tubes were scanned and the period of each strain was calculated (2).

Generation of H3K36R and H3K9QK14QK18Q Mutant Strains—H3K36R and *H3K9QK14QK18Q* mutant strains were generated as described previously (36). A knock-in cassette containing the mutated histone H3 gene and a hygromycin resistance gene (*hph*) inserted downstream of the 3′-untranslated region (UTR) of the H3 gene was transformed into a *ku70^{RIP}* genetic background strain and hygromycin-resistant transformants were selected by hygromycin B. The homokaryotic strains were obtained by microconidia purification and confirmed by DNA sequencing. The *H3K9QK14QK18Q wc-1^{RIP}* double mutants were obtained by crossing.

Generation of Antiserum against SET-2—GST-SET-2 (containing SET-2 amino acids 308–568) fusion protein was expressed in BL21 cells and the recombinant protein was purified and used as the antigen to generate rabbit polyclonal antiserum as described previously (25, 36, 37).

Chromatin Immunoprecipitation—ChIP assay was performed as described previously (1, 25, 36, 38). Briefly, *Neurospora* tissues under the experimental conditions were fixed with 1% formaldehyde for 15 min at 25 °C with shaking, the fixation reaction were stopped by adding 125 mM glycine with shaking for another 5 min. The cross-linked tissues are grinded and resuspended at 1 g/8 ml in lysis buffer containing protease inhibitors (1 mM PMSF, 1 μg/ml leupeptin, and 1 μg/ml pepstatin A). Chromatin was sheared by sonication to ~200-500-bp fragments. 1 ml of protein (2 mg/ml) was used for each immunoprecipitation reaction, and 10 μl was kept as the input DNA. ChIP assay was carried out with 10 μl of SET-2 antibody, 5 μl of H3 antibody (CST 2650), 3 μl of H3K36me3 antibody (abcam ab9050), 4 μl of H3ac antibody (Millipore 06-599) or 10 μl of H4ac antibody (Millipore 06-598). Immunoprecipitated DNA was quantified using real-time PCR. The primer sequences used are C-Box (5′-GTCAAGCTCGTACCCACATC-3′ and 5′-CCGAAAGTATCTTGAGCCTCC-3′), PLRE (5′-CGGACGACGGCTGGCCAATTAG-3′ and 5′-TCGTGCTCTCTTGCTCACTTTCC-3′), TSS (5′- GAGGAACCAGAACGTAGCAG-3′ and 5′-GCAGGATAAACGGAGAAATGAC-3′), ORF 5′ (5′-TTACTTCATCTTCCGCACTGG-3′ and 5′-GGCAGGGTTACGATTGGATT-3′), ORF m (5′-GGACACCTTTCATTACAAACCG-3′ and 5′-TCCGCTAAAATCCCACTTCG-3′), ORF 3′ (5′-GATACCGAGACTGATGTGCG-3′ and 5′-AGCATGTCCACCTCTTTTCC-3′), 3′ UTR (5′-GAGAGCAAAAGGAACGCATTG-3′ and 5′-CTCCCCTGAAAATGGCAAAAG-3′), *ncu05093* (5′-

