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Role for Protein Kinase A in the *Neurospora* Circadian Clock by Regulating White Collar-Independent *frequency* Transcription through Phosphorylation of RCM-1

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Rhythmic activation and repression of clock gene expression is essential for the eukaryotic circadian clock functions. In the *Neurospora* circadian oscillator, the transcription of the *frequency* (*frq*) gene is periodically activated by the White Collar (WC) complex and suppressed by the FRQ-FRH complex. We previously showed that there is WC-independent *frq* transcription and its repression is required for circadian gene expression. How WC-independent *frq* transcription is regulated is not known. We show here that elevated protein kinase A (PKA) activity results in WC-independent *frq* transcription and the loss of clock function. We identified RCM-1 as the protein partner of RCO-1 and an essential component of the clock through its role in suppressing WC-independent *frq* transcription. RCM-1 is a phosphoprotein and is a substrate of PKA *in vivo* and *in vitro*. Mutation of the PKA-dependent phosphorylation sites on RCM-1 results in WC-independent transcription of *frq* and impaired clock function. Furthermore, we showed that RCM-1 is associated with the chromatin at the *frq* locus, a process that is inhibited by PKA. Together, our results demonstrate that PKA regulates *frq* transcription by inhibiting RCM-1 activity through RCM-1 phosphorylation.

Circadian clocks allow prokaryotic and eukaryotic organisms to regulate their daily molecular, cellular, behavioral, and physiological activities. The eukaryotic circadian clocks are composed of autoregulatory negative-feedback loops, including positive and negative elements that form the core circadian oscillators (1–6). Rhythmic transcriptional activation of the negative elements by the positive elements and rhythmic repression of positive elements by the negative elements are thought to be the main molecular basis for the generation of endogenous rhythmicity in eukaryotic clock systems. In addition to regulation at the transcriptional level, posttranslational modification of clock proteins by phosphorylation plays essential roles in clock functions (7–10).

In the filamentous fungus *Neurospora crassa*, the core circadian negative feedback loop is composed of the positive elements, White Collar 1 (WC-1) and White Collar 2 (WC-2), and the negative elements, FREQUENCY (FRQ) and its partner FRQ-interacting RNA helicase (FRH) (2, 3, 11, 12). WC-1 and WC-2, two Per-Arnt-Sim (PAS) domain-containing transcription factors, form the WC heterodimeric complex that binds to the Clock (C)-box of *frq* promoter to activate *frq* transcription (13–18). On the other hand, FRQ and FRH form the FFC complex to inhibit *frq* transcription by suppressing the activity of the WC complex by promoting WC phosphorylation (19–24). FRQ is progressively phosphorylated over time before its degradation through the ubiquitin–proteasome pathway mediated by FWD-1 (21, 25–28). Genetic analyses show that the FRQ-dependent phosphorylation of WC-1 and WC-2 that suppresses White Collar complex (WCC) activity is mostly mediated by CKI and CKII (21).

WC complex was long thought to be the only transcriptional activator of *frq* transcription. However, we recently discovered that *frq* transcription can occur in a WC-independent manner, and the suppression of the WC-independent *frq* transcription by RCO-1 is essential for circadian clock function (29). The homolog of RCO-1 in *Saccharomyces cerevisiae* is Tup1, a component of the Tup1-Ssn6 complex that represses gene expression (30, 31). Even though our previous results suggest that RCO-1 regulates the chromatin status at the *frq* locus, RCO-1 does not appear to be associated with the *frq* locus. Therefore, the mechanism of how RCO-1 represses WC-independent *frq* transcription and how it affects the chromatin status are not clear.

The cyclic AMP (cAMP)-dependent protein kinase A (PKA) was previously shown to act as a FRQ-independent WC kinase and serve as a priming kinase for the casein kinase-dependent WC phosphorylation, a process that inhibits WC complex activity and stabilize WC proteins (32). However, this interpretation cannot fully explain the molecular phenotype of the *mcb* mutant, in which PKA activity is high as a result of the mutated PKA regulatory subunit. Despite the dramatically reduced WC complex binding activity at the *frq* promoter in the *mcb* mutant, FRQ expression levels are at an intermediate level in constant darkness (DD) (32). These results suggest that PKA may have another role in regulating FRQ expression.

We show here that elevated PKA activity in *Neurospora* induces WC-independent *frq* transcription. Protein purification of RCO-1 identified RCM-1/Ssn6p as the protein partner of RCO-1 that is

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essential for clock function and is also required for suppressing WC-independent *frq* transcription. In addition, we demonstrated that RCM-1 is a substrate of PKA and the PKA-dependent phosphorylation of RCM-1 regulates the transcription repression activity of RCM-1 on *frq* expression. Furthermore, RCM-1 is associated with DNA at the *frq* locus, suggesting a mechanism for how the RCM-1/RCO-1 complex functions to suppress *frq* transcription.

MATERIALS AND METHODS

Strains and culture conditions. The 87-3 (*bd*, *a*) and 301-6 (*bd*, *his-3*, *A*) strains were used as the wild-type strains in the present study. The *bd* $ku70^{RIP}$ strain generated previously (21) was used as the host strain for creating the *pkac-1* or *pde-2* knockout mutant. The *bd* $pkac\text{-}1^{KO}$ or $pde\text{-}2^{KO}$ strain was created by deleting the entire PKAC-1 or PDE-2 open reading frame (ORF) through homologous recombination using a protocol described previously (33). An *mcb* strain (FGSC 7094) was obtained from the Fungal Genetic Stock Center and crossed with a *bd* strain to create the *bd mcb* strain. The $rcm\text{-}1^{RIP}$ strains were created by the repeat-induced point mutation (RIP) method (34). A positive transformant (*bd*, *A*) with a plasmid containing *qa-2* promoter driven the RCM-1 ORF and its 3′ untranslated region (3′-UTR) at its *his-3* locus was crossed with a wild-type strain (*bd*, *a*) to get the $rcm\text{-}1^{RIP}$ strain. The $rco\text{-}1^{KO}$, $wc\text{-}1^{KO}$, $wc\text{-}2^{KO}$, and *frq9* strains generated previously (14, 18, 29, 35) were also used in the present study. The newly created *mcb* $wc\text{-}1^{KO}$, *mcb* $wc\text{-}2^{KO}$, $pde\text{-}2^{KO}$ $wc\text{-}2^{KO}$, $rcm\text{-}1^{5E}$ $wc\text{-}2^{KO}$, $rcm\text{-}1^{RIP}$ $wc\text{-}1^{KO}$, $rcm\text{-}1^{RIP}$ $wc\text{-}2^{KO}$, $rcm\text{-}1^{RIP}$ *frq9*, and $rco\text{-}1^{KO}$ *frq9* double mutants were obtained by crossing.

The medium for race tube assays contained 1× Vogel's salts, 0.1% glucose, 0.17% arginine, 50 ng of biotin/ml, and 1.5% agar with or without quinic acid (QA). Liquid culture conditions were as described previously (36, 37).

Luciferase reporter assay. The luciferase reporter assay was performed as reported previously (29, 38, 39). The 87-3 (*bd*, *a*), *frq-luc* strain was used as the control strain. The $rcm\text{-}1^{RIP}$ strain was crossed with the 87-3 (*bd*, *a*), *frq-luc* strain to obtain the $rcm\text{-}1^{RIP}$, *frq-luc* strain.

Creation of the *rcm-1* knock-in strains. To create the $rcm\text{-}1^{KI\text{-}WT}$ strain, a wild-type knock-in cassette with a hygromycin resistance gene (*hph*) was inserted into downstream of the *rcm-1* 3′-UTR (see Fig. 7A). The cassette was then transformed into the *bd* $ku70^{RIP}$ strain to select for *hph*-resistant transformants. The homokaryotic strains were obtained by microconidial purification and confirmed by DNA sequencing (21). Using this method, we created four *rcm-1* knock-in strains at the endogenous locus by homologous recombination: the $rcm\text{-}1^{KI\text{-}WT}$, $rcm\text{-}1^{2A}$, $rcm\text{-}1^{5A}$, and $rcm\text{-}1^{5E}$ strains. In the $rcm\text{-}1^{2A}$ strain, S718 and S733 were mutated to alanine residues, whereas five identified RCM-1 phosphorylation sites (S718, S733, S630, S682, and T536) were mutated to alanine residues in the $rcm\text{-}1^{5A}$ strain or to glutamic acids to mimic phosphorylation at these residues in the $rcm\text{-}1^{5E}$ strain.

Generation of antiserum against PKAC-1 or RCM-1. The glutathione *S*-transferase (GST)/PKAC-1 fusion protein (containing PKAC-1 amino acids 1 to 318) or GST/RCM-1 (containing RCM-1 amino acids 480 to 883) was expressed in BL21 cells, and the recombinant proteins were purified and used as the antigens to generate rabbit polyclonal antisera as described previously (36, 37). RCO-1 antiserum was generated previously (29).

Protein and RNA analyses. Protein extraction, quantification, Western blot analysis, and immunoprecipitation assays were performed as previously described (40, 41). Nuclear and cytoplasmic protein extracts were prepared as previously described (23). For Western blot analyses, equal amounts of total protein (40 μg) were loaded in each protein lane. After electrophoresis, proteins were transferred onto polyvinylidene difluoride membrane, and Western blot analysis was performed. To monitor PKA phosphorylation *in vivo*, substrate proteins were precipitated under denaturing conditions, with protease and phosphatase inhibitors present at all steps. The level of PKA phosphorylation was then assessed by Western blotting with an anti-PKA substrate antibody (Cell Signaling, catalog no. 9621). RNA was extracted as described previously and then analyzed by Northern blotting (29). For Northern blot analysis, equal amounts of total RNA (20 μg) were loaded onto agarose gels for electrophoresis, and the gels were blotted and probed with RNA probes specific for *frq* or *ccg-1*. For Western and Northern blot analyses, each experiment was independently performed at least three times.