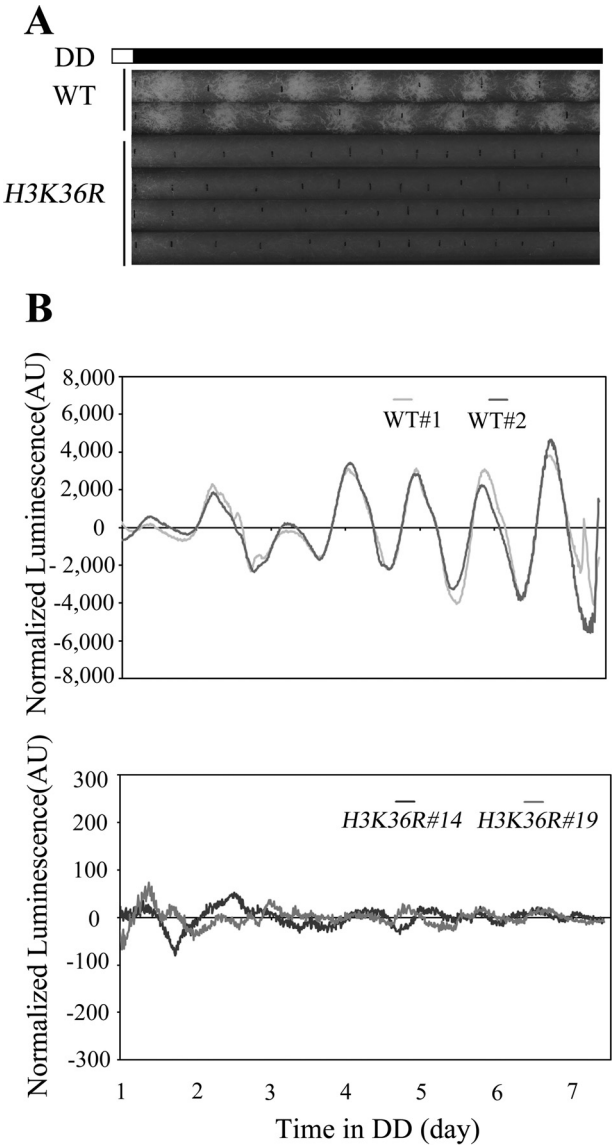

FIGURE 2. **H3K36 methylation is required for circadian rhythm.** Race tube assay of the wild-type and *H3K36R* strains is shown in *A*, *vertical black lines* on race tubes mark daily growth fronts of the strains. The conidiation rhythm in *H3K36R* strains is lost compared with wild-type strains. Four independent *H3K36R* strains were shown in the figure. *B*, analyses of luciferase activity in wild-type, *frq*-luc, and *H3K36R*, *frq*-luc strains show that the luciferase activity rhythm is impaired in the *H3K36R*, *frq*-luc strains. Three independent *H3K36R*, *frq*-luc strains were used to test luciferase activity, and the result of two strains was shown in the figure. The low normalized luciferase signal levels in the *H3K36R*, *frq*-luc strain reflect the low-amplitude fluctuation of luciferase activity. Different strains' conidia suspension was placed on AFV medium and luminescence was recorded in real time using a LumiCycle. Raw data were normalized to subtract the baseline calculated by LumiCycle analysis software.