***In vitro* phosphorylation.** For the *in vitro* phosphorylation assay, GST/RCM-1 fusion protein were purified from *Escherichia coli* BL21 cells and incubated with bovine PKA (Promega) or partially purified Myc/His/PKAC-1 from *Neurospora*. Standard reaction mixtures (total 30 μl) were set up containing 5 μg of substrate and 0.5 μg of kinase in the reaction buffer containing 20 mM Tris-HCl (pH 7.4), 10 mM $MgCl_2$, 25 μM ATP, and 3 μCi of [γ-^{32}P]ATP for 30 min at 30°C with or without cAMP (1 μM). The proteins were separated by SDS-PAGE, and the level of phosphorylation was assessed by autoradiography. Each experiment was independently performed at least three times.

Purification of RCM-1 from *N. crassa*, followed by MS/MS. RCM-1 was immunoprecipitated using RCM-1 antibody from wild-type strain in the presence of protease and phosphatase inhibitors in the extraction buffer. The precipitates were analyzed by SDS-PAGE (4 to 20%), which was subsequently stained with colloidal blue. The colloidal blue-stained RCM-1 protein bands were excised from SDS gels and subjected to in-gel digestion with trypsin. The phosphopeptide sequences and sites of phosphorylation were identified by nano-electrospray tandem mass spectrometry (MS/MS).

ChIP analyses. Chromatin immunoprecipitation (ChIP) assays were performed as described previously, and 1 ml of protein (2 mg/ml) was used for each immunoprecipitation reaction (29). The ChIPs were carried out with 2 μl of WC-2 antibody or RCM-1 antibody. Immunoprecipitated DNA was quantified using real-time PCR. The primer sets used are listed in a previously published study (29). Occupancies were normalized to a sample of input DNA (10 μl), and data are presented as the percentage of input DNA. Each experiment was independently performed at least three times.

RESULTS

MCB, the regulatory subunit of PKA, is required for suppressing WC-independent *frq* transcription. To identify new components that are involved in the regulation of the WC-independent *frq* transcription, we generated double mutants by crossing the wc^{KO} single mutants with some of the known *Neurospora* clock mutants. *mcb* encodes the regulatory subunit of PKA that inhibits PKA activity. A previously isolated mutant, *mcb*, had high PKA activity due to a small deletion in the *mcb* regulatory region that results in low *mcb* expression levels (32). In this *mcb* mutant, the circadian oscillations of FRQ protein levels and FRQ phosphorylation patterns were completely abolished and WC binding to the *frq* promoter in DD was dramatically reduced, an observation consistent with a role for PKA in phosphorylating WCC to inhibit its activity. However, the levels of FRQ expression were constant at an intermediate level in the *mcb* strain (32), suggesting another unknown role of PKA in regulating *frq* expression. To test this hypothesis, we created *mcb* $wc\text{-}1^{KO}$ and *mcb* $wc\text{-}2^{KO}$ double mutants and determined the expression levels of *frq* and FRQ in these strains that were cultured in constant light (LL). Western blot analyses showed that no WC-1 or WC-2 signals and decreased levels of MCB proteins were detected in the *mcb* $wc\text{-}1^{KO}$ or *mcb* $wc\text{-}2^{KO}$ double mutants, respectively, confirming their genetic identity (Fig. 1A). In contrast to the almost undetectable FRQ expression levels in the $wc\text{-}1^{KO}$ or $wc\text{-}2^{KO}$ single mutant, the *mcb* wc^{KO} double mutants expressed significant amounts of FRQ protein. For cultures grown in DD (Fig. 1B), constant FRQ ex-

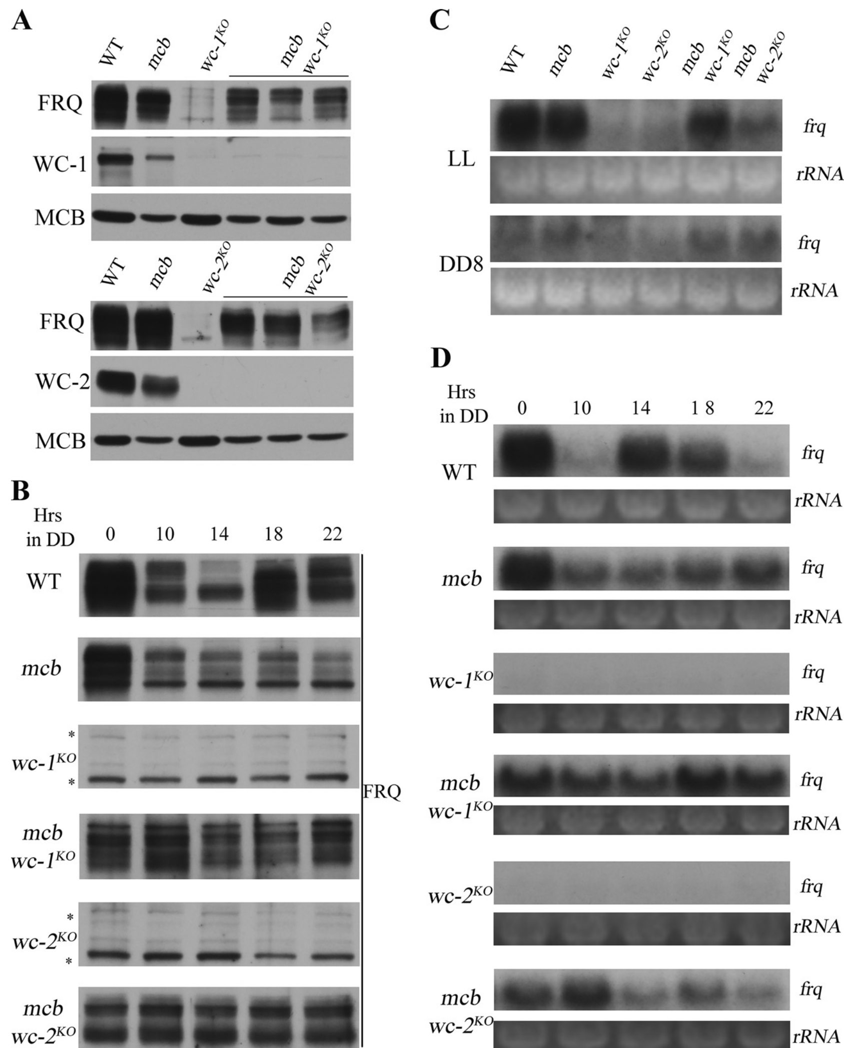

FIG 1 MCB is required for suppressing WC-independent *frq* transcription. (A) Western blot analyses were performed with antibodies against FRQ, WC-1, WC-2, or MCB in wild-type, *mcb*, *wc-1^{KO}*, *wc-2^{KO}*, *mcb wc-1^{KO}*, and *mcb wc-2^{KO}* strains. Three different *mcb wc-1^{KO}* and *mcb wc-2^{KO}* strains were used. (B) Western blot analysis of FRQ in wild-type, *mcb*, *wc-1^{KO}*, *wc-2^{KO}*, *mcb wc-1^{KO}*, and *mcb wc-2^{KO}* strains. Asterisks indicate nonspecific bands. (C) Northern blot analysis of *frq* mRNA in wild-type, *mcb*, *wc-1^{KO}*, *wc-2^{KO}*, *mcb wc-1^{KO}*, and *mcb wc-2^{KO}* strains in LL and DD8. (D) Northern blot analysis of *frq* mRNA in wild-type, *mcb*, *wc-1^{KO}*, *wc-2^{KO}*, *mcb wc-1^{KO}*, and *mcb wc-2^{KO}* strains at different time points in DD.

pression at an intermediate levels were observed at different time points in the *mcb wc^{KO}* double mutants. Similar results were also seen in the Northern blot analyses, which showed intermediate levels of *frq* mRNA in the *mcb wc^{KO}* double mutants in DD that are comparable to those in the *mcb* single mutant in DD (Fig. 1C and D), suggesting that FRQ expression in the *mcb* single mutant is mostly WC independent in DD. Together, these results demonstrate that MCB is required for the suppression of WC-independent *frq* transcription.

Elevated PKA activity results in WC-independent *frq* transcription. In *Neurospora*, *pkac-1* (NCU00682) and *pkac-2* (NCU06240) encode the PKA catalytic subunits, and PKAC-1 is the major catalytic subunit (32, 42). To determine whether the WC-independent *frq* transcription in *mcb* mutant is due to the increase of PKA activity but not due to a role of MCB in another process, we introduced a QA-inducible Myc-His-tagged PKAC-1 construct into the wild type or a *pkac-1^{KO}*, *wc-1^{KO}*, or *wc-2^{KO}* single mutant strain. Race tube assay showed that the induction of Myc/His/PKAC-1 expression in the *pkac-1^{KO}* strain by QA (10^{-2} M) was able to rescue the growth and circadian conidiation defects of the *pkac-1^{KO}* strain (Fig. 2A), indicating that Myc-His-tagged PKAC-1 was functional. Interestingly, race tube assay showed that the induction of Myc/His/PKAC-1 expression by QA (10^{-2} M) in the wild-type strain significantly reduces the conidiation rhythm (Fig. 2B), a phenotype that is similar to but not as severe as the *mcb* mutant (Fig. 2E). As shown in Fig. 2C and D, the induction of Myc-His-PKAC-1 in the *wc-1^{KO}* or *wc-2^{KO}* strain results in FRQ and *frq* expression in the *wc-1^{KO}* or *wc-2^{KO}* strain in LL and DD. It should be noted that the *qa-2* promoter is not a strong promoter that can only express Myc/His/PKAC-1 to a level that is similar to the endogenous PKAC-1 in these *wc^{KO}* mutants (Fig. 2C). This may explain the modest levels of *frq* and FRQ expression in the *wc^{KO}* mutants after induction.

pde-1 (NCU00237) and *pde-2* (NCU00478) encode cAMP

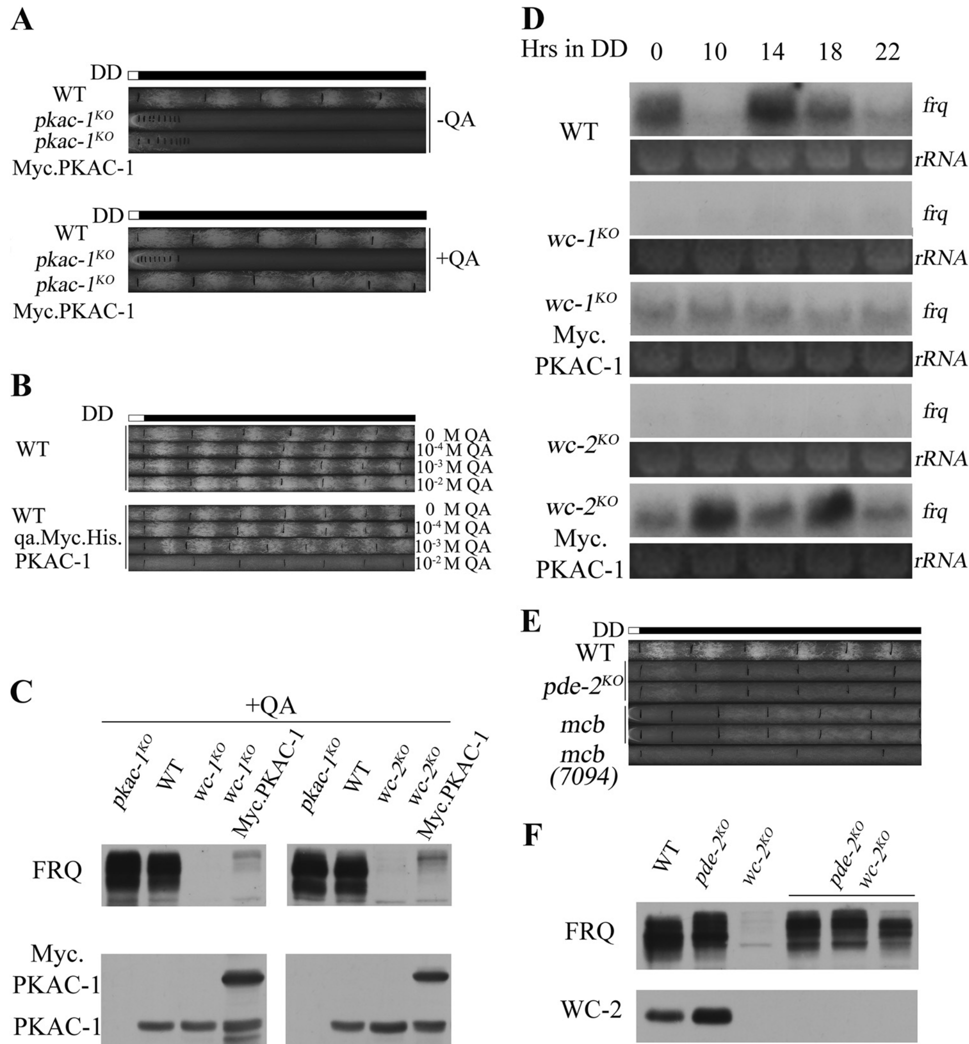

FIG 2 Elevated PKA activity results in WC-independent *frq* transcription. (A) Circadian conidiation rhythm of the wild-type and *pkac-1^{KO}* strains and the *pkac-1^{KO}*, qa-Myc-His-PKAC-1 transformants in the race tube in the presence of QA (10^{-2} M). (B) Race tube assays of wild-type and wild-type, qa-Myc-His-PKAC-1 strains in the presence of QA in DD. (C) Western blot analyses were performed using antibodies against FRQ or PKAC-1 in wild-type, *pkac-1^{KO}*, *wc-1^{KO}*, and *wc-2^{KO}* strains and in *wc-1^{KO}*, qa-Myc-His-PKAC-1, and *wc-2^{KO}*, qa-Myc-His-PKAC-1 transformants in the presence of QA (10^{-2} M). (D) Northern blot analysis of *frq* mRNA in wild-type, *wc-1^{KO}*, and *wc-2^{KO}* strains and in *wc-1^{KO}*, qa-Myc-His-PKAC-1, and *wc-2^{KO}*, qa-Myc-His-PKAC-1 transformants in the presence of QA (10^{-2} M). (E) Race tube assays of wild-type, *mcb*, and *pde-2^{KO}* strains in DD. (F) Western blot analyses were performed using antibodies against FRQ or WC-2 in wild-type, *pde-2^{KO}*, *wc-2^{KO}*, and *pde-2^{KO} wc-2^{KO}* strains. Three independent *pde-2^{KO} wc-2^{KO}* strains were used.

phosphodiesterase in *Neurospora*, and PDE-2 is the major high-affinity enzyme that can inhibit PKA activity (43, 44). To further confirm our conclusion, we created a *pde-2^{KO}* strain. As shown by race tube assay, the circadian conidiation rhythm was initially dampened and later entirely abolished in the *pde-2^{KO}* strain, which is similar to that of the *mcb* mutant, suggesting that the deletion of *pde-2* resulted in elevated PKA activity (Fig. 2E). To examine whether the WC-independent *frq* transcription in the *pde-2^{KO}* strain is also impaired, we generated *pde-2^{KO} wc-2^{KO}* double mutants. As shown in Fig. 2F, nearly wild-type levels of FRQ expression were seen in the *pde-2^{KO} wc-2^{KO}* double mutants in LL. Taken together, these results demonstrate that PKA promotes WC-independent *frq* transcription.

RCM-1 is the partner of RCO-1 and is required for suppressing WC-independent *frq* transcription. In *S. cerevisiae*, the homolog of RCO-1, Tup1, forms a complex with Ssn6 to repress gene expression (30, 31). To examine whether the *Neurospora* Ssn6 homolog RCM-1 (NCU06842) has a similar role, we generated a RCM-1 antibody and used it to purify RCM-1 from *Neurospora* by immunoprecipitation. As shown by colloidal blue-stained SDS-PAGE gel in Fig. 3A, RCM-1 coprecipitated with another protein, which was identified as RCO-1 by mass spectrometry analysis. These results confirm that RCO-1 and RCM-1 form a complex, as previously shown by Sancar et al. and as suggested by Olmedo et al. (45, 46).

To examine the role of RCM-1 in the clock, we generated *rcm-1* disruption mutants by repeat-induced point mutation (RIP) (34). As shown by Western blotting in Fig. 3B, the expression of RCM-1 was abolished in the *rcm-1^{RIP}* strains. DNA sequencing revealed that they contained many G-C to A-T point mutations in the *rcm-1* gene, including several premature stop codons in the RCM-1 ORF (Fig. 3C). Race tube assays showed that the *rcm-1^{RIP}*