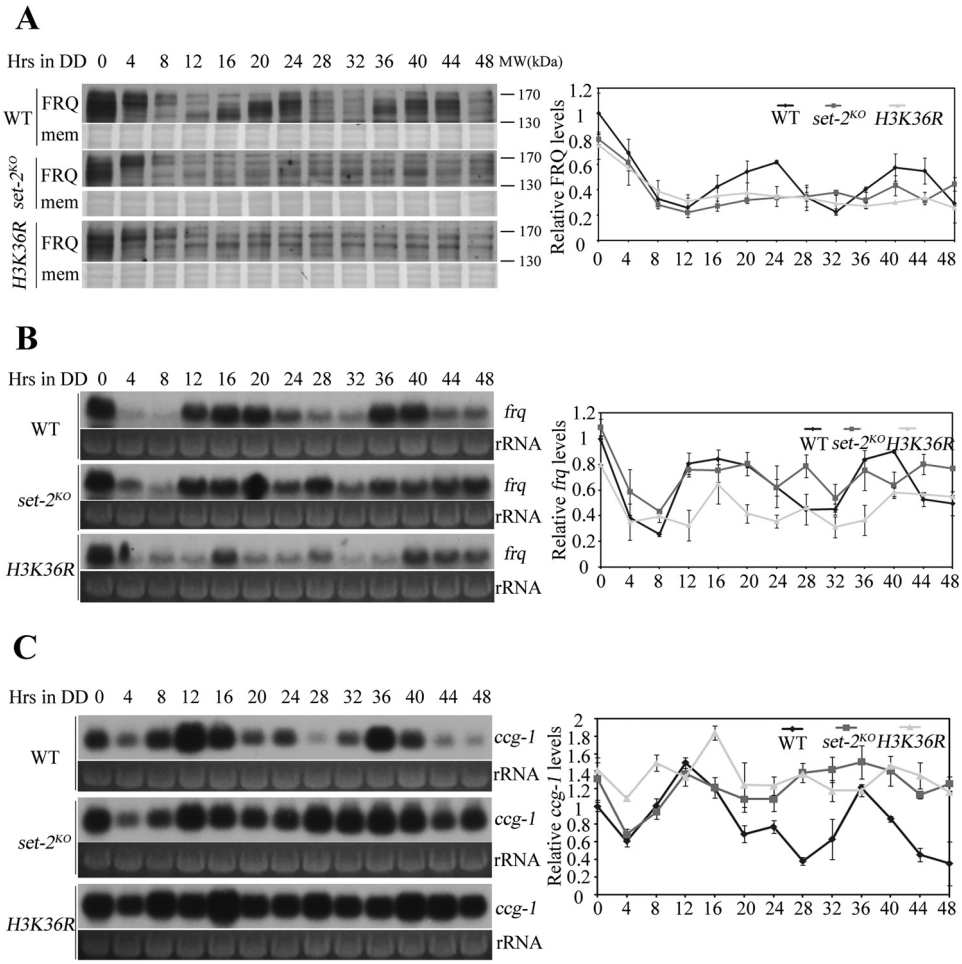

FIGURE 3. **H3K36 methylation is essential for the *Neurospora* circadian clock.** *A*, Western blot analysis shows that the circadian oscillation of FRQ in *set-2*^{KO} and *H3K36R* is impaired when compared with wild-type strain. Samples were grown in constant darkness (*DD*) for indicated hours before harvest. The Coomassie Blue-stained membranes (mem) represent total protein in each sample and act as a loading control. Quantification of the FRQ protein is shown on the right side of Western blot. Northern blot analysis shows that *frq* (*B*) and *ccg-1* (*C*) transcription are arrhythmic in *set-2*^{KO} and *H3K36R* strains. Ribosome RNA (rRNA) bands stained by ethidium bromide shown below the Northern blot act as a loading control for each sample. Quantification of *frq* and *ccg1* are shown on the right side of Northern blot. These experiments were performed at least three times, and one representative result is shown here.

CAAACACAGCAACACTCCAG-3′ and 5′-CAGCCAATACCTCTATCCCAG-3′). ChIP-qPCR (quantitative PCR) data were normalized by the input and presented as percentage of input DNA. Each experiment was independently performed at least three times.

Protein and RNA Analyses—Protein extraction, quantification and Western blot analysis were performed as described previously (39, 40). Western blot analyses were performed by using antibodies against the proteins of interest. Equal amounts of total protein (40 μg) were loaded in each protein lane. After electrophoresis, proteins were transferred onto PVDF membrane, and Western blot analysis was performed. RNA was extracted as described previously and then analyzed by Northern blotting (26). For Northern blot analysis, equal amounts of total RNA (20 μg) were loaded onto 1.3% agarose gels for electrophoresis, the RNAs were transferred to nylon membrane. The membranes were probed with RNA probes specific for *frq* or *ccg-1 gene.*

Luciferase Reporter Assay—The luciferase reporter assay was performed as reported previously (41). The 87-3 (*bd*, *a*), *frq-luc* strain was used as control strain in this study. The *rpd-3*^{KO} or *H3K9QK14QK18Q* strains were crossed with the 87-3 (*bd*, *a*), *frq-luc* strain to obtain the *rpd-3*^{KO}, *frq-luc* strain and *H3K9QK14QK18Q*, *frq-luc* strain. The luciferase reporter construct was transformed into *H3K36R* (*bd*, *a*) strain to obtain *H3K36R, frq-luc* strain. LumiCycle (ACTIMETRICS) and the AFV (autoclaved FGS-Vogel's) medium (1×FGS, 1×Vogel's medium, 50 μg/liter biotin, and 1.8% agar) containing 50 μM firefly luciferin (BioSynt L-8200 D-luciferin firefly [synthetic] potassium salt) were used for the luciferase assay. Conidia suspensions were placed on AFV medium and grown in constant light (LL) overnight to synchronize the clock. The cultures were then transferred to constant darkness, and luminescence was recorded in real time using a LumiCycle after 1 day in DD (under our experimental condition, luciferase signals are highly variable during the first day in the LumiCycle and become stabilized afterward, which is likely due to an artifact caused by the light-dark transfer of the cultures). The data were then normalized with LumiCycle Analysis software by subtracting the baseline luciferase signal, which increases as cell grows.

Results

SET-2 Is Rhythmically Recruited to frq Gene and Results in Rhythmic H3K36me3 at frq Locus—Our previous results showed that SET-2 plays an important role in *Neurospora* circadian system (1). However, the underlying mechanism is not clear. Several studies in yeast exhibited that Set2 is recruited to the open reading frame (ORF) of actively transcribed genes by interacting with phosphorylated CTD of RNA polymerase II (27, 42). To test whether SET-2 directly regulates *frq* transcription, chromatin immunoprecipitation (ChIP) assay was performed by using the SET-2-specific polyclonal antibody (Fig. 1*A*). As there are nonspecific bands recognized by SET-2 antibody, *set-2*^{KO} strain was used as the negative control in the ChIP assay. At DD16 (constant darkness), a time point when *frq* transcription peaks, the signal of SET-2 toward the 3′-end of *frq* gene in the wild-type strain was significantly higher than that in the *set-2*^{KO} strain, while no SET-2 enrichment was detected at the promoter regions of *frq* and *ncu05093* genes (used as a negative control here) in both strains. These results indicate that SET-2 is recruited to *frq* locus during transcription (Fig. 1, *B* and *C*). Moreover, the enrichment of SET-2 at the 3′ region of *frq* ORF was rhythmic in different circadian time points (Fig. 1*D*). In agreement with these results, H3K36 trimethylation (H3K36me3) was rhythmic at increased levels toward the 3′ end of *frq* in the wild-type strain in DD (Fig. 1, *E* and *F*). These results suggest that SET-2 is enriched at *frq* locus and directly regulates *frq* transcription.