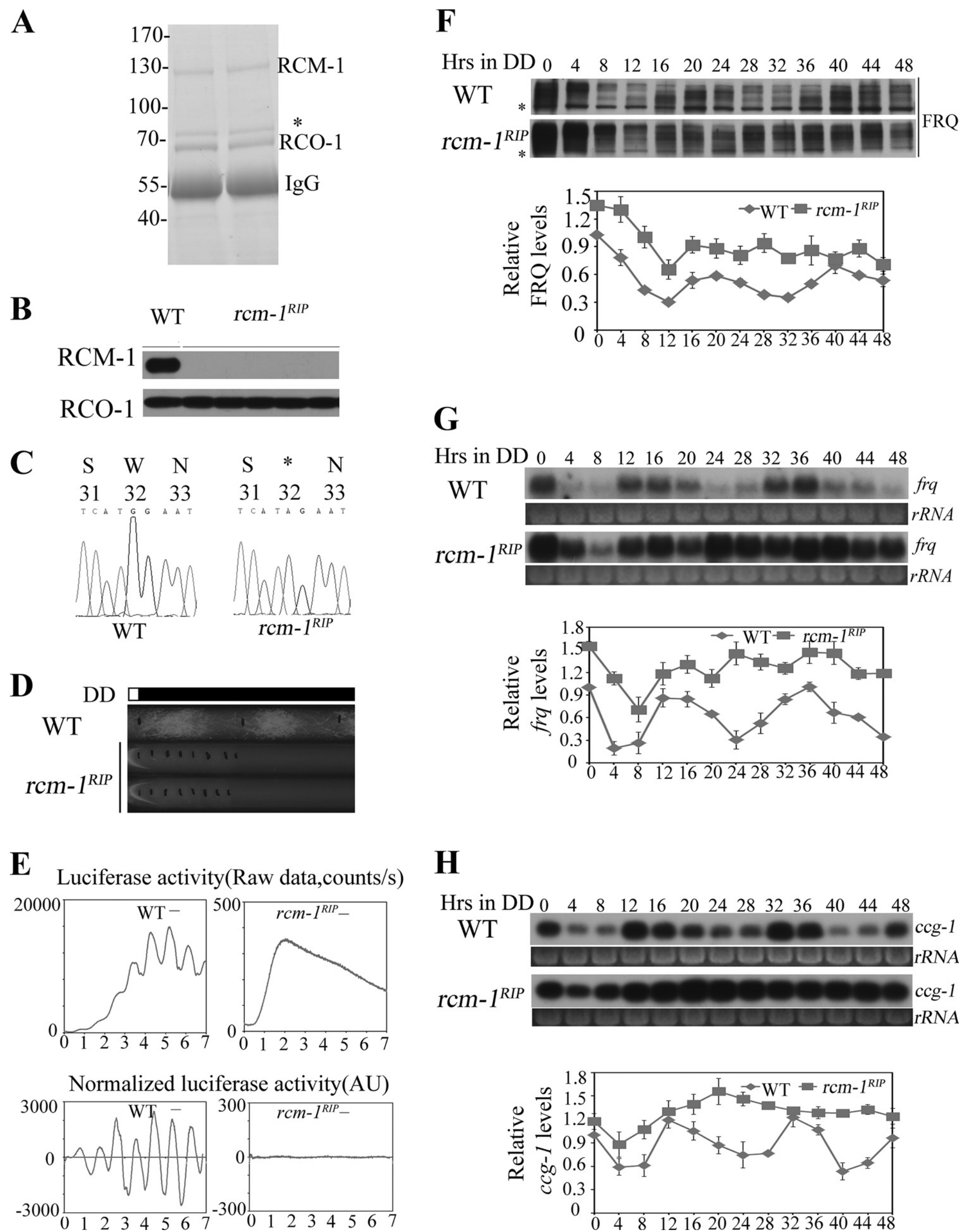

FIG 3 RCM-1 is required for rhythmic *frq* transcription. (A) The colloidal blue-stained SDS-PAGE gel showing the purification of the RCM-1/RCO-1 complex from the wild-type strain. The asterisk indicates a nonspecific band. (B) Western blot analysis showing that there is no RCM-1 signal in *rcm-1*^{RIP} strains, while the levels of RCO-1 protein are not affected by mutation of *rcm-1*. (C) DNA sequencing showing that the *rcm-1*^{RIP} strain contains many G-C to A-T point mutations in the *rcm-1* allele, including premature stop codon (a replacement of a TGG codon by a TAG stop codon at nucleotide +32) in the RCM-1 ORF of one *rcm-1* allele. (D) Race tube assays of wild-type and *rcm-1*^{RIP} strains in DD. (E) Luciferase reporter assay showing the *frq* promoter activity in WT (left) and *rcm-1*^{RIP} (right) strains grown in DD for several days. Raw data were normalized to subtract the baseline calculated by LumiCycle analysis software (38, 39). (F) Western blot analysis of FRQ protein in wild-type and *rcm-1*^{RIP} strains grown in the dark for the indicated times. The asterisks indicate nonspecific bands. (G) Northern blot analysis of *frq* mRNA in wild-type and *rcm-1*^{RIP} strains. (H) Northern blot analysis of *ccg-1* mRNA in wild-type and *rcm-1*^{RIP} strains.

strains exhibited a slow-growth phenotype and lacked a conidiation rhythm (Fig. 3D), which are similar to those of the *rco-1*^{KO} mutants (29). To confirm the lack of circadian rhythm at the molecular level, we introduced a *frq* promoter driven luciferase reporter into the *rcm-1*^{RIP} strain (38). As shown in Fig. 3E, the robust circadian rhythm of luciferase activity seen in a wild-type strain was abolished in the *rcm-1*^{RIP} strain.

Western blot of FRQ in DD showed that, after the initial light/dark transition, FRQ levels stayed high after DD16 and there was no obvious rhythm of FRQ phosphorylation profile (Fig. 3F). Northern blot analysis of *frq* and *ccg-1* mRNA showed that the circadian rhythms of *frq* and *ccg-1* mRNA were also abolished in the *rcm-1*^{RIP} strain and the levels of *frq* mRNA were constantly high (Fig. 3G and H), a finding consistent with a role for RCM-1 in suppressing *frq* expression. Together, these results indicate that RCM-1, like RCO-1, is required for a functional clock.

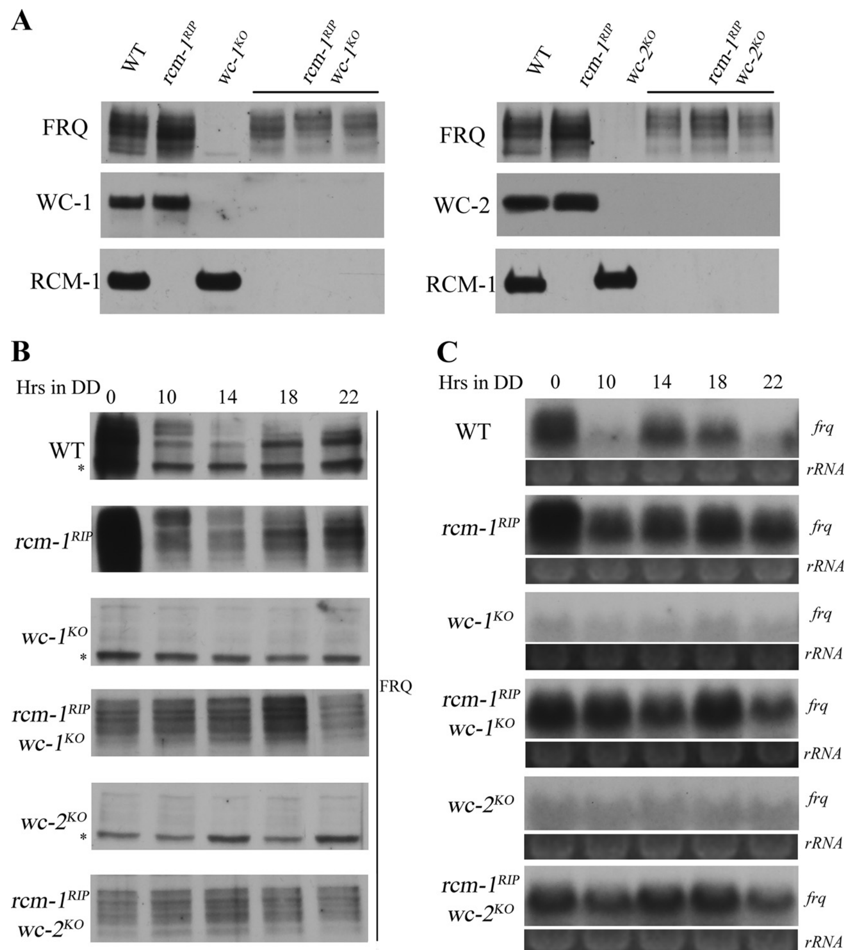

FIG 4 RCM-1 is required for suppressing WC-independent *frq* transcription. (A) Western blot analyses were performed with antibodies against FRQ, WC-1, WC-2, or RCM-1 in wild-type, *rcm-1^RIP^*, *wc-1^KO^*, *wc-2^KO^*, *rcm-1^RIP^ wc-1^KO^*, and *rcm-1^RIP^ wc-2^KO^* strains. (B) Western blot analysis of FRQ protein in wild-type, *rcm-1^RIP^*, *wc-1^KO^*, *wc-2^KO^*, *rcm-1^RIP^ wc-1^KO^*, and *rcm-1^RIP^ wc-2^KO^* strains. The asterisks indicate nonspecific bands. (C) Northern blot analysis of *frq* mRNA in wild-type, *rcm-1^RIP^*, *wc-1^KO^*, *wc-2^KO^*, *rcm-1^RIP^ wc-1^KO^*, and *rcm-1^RIP^ wc-2^KO^* strains.

WC proteins are required to function as a complex for their clock and light responses in *Neurospora* (15). To examine the role of RCM-1 in suppressing WC-independent *frq* transcription, we generated $rcm\text{-}1^{RIP}$ $wc\text{-}1^{KO}$ or $rcm\text{-}1^{RIP}$ $wc\text{-}2^{KO}$ double mutants. Western blot analyses using the WC and RCM-1 antibodies confirmed the molecular identifies of these mutants (Fig. 4A). As expected, nearly normal levels of FRQ expression were observed in both $rcm\text{-}1^{RIP}$ $wc\text{-}1^{KO}$ and $rcm\text{-}1^{RIP}$ $wc\text{-}2^{KO}$ double mutants in LL and DD (Fig. 4B). Similarly, high levels of *frq* mRNA were also observed in these double mutants (Fig. 4C), indicating that RCM-1 is required for suppressing WC-independent transcription of *frq*.

RCM-1 is a substrate of PKA. The regulation of WC-independent transcription of *frq* by PKA activity prompted us to examine whether PKA can exert its effect by phosphorylating the RCO-1/RCM-1 complex. Consistent with this hypothesis, we found that both RCM-1 and RCO-1 are phosphoproteins in *Neurospora* as indicated by protein mobility shifts in the presence of phosphatase inhibitors and after lambda phosphatase treatment (Fig. 5A). To examine whether RCO-1 and RCM-1 proteins are substrates of PKA, we performed immunoprecipitation assays to check whether PKAC-1 interacts with RCO-1 or RCM-1 in *Neurospora*. As shown in Fig. 5B, Myc/His/PKAC-1 expressed in the $pkac\text{-}1^{KO}$ strain coprecipitated with RCM-1, whereas it only interacted weakly with RCO-1, suggesting that RCM-1 but not RCO-1 interacts with PKAC-1. It is possible that PKAC-1 prefers to interact with the monomeric RCM-1 or that the the interaction between PKAC-1 and RCO-1 is transient.

We then examined whether RCM-1 can be phosphorylated by PKA *in vitro*. A series of GST/RCM-1 recombinant proteins were purified from *E. coli* BL21 cells and were used in *in vitro* kinase assays (Fig. 5C). We found that the C-terminal region of RCM-1 could be efficiently phosphorylated by both the bovine PKA and the *Neurospora* PKAC-1 (Fig. 5C and D). In addition, RCO-1 was only weakly phosphorylated by the *Neurospora* PKA (Fig. 5D) compared to RCM-1 or FRQ, suggesting that RCO-1 is not an efficient substrate for PKA.

To confirm that PKA is involved in phosphorylating RCM-1 *in vivo*, we took advantage of a commercially available antibody (Cell Signaling Technologies) that specifically recognizes the phosphorylated forms of PKA target sites. This antibody recognizes phosphorylated serine or threonine residues that are preceded by an

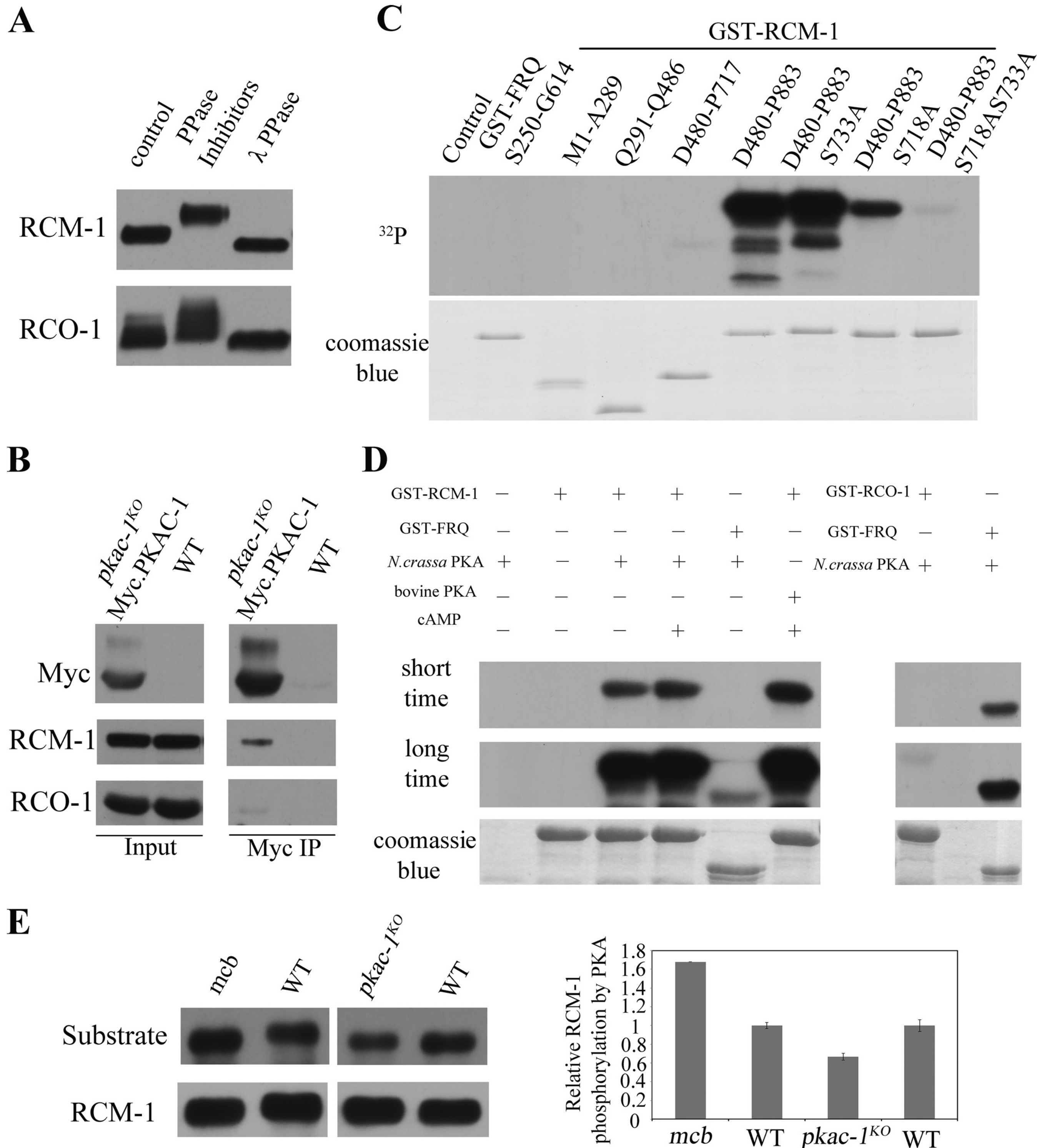

FIG 5 RCM-1 is a substrate of PKA. (A) Western blot analyses showing the phosphorylation profiles of RCM-1 and RCO-1 in the presence of phosphatase inhibitors and after treatment with λ phosphatase. (B) Immunoprecipitation (IP) assay showing that RCM-1 interacts with Myc/PKAC-1. The Myc-tagged PKAC-1 was immunoprecipitated from *Neurospora* extracts, and the relative levels of the associated RCM-1 and RCO-1 were assessed by Western blotting using their specific antibodies. (C) *In vitro* kinase assay showing that purified *Neurospora* PKA phosphorylates RCM-1 at both S718 and S733. Different portions of RCM-1 were fused to GST and purified from *E. coli* BL21 cells and GST-FRQ (S250-G614) was used as a positive control. These proteins were incubated with *Neurospora* PKAC-1 and [γ-^{32}P]ATP for 30 min at 30°C. The reaction products were then separated using SDS-PAGE, and the level of PKA phosphorylation was assessed by autoradiography. (D) *In vitro* kinase assay showing that the C-terminal fragment of RCM-1 (D480-P883) is phosphorylated by either the bovine or *Neurospora* PKA, and this phosphorylation is increased after the addition of cAMP. Full-length RCO-1 is phosphorylated weakly by the *Neurospora* PKA compared to those of the positive control GST-FRQ (S250-G614). (E) Western blot analyses showing hyperphosphorylation of RCM-1 in the *mcb* strain with elevated PKA activity and hypophosphorylation of RCM-1 in the *pkac-1^{KO}* strain with decreased PKA activity.

arginine residue at the −3 position. It is a useful tool in identifying substrates of AGC family kinases, including PKA and PKC. We immunoprecipitated RCM-1 proteins using RCM-1 antibody and then examined whether the RCM-1 proteins could be recognized by the PKA substrate-specific antibody in Western blot analysis. As shown in Fig. 5E, the immunoprecipitated wild-type RCM-1 was indeed recognized by this PKA-substrate specific antibody. More importantly, the level of the PKA phosphorylated RCM-1 decreased in the *pkac-1*^{KO} mutant (note that PKAC-1 is one of the two PKA catalytic subunits in *Neurospora* and other AGC family kinases may also phosphorylate those sites) but increased in the *mcb* mutant. Together, these results strongly suggest that RCM-1 is directly phosphorylated by PKA.

Identification of RCM-1 phosphorylation sites *in vivo*. Bioinformatics analysis (http://mendel.imp.ac.at/sat/pkaPS) of RCM-1 protein revealed two PKA consensus sites (S718 and S733) and other nonconsensus sites (Fig. 6A). To identify phosphorylated residues of RCM-1 *in vivo*, we affinity purified RCM-1 in the presence of phosphatase inhibitors from the wild-type strain grown in LL. The colloidal blue-stained protein band corresponding to RCM-1 was excised from SDS-PAGE gels and subjected to trypsin digestion. The resulting peptides were then analyzed by nano-electrospray MS/MS. Our MS analyses (sequence coverage of 63.69%) led to identification of eight RCM-1 phosphorylation sites (Fig. 6B). Figure 6C shows two representative results of the MS/MS analysis for two of the phosphopeptides of RCM-1, which carry phosphorylated serine-718 and threonine-720 or a single phosphorylated serine-733. Among the sites identified by MS analyses, Ser-718, Ser-733, Ser-630, Thr-536, and Ser-682 are also potential PKA phosphorylation sites predicted by bioinformatics analysis. In addition, the peptides containing these eight phosphorylation sites are located in C terminus of RCM-1 (Fig. 6B), an observation consistent with our *in vitro* kinase assay results (Fig. 5C).

To further confirm the MS results, we created *rcm-1*^{2A} mutants in which S718 and S733 were mutated to alanine residues and used the PKA substrate-specific antibody to examine the phosphorylation of RCM-1. As shown in Fig. 6D, the levels of RCM-1 recognized by the PKA substrate-specific antibody were almost completely abolished in the *rcm-1*^{2A} mutants, indicating that S718 and S733 are the major PKA sites on RCM-1. Taken together, these results indicate that PKA is a major kinase that phosphorylates RCM-1.