SET-2-mediated H3K36me Is Required for Neurospora Circadian Rhythm—To explore whether the effect of SET-2 on circadian clock is mediated by histone methylation, histone *H3K36R* mutant strains were generated by substituting H3 lysine 36 with arginine at its endogenous locus (14, 36). Comparison of the *H3K36R* strain to wild-type on race tubes indicated an apparent defect of conidiation formation and a reduced hyphae growth rate in *H3K36R* mutants (Fig. 2*A*). To further define the function of H3K36 methylation, we introduced a *frq* promoter-driven luciferase reporter into the *H3K36R* strain to examine the *frq* promoter activity (43). As in the *set-2*^{KO} mutant (1), the robust bioluminescence rhythm of the wild-type was abolished in *H3K36R* strains (Fig. 2*B*). These results suggest that H3 lysine 36 is the crucial physiological substrate of SET-2, and the methylation on this residue is required for the *Neurospora* circadian rhythm.

To determine the role of H3K36 methylation in circadian clock, the circadian rhythmicity in *set-2*^{KO} and *H3K36R* strains were molecularly analyzed by examining the expression of FRQ protein and *frq* mRNA in constant darkness (DD). As shown in Fig. 3*A*, the wild-type strain exhibited robust rhythms at FRQ protein level and FRQ phosphorylation profile, but both rhythms were severely disrupted either in *set-2*^{KO} or in *H3K36R* strains. Same as FRQ protein results, the circadian rhythm of *frq* mRNA abundance was abolished in the *set-2*^{KO} and *H3K36R* mutants (Fig. 3*B*). Defects of the core circadian oscillator would affect the rhythmic expression of genes in the output pathway. Indeed, the rhythm of clock-controlled gene *ccg-1* was eliminated in *set-2*^{KO} and *H3K36R* strains (Fig. 3*C*). Taken together,

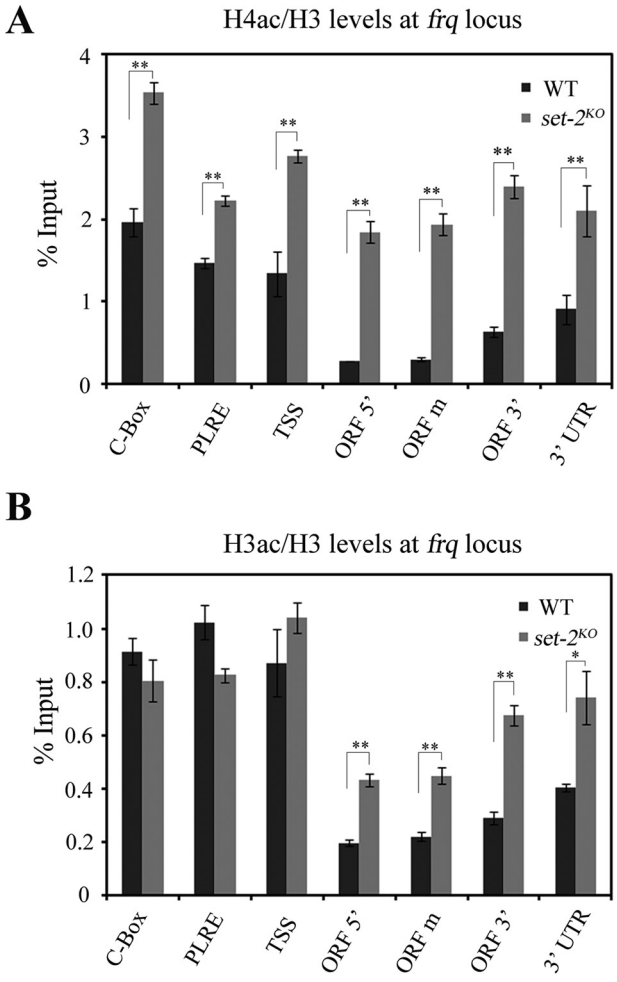

FIGURE 4. **SET-2 directs the histone acetylation state of *frq* ORF.** ChIP analyses of H4ac (*A*) and H3ac (*B*) occurring at the *frq* locus. Both H4ac and H3ac levels at *frq* ORF region are increased in *set-2*^{KO} strains compared with wild-type. ChIP experiment was performed on chromatin isolated at DD16.The amount of DNA associated with H4ac or H3ac was determined by qPCR. Significance was assessed by using a two-tailed *t* test. *, $p < 0.05$; **, $p < 0.01$. Error bars show the mean ± S.D. ($n = 3$).

these data demonstrate that H3K36 methylation is required for sustained clock rhythmicity in *Neurospora*.