Phosphorylation of RCM-1 by PKA results in WC-independent *frq* transcription. To test whether the observed derepression of WC-independent *frq* transcription by elevated PKA activity was due to phosphorylation of RCM-1 by PKA at the identified phosphorylation sites, we created *rcm-1* knock-in strains at the endogenous locus by homologous recombination, i.e., the *rcm-1*$^{KI\text{-}WT}$, *rcm-1*^{5A}, and *rcm-1*^{5E} strains (21). In the *rcm-1*$^{KI\text{-}WT}$ strain, a wild-type knock-in cassette with a hygromycin resistance gene (*hph*) was inserted downstream from *rcm-1* 3′-UTR, while five identified RCM-1 phosphorylation sites (S718, S733, S630, S682, and T536) were mutated to alanine residues in the *rcm-1*^{5A} or to glutamic acids to mimic phosphorylation at these residues in the *rcm-1*^{5E} strains (Fig. 7A). Mutation of these phosphorylation sites in the *rcm-1*^{5E} strain had no effect on the interaction between RCM-1 and RCO-1 (Fig. 7B), which is consistent with previous studies that TPR (tetratricopeptide repeat) domain in the N terminus of Ssn6 is required for its interaction with Tup1 in *S. cerevisiae* (47, 48).

Race tube assays showed that both WT and *rcm-1*$^{KI\text{-}WT}$ strains exhibit robust conidiation rhythms (Fig. 7C), whereas the *rcm-1*^{5A} strain exhibited a modestly short period with low-amplitude conidiation rhythms (Fig. 7C), and the *rcm-1*^{5E} strain exhibited a severely dampened low-amplitude conidiation rhythm (Fig. 7C). This result suggests that the proper phosphorylation of RCM-1 at these sites is important for its role in the *Neurospora* circadian clock. We then investigated the circadian rhythms of these strains at the molecular levels. As shown in Fig. 7D, while robust oscillations of FRQ amounts and its phosphorylation profiles in DD were seen in the wild-type or *rcm-1*$^{KI\text{-}WT}$ strain, such oscillations were damped in the *rcm-1*^{5E} strain. Moreover, the circadian rhythm of *ccg-1* mRNA was also severely damped in the *rcm-1*^{5E} strain (Fig. 7E). It should be noted that the circadian rhythms are not completely abolished in the *rcm-1*^{5E} strain, suggesting the existence of other functional phosphorylation sites. Taken together, these results suggest that these phosphorylation events on RCM-1 negatively regulate the function of RCM-1 in the *Neurospora* circadian clock.

To examine whether mimicking phosphorylation in the *rcm-1*^{5E} strain leads to WC-independent *frq* transcription, we created *rcm-1*^{5E} *wc-2*^{KO} double mutants and compared the expression of FRQ to those in the wild type and the *wc-2*^{KO} single mutant. As expected, significant levels of FRQ expression were observed in LL and DD in the *rcm-1*^{5E} *wc-2*^{KO} strain (Fig. 7F). Similarly, near normal levels of *frq* mRNA were seen in the *rcm-1*^{5E} *wc-2*^{KO} strain (Fig. 7G). These results suggest that the phosphorylation of these PKA sites on RCM-1 inhibits its function and leads to WC-independent *frq* transcription. Therefore, PKA regulates WC-independent *frq* transcription, at least in part, through its direct phosphorylation of RCM-1 protein.

RCM-1 associates with the *frq* locus, and its phosphorylation reduces its binding. How does PKA inhibit RCM-1 activity? We previously showed that RCO-1 regulates the chromatin status at the *frq* locus, but how RCO-1 acts is not known since RCO-1 was not found to be associated with chromatin at the *frq* locus (29). The identification of RCM-1 as the partner of RCO-1 (45, 46) prompted us to examine whether the complex acts to regulate the chromatin structure at the *frq* locus via RCM-1. ChIP assays using the RCM-1 antibody showed that RCM-1 is enriched throughout the *frq* locus with a peak at the C-box of *frq* promoter (Fig. 8A), suggesting that the RCO-1/RCM-1 complex regulates the chromatin structure at the *frq* locus via the recognition of chromatin by RCM-1. Importantly, levels of RCM-1 enrichment at the *frq* locus were dramatically reduced in the *mcb* strain in different time points in DD (Fig. 8B), suggesting that elevated PKA activity reduces RCM-1 association at the *frq* promoter. Moreover, we examined the phosphorylation of RCM-1 in different time points using the PKA substrate-specific antibody. As shown in Fig. 8C, the levels of RCM-1 recognized by the PKA substrate-specific antibody were rhythmic at different time points in DD. Importantly, the enrichment of RCM-1 at the chromatin was low when it was hyperphosphorylated and was high when it was hypophosphorylated, suggesting that the PKA-mediated phosphorylation of RCM-1 inhibits its association at the *frq* promoter.

Phosphorylation may also regulate protein stability and cellular localization. However, we found that the stability of RCM-1 in

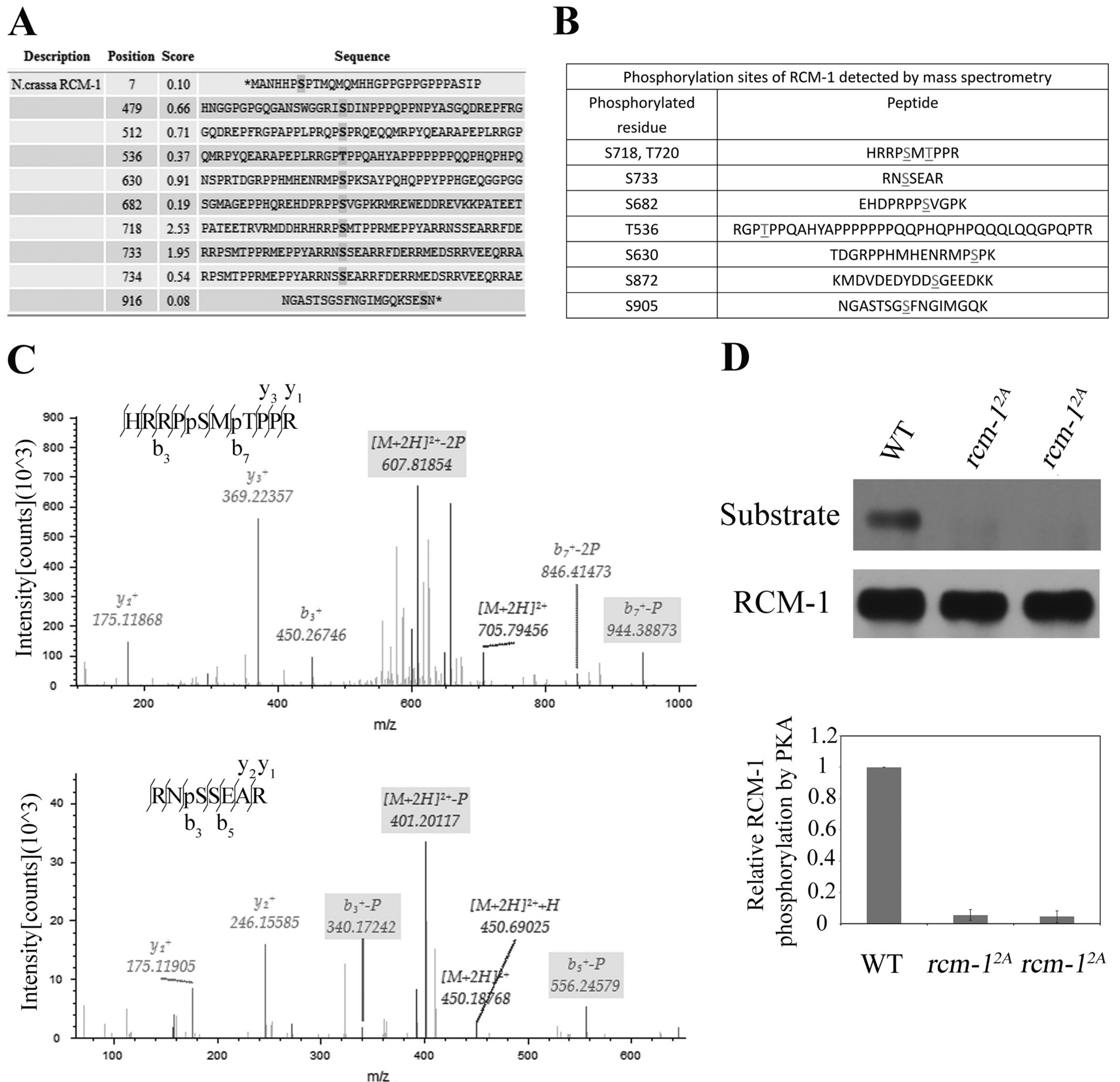

FIG 6 Identification of RCM-1 phosphorylation sites *in vivo*. (A) PKA phosphorylation sites on RCM-1 are predicted using a database (http://mendel.imp.ac.at/sat/pkaPS). Among them, S718 and S733 are PKA consensus sites. (B) Phosphorylation sites of RCM-1 detected by mass spectrometry. (C) Identification of RCM-1 phosphorylation sites by mass spectrometry. The mass spectrometry of the productions resulting from collision-induced dissociation of two representative phosphorylated RCM-1 peptides are shown. The upper panel shows the results of an analysis of a trypsin fragment containing S718 and T720 of RCM-1; the lower panel shows the results of an analysis of a trypsin fragment containing S733 of RCM-1. (D) RCM-1 was recognized by an anti-PKA substrate antibody in an S718- and S733-dependent manner.

the *mcb* or *pkac-1*^{KO} strain was not affected (Fig. 8D). In addition, the nuclear localization of RCM-1 was similar in wild-type and *mcb* strains (Fig. 8E). These results suggest that PKA does not play a significant role in regulating RCM-1 stability and cellular localization. Therefore, PKA represses RCM-1 activity mainly through inhibition of RCM-1 chromatin association activity by phosphorylation of RCM-1.