Deletion of SET-2 Results in Hyperacetylation of frq Open Reading Frame (ORF)—Why H3K36me3 is required for proper *frq* transcription? In *Saccharomyces cerevisiae*, Set2-mediated H3K36me3 maintains a hypoacetylation state of transcribed genes (28, 29, 31, 32), especially for genes with long sequence and transcribed less often (44). Based on these studies, we wondered whether SET-2 affects the histone acetylation level at *frq* locus. As shown in Fig. 4, *A* and *B*, deletion of *set-2* gene resulted in increased H3 and H4 acetylation levels over *frq* ORF, especially atits 3′ end.These results suggest that SET-2-mediated H3K36 methylation suppresses histone acetylation at *frq* ORF region.

Rpd3S-mediated Deacetylation of frq Coding Region Suppresses WC-independent frq Expression—The deacetylase complex Rpd3S can remove histone acetylation at the coding region of transcribed genes through recognizing histone H3K36me (28, 29). To explore whether H3K36me-dependent deacetylation of *frq* coding region is required for proper *frq* transcription,

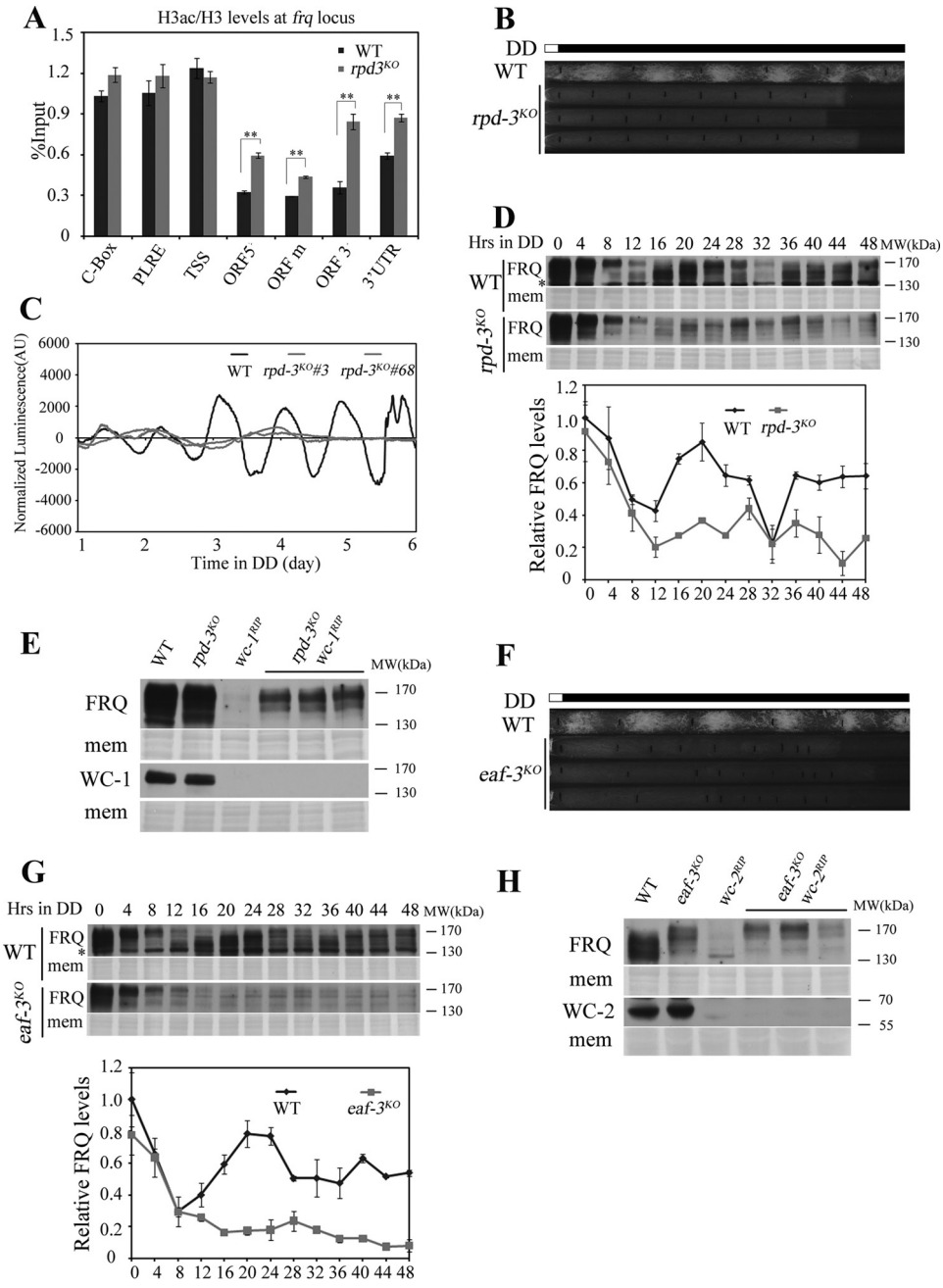

FIGURE 5. **Rpd3S-mediated deacetylation of *frq* coding region suppresses WC-independent *frq* expression.** *A*, H3ac at the *frq* locus was examined by ChIP analysis in wild-type and *rpd-3*^{KO} strains at DD16. The H3 acetylation levels at *frq* ORF region are higher in *rpd-3*^{KO} strain than wild-type. Significance was assessed by using a two-tailed *t* test. *, $p < 0.05$; **, $p < 0.01$. Error bars show the mean ± S.D. ($n = 3$). *B*, conidiation rhythmicity is lost in *rpd-3*^{KO} strains in the race tube assay. *Vertical black lines* on race tubes mark daily growth fronts of the strains. Three independent *rpd-3*^{KO} strains run on race tube were shown in the figure. *C*, luciferase reporter assay showing the normalized *frq* promoter activity of wild-type, *frq-luc* and *rpd-3*^{KO}, *frq-luc* strains. Three independent *rpd-3*^{KO}, *frq-luc* strains were used to test luciferase activity and the result of two strains was shown in the figure. *D*, Western blot analysis shows the circadian oscillation of FRQ in *rpd-3*^{KO} is impaired when compared with wild-type strain. Samples were grown in darkness (*DD*) for indicated hours before harvest. The Coomassie Blue-stained membranes (*mem*) represent total protein in each sample. *Asterisks* indicate nonspecific bands. The experiment was performed independently three times and one representative result was shown in the figure. *E*, Western blot analyses were performed using antibodies against FRQ or WC-1 in the wild-type, *rpd-3*^{KO}, *wc-1*^{RIP}, and *rpd-3*^{KO} *wc-1*^{RIP} strains. Samples were grown under constant light before harvest and three different *rpd-3*^{KO} *wc-1*^{RIP} double mutants were used. The Coomassie Blue-stained membranes (mem) represent total protein in each sample. *F*, race tube assay of wild-type and *eaf-3*^{KO} strains. Three independent *eaf-3*^{KO} strains were shown in the figure. *G*, Western blot analysis showing the circadian oscillation of FRQ in the wild-type and *eaf-3*^{KO} strains. Both FRQ protein and phosphorylation rhythm in wild-type are abolished in the *eaf-3*^{KO} strain. Samples were grown in constant darkness (*DD*) for indicated hours before harvest. The Coomassie Blue-stained membranes (mem) represent total protein in each sample. *Asterisks* indicate nonspecific bands. The experiment was performed independently three times and one representative result was shown in the figure. *H*, Western blot analyses were performed using antibodies against FRQ or WC-2 in the wild-type, *eaf-3*^{KO}, *wc-2*^{RIP}, and *eaf-3*^{KO} *wc-2*^{RIP} strains. Samples were grown under constant light before harvest and three different *eaf-3*^{KO} *wc-2*^{RIP}double mutants were used. The Coomassie Blue-stained membranes (mem) represent total protein in each sample.

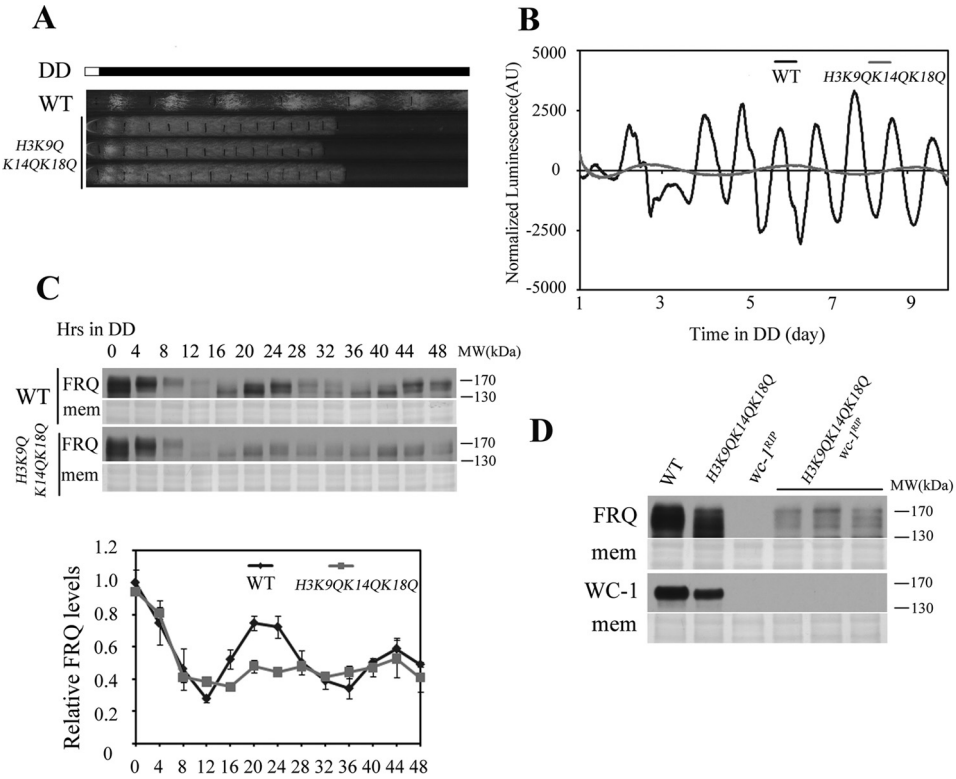

FIGURE 6. **The mimic acetylation states of histone H3 regulate *frq* transcription.** *A*, *H3K9QK14QK18Q* strain lost the robust conidiation rhythm compared with WT in the race tube assay. Three independent *H3K9QK14QK18Q* strains were shown in the figure. *B*, analyses of luciferase activity in wild-type, *frq-luc* and *H3K9QK14QK18Q, frq-luc* strains. Three independent *H3K9QK14QK18Q, frq-luc* strains were used to test the luciferase activity and all strains lost the rhythmic luciferase activity compared with wild-type. One wild-type, *frq-luc* and one *H3K9QK14QK18Q, frq-luc* strain result was shown in the figure. *C*, Western blot analysis showing the circadian oscillation of FRQ in the wild-type and *H3K9QK14QK18Q* mutant strains. Samples were grown in constant darkness (*DD*) for indicated hours before harvest. The Coomassie Blue-stained membranes (*mem*) represent total protein in each sample. The experiment was performed independently at least three times, and one representative result was shown in the figure. *D*, Western blot analyses were performed using antibodies against FRQ or WC-1 in the wild-type, *H3K9QK14QK18Q*, *wc-1*^{RIP}, and *H3K9QK14QK18Q wc-1*^{RIP} strains. Samples were grown on constant light before harvest and three different *H3K9QK14QK18Q wc-1*^{RIP} strains were used. The Coomassie Blue-stained membranes (mem) represent total protein in each sample.

we deleted the histone deacetylase gene *rpd-3* (*ncu00824*) from the *Neurospora* genome. As illustrated in the *set-2*^{KO} strain, the histone H3 acetylation levels were elevated after deletion of *rpd-3* (Fig. 5*A*). The race tube assay showed that disruption of *rpd-3* gene resulted in loss of circadian conidiation rhythm (Fig. 5*B*). We also noticed the slow growth rate of *rpd-3*^{KO} mutants with sparse conidia. So the luciferase reporter driven by *frq* promoter was introduced into the *rpd-3*^{KO} strain to confirm its phenotype at the molecular level. Fig. 5*C* showed that the robust circadian rhythm of luciferase activity in the wild-type was abolished in *rpd-3*^{KO} strains. Western blot analysis confirmed the disrupted clock function of the *rpd-3*^{KO} mutant as both the FRQ protein level and FRQ phosphorylation profile rhythms were lost (Fig. 5*D*). The impaired circadian phenotype prompts us to detect WC-independent *frq* expression in the *rpd-3*^{KO} strain. The *rpd-3*^{KO} *wc-1*^{RIP} double mutant strains were generated, and FRQ expression was observed in these double mutants but not in *wc-1*^{RIP} single mutant (Fig. 5*E*). These results indicate that histone deacetylase RPD-3 is required for suppressing WC-independent *frq* expression.

In budding yeast, the recognition of methylated H3K36 by Rpd3S complex requires the Eaf3 subunit (28, 30). To examine whether the suppression of WC-independent *frq* expression by RPD-3 is dependent on Rpd3S complex, the *eaf-3*^{KO} (*ncu06787*) strain was created. Like *rpd-3*^{KO} strains, the conidiation rhythm was abolished in the *eaf-3*^{KO}mutants (Fig. 5*F*), along with the loss of rhythmic FRQ expression (Fig. 5*G*). In addition, FRQ protein was detectable in *eaf-3*^{KO} *wc-2*^{RIP} double mutant strains (Fig. 5*H*), suggesting that the Rpd3S complex is involved in suppressing WC-independent *frq* expression.