Derepression of WC-independent *frq* transcription results in reduced binding of WC-2 at the C-box of *frq* promoter. How does the derepression of WC-independent *frq* transcription affect the circadian expression of *frq*? In wild-type strain, the circadian rhythm of *frq* transcription is driven by the WC complex through its rhythmic binding of the *frq* promoter at the C-box. ChIP assays using WC-2 antibody showed that the enrichment of WC-2 at the

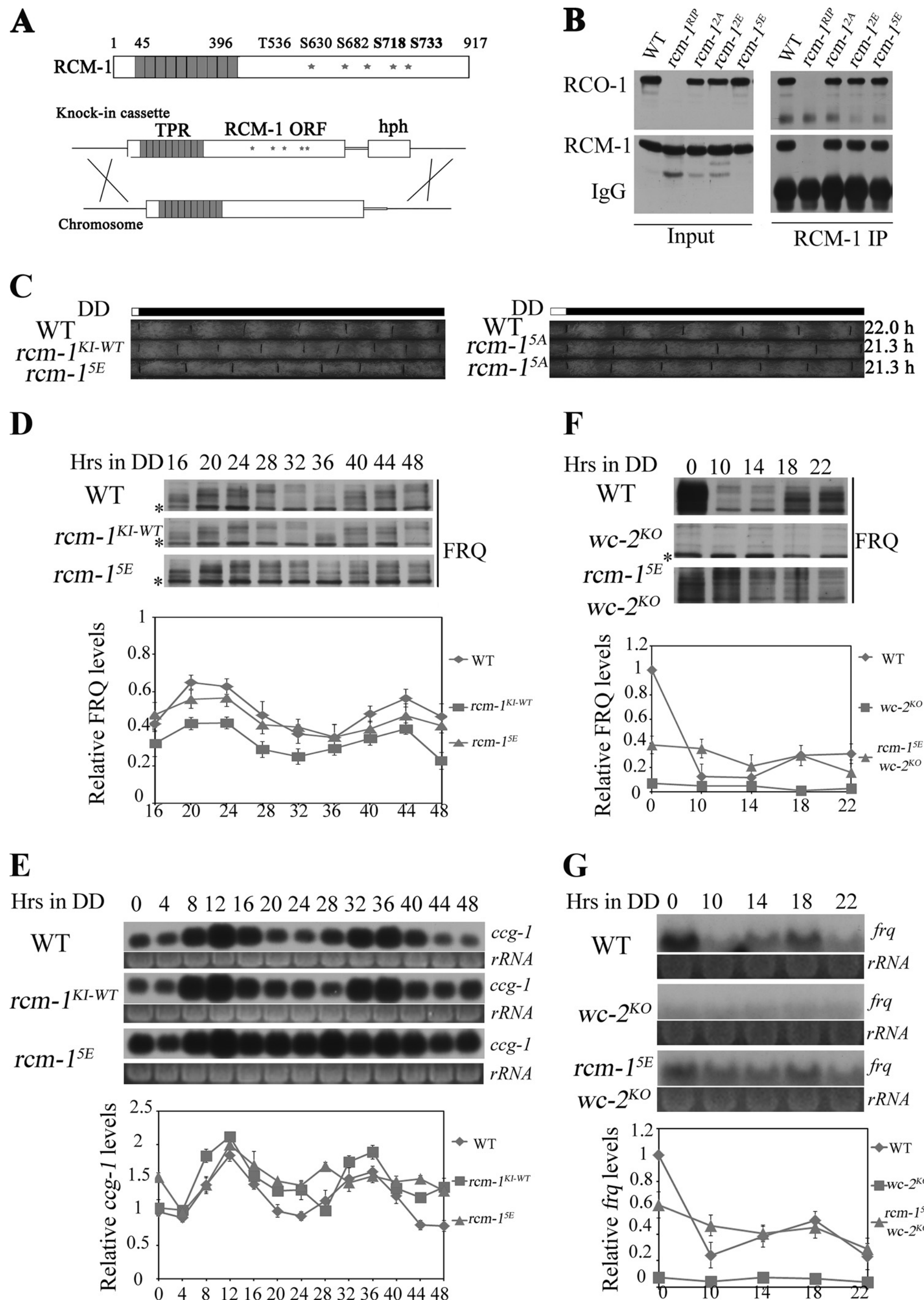

FIG 7 Phosphorylation of RCM-1 results in WC-independent *frq* transcription. (A) RCM-1 contains 10 TPR (tetratricopeptide repeat) motifs in the N-terminal region and different phosphorylation sites in the C-terminal region. The asterisks in the RCM-1 ORF indicate the location of the mutation sites. The endogenous *rcm-1* gene was replaced with a knock-in cassette containing the mutated *rcm-1* gene by homologous recombination and *hph*-resistant transformants were selected. (B) Coimmunoprecipitation (Co-IP) experiments showing that RCM-1 interacts with RCO-1 and the interactions are not affected in the *rcm-1* knock-in strains. (C) Race tube assays showing the circadian conidiation rhythm in wild-type, *rcm-1^{KI-WT}*, *rcm-1^{5E}*, and *rcm-1^{5A}* strains. (D) Western blot analysis of FRQ protein in wild-type, *rcm-1^{KI-WT}* and *rcm-1^{5E}* strains grown in the dark for the indicated times. The asterisks indicate nonspecific bands. (E) Northern blot analysis of *ccg-1* mRNA in wild-type, *rcm-1^{KI-WT}*, and *rcm-1^{5E}* strains. (F) Western blot analysis of FRQ in wild-type, *wc-2^{KO}*, and *rcm-1^{5E} wc-2^{KO}* strains. The asterisks indicate nonspecific bands. (G) Northern blot analysis of *frq* mRNA in wild-type, *wc-2^{KO}*, and *rcm-1^{5E} wc-2^{KO}* strains.

C-box was dramatically decreased at different time points in DD and lacked an obvious rhythm in both *rcm-1^{RIP}* and *mcb* mutants (Fig. 9A), indicating that the transcriptional activity of WC complex is impaired. How is WC complex activity inhibited when WC-independent *frq* transcription is derepressed? FRQ is known to inhibit the WC complex activity by promoting its phosphorylation (20, 21, 49). Thus, we compared WC-1 and WC-2 phosphorylation profiles between wild-type and *rcm-1^{RIP}* strains at

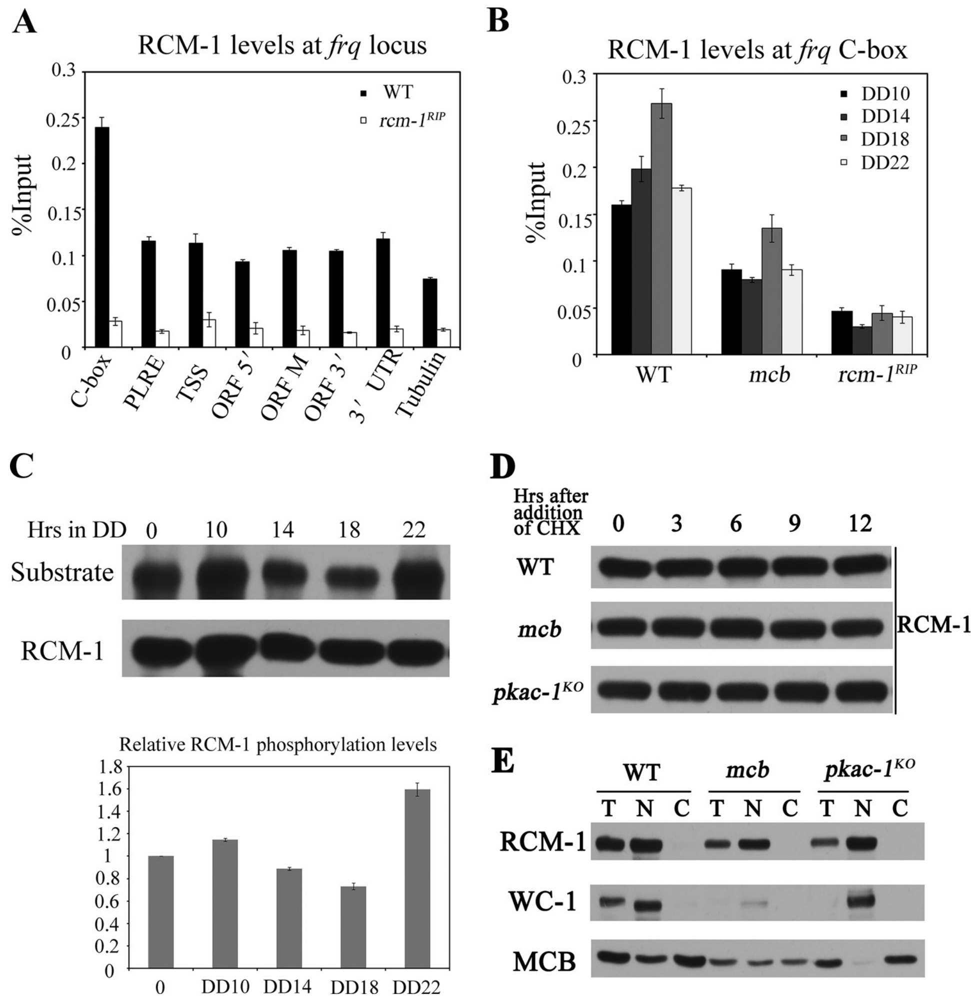

FIG 8 The association of RCM-1 with chromatin at the *frq* locus is affected by PKA-mediated phosphorylation. (A) ChIP analysis showing the recruitment of RCM-1 at different regions of the *frq* locus in wild-type and *rcm-1^{RIP}* strains at DD18. (B) ChIP analysis showing the enrichment of RCM-1 at the C-box of the *frq* promoter in wild-type, *mcb*, and *rcm-1^{RIP}* strains at the indicated time points. (C) Western blot analysis showing PKA-mediated phosphorylation of RCM-1 in different time points in constant darkness (DD). (D) Western blot analysis showing that RCM-1 is stable in wild-type, *mcb*, and *pkac-1^{KO}* strains. Protein stability was determined by measuring RCM-1 levels after the addition of CHX (10 μg/ml). (E) Western blot analysis showing the localization of RCM-1 in wild type, *mcb*, or *pkac-1^{KO}* strains. RCM-1 is localized in the *Neurospora* nuclei. Total (T), nuclear (N), and cytosolic (C) protein extracts were isolated from wild type, *mcb*, or *pkac-1^{KO}* strains, separated by SDS-PAGE, and hybridized with RCM-1-specific antibody. As controls, we used antibodies against the nuclear protein WC-1 and against cytosolic protein MCB.

different time points in DD. As shown in Fig. 9B, the phosphorylations of WC-1 and WC-2 were markedly increased in the *rcm-1^{RIP}* strain at these time points. The hyperphosphorylated WC proteins in the *rcm-1^{RIP}* strain suggest that constitutive WC-independent FRQ expression promotes WC phosphorylation.