Sustained Hyperacetylation of Histone H3 Results in Arrhythmic Circadian Clock—Previous study has shown that *Neurospora* RPD-3 affects the acetylation levels of histone H3 on Lys-9, Lys-14, and Lys-18 (45). To test whether histone acetylation at these sites is essential for circadian clock, the histone H3 lysine 9, 14, and 18 were substituted with glutamine to mimic hyperacetylated histone H3. In the race tube assay, *H3K9QK14QK18Q* mutant showed arrhythmic conidiation formation compared with wild-type strain (Fig. 6*A*). The luciferase assay showed that the rhythmic *luciferase* transcription driven by *frq* promoter in *H3K9QK14QK18Q* strains were dramatically impaired (Fig. 6*B*). Consistent with the luciferase data, rhythmic FRQ protein expression profile was also dampened (Fig. 6*C*), indicating that *H3K9QK14QK18Q* mutation affects *Neurospora* circadian clock. Similar to that in *rpd-3*^{KO} *wc-1*^{RIP} double mutants (Fig. 5*E*), WC-independent *frq* expression was detected in *H3K9QK14QK18Q wc-1*^{RIP} strains (Fig. 6*D*). These results suggest that histone acetylation is important in maintaining *Neurospora* circadian clock by regulating WC-independent *frq* expression.

Discussion

Rhythmic *frq* transcription is not only a consequence but also a major basis for the circadian negative feedback loop in *Neurospora*. Our previous work has shown that there is WC-independent *frq* transcription in *rco-1*^{KO} mutants (1). Moreover, the constitutive transcription of WC-independent *frq* interferes with the negative feedback loop and disrupts the circadian oscillator in *rco-1*^{KO} mutants. Therefore, it is necessary to repress WC-independent *frq* transcription for normal clock function. Obviously, it is of great importance to find out how this is achieved in the wild-type strain.

In this study, we found that maintaining a proper histone acetylation status at *frq* locus is essential for the repression of the WC-independent *frq* transcription. First, loss of SET-2 led to increased histone acetylation levels at the *frq* locus and WC-independent *frq* transcription (1). Second, deletion of *rpd-3*, subunit of the Rpd3S complex, also resulted in hyperacetylation at *frq* locus. Last, *frq* could be expressed independent of WC complex in the *H3K9QK14QK18Q* mutant, which mimics hyperacetylation of histone H3. In mammals, histone acetylation was found to be important in regulating circadian gene transcription (46–49). However, what we found here is different from those studies. Previous studies mainly focus on the rhythmic changes of histone acetylation at the promoter region of clock genes (46–51), but in our system the histone acetylation at ORF of the clock gene is important for its transcription.

In yeast, Set-2 regulates the histone acetylation level of transcribed gene through different pathways. One is by recruiting the Rpd3S complex, and another one is by suppressing histone exchange through chromatin remodelers Isw1 (imitation switch protein 1) and Chd1 (chromo-domain helicase DNA-binding protein 1) (28, 29, 31, 32). In *Neurospora*, SET-2 functions through similar pathways to maintain a proper histone acetylation status at *frq* locus during transcription. As shown in this work, deletion of *rpd-3* led to increased histone acetylation levels and WC-independent *frq* expression. Our previous study showed that chromo-domain helicase DNA-binding protein 1 (CHD-1) was also required for the repression of the WC-independent *frq* transcription in *Neurospora* (1), suggesting that histone exchange is also important in maintaining hypoacetylated histone pattern at *frq* locus. These results suggest that the Rpd3S complex and CHD-1 may work together to maintain a proper acetylation status across *frq* ORF.

The SET-2 protein and its activity are conserved across species. Obviously, *frq* is not the only gene that SET-2 directly regulated. In 2005, Adhvaryu *et al.* have found that SET-2 is required for *Neurospora* growth and development (26), indicating that SET-2 regulate genes in these processes. Furthermore, previous study in *Saccharomyces cerevisiae* has shown that ~25% of the total genome displayed a significant increase of H4ac following deletion of Set-2. The genes with longer sequence and less often transcribed display a stronger dependence on the Set2-Rpd3S pathway (44). These studies indicate that SET-2 may have a global effect on gene regulation. In this study, our results showed that similar yeast SET-2 pathway exists in *Neuropora*, but the function of this pathway on *frq* transcription is quite different from those described in yeast. One major function of the Set-2 pathway in yeast is to suppress cryptic transcription originated from transcribed ORF. However, cryptic transcripts from *frq* locus were not found in the *set-2*^{KO} mutants (21). Instead, SET-2 is required to regulate the transcription of the full-length *frq* gene, which is supposed to be transcribed from its normal promoter (based on the length of *frq* mRNA in wild-type and *set-2*^{KO} mutant strains).

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