WCC activity is also regulated by its stability (23). We examined WC-1 and WC-2 stability in the *rcm-1^{RIP}* strain after the addition of cycloheximide (CHX) in LL and found that the stability of WC-1 and WC-2 were not affected (Fig. 9C). This result further suggested that WC-independent FRQ expression regulates WC activity mainly through phosphorylation.

To exclude the possibility that RCM-1 or RCO-1 regulates WC activity independent of FRQ, we generated *rcm-1^{RIP} frq9* and *rco-1^{KO} frq9* double mutants. In *frq9* mutants, a frameshift mutation causes production of a truncated FRQ protein and the loss of circadian negative-feedback loop (35). ChIP assays with WC-2 antibody showed that even though the enrichment of WC-2 at the *frq* C-box was dramatically decreased in an *rcm-1^{RIP}* or an *rco-1^{KO}* single mutant, the WC-2 enrichment in the *rcm-1^{RIP} frq9* and *rco-1^{KO} frq9* double mutants were comparable to that of the *frq9* single mutant (Fig. 9D). These results indicate that the effect of RCM-1 or RCO-1 on WC activity is dependent on FRQ. Together, our results suggest that constitutive WC-independent FRQ expression promotes WC phosphorylation, which results in reduced binding of WC-2 at the C-box of *frq* promoter and abolishes the rhythmic transcription of *frq* driven by the WC complex. Thus, the constant expression of WC-independent FRQ leads to inhibition of WC activity.

DISCUSSION

Suppression of the WC-independent *frq* transcription is required for the *Neurospora* circadian clock function. In the present study, we showed that RCO-1 and RCM-1 function as a complex (45, 46) to regulate WC-independent *frq* transcription and that the PKA

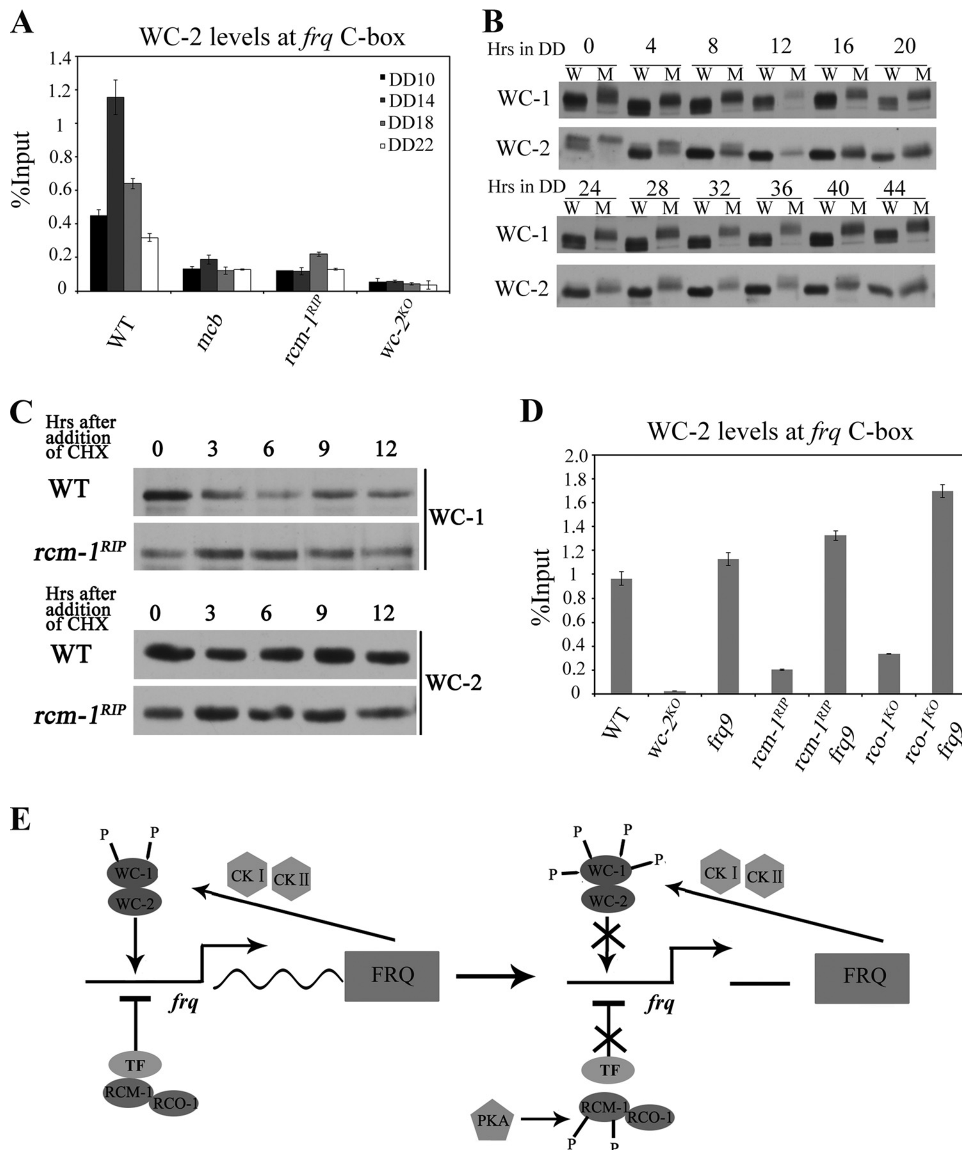

FIG 9 WC-independent *frq* transcription results in reduced binding of WC-2 at the C-box of *frq* promoter. (A) ChIP analysis showing the enrichment of WC-2 at the C-box of the *frq* promoter in wild-type, *mcb*, *rcm-1^{RIP}*, and *wc-2^{KO}* strains at the indicated times. (B) Western blot analyses showing the phosphorylation profiles of WC-1 and WC-2 in wild-type and *rcm-1^{RIP}* strains at the indicated DD time points. W, wild type; M, *rcm-1^{RIP}* mutants. (C) Western blot analyses showing that WC-1 and WC-2 are stable in wild-type and *rcm-1^{RIP}* strains. Protein stability was determined by measuring WC-1 and WC-2 levels after the addition of CHX (10 μg/ml). (D) ChIP analysis showing the recruitment of WC-2 at the C-box of the *frq* promoter in wild-type, *wc-2^{KO}*, *rcm-1^{RIP}*, *rco-1^{KO}*, *rcm-1^{RIP} frq9*, and *rco-1^{KO} frq9* strains at DD14. (E) Model for the role of PKA in the *Neurospora* circadian clock by phosphorylating RCM-1. RCO-1-RCM-1 complex is recruited to the *frq* locus by an unknown transcription factor for suppressing WC-independent *frq* transcription, and disruption of *rcm-1* or *rco-1* results in constitutive WC-independent *frq* transcription. PKA directly phosphorylates RCM-1, which may disrupt the interaction between RCM-1 and the transcription repressor and relieve RCO-1/RCM-1-mediated repression of WC-independent *frq* transcription. The accumulation of WC-independent and WC-dependent FRQ mediates WC phosphorylation through CKI and CKII, resulting in the dissociation of WCC from *frq* promoter and inactivation of WC-dependent *frq* transcription.

signaling pathway is a key regulator of this process by phosphorylating and inhibiting RCM-1. Our results demonstrated that the activation of the PKA pathway induces *frq* transcription in a WC-independent manner, which interferes with the circadian negative feedback loop. Our findings identified RCM-1 as a direct target of PKA. PKA interacts and phosphorylates RCM-1, and the phosphorylation of RCM-1 relieves RCO-1/RCM-1-mediated repression of WC-independent *frq* transcription. Thus, PKA, in addition to its previously identified role in phosphorylating WCC directly (32), is a critical regulator of *frq* transcription by regulating RCO-1-RCM-1 activity.

In *S. cerevisiae*, Ssn6 and Tup1 are known to be phosphorylated (50, 51). However, the role of their phosphorylation on their function is not clear. In the present study, we demonstrate that PKA regulates WC-independent *frq* transcription by phosphorylating RCM-1. Several lines of evidence presented here indicate that RCM-1 is a direct substrate of PKA. First, immunoprecipitation assays showed that PKA and RCM-1 associate with each other in

Neurospora (Fig. 5B). Second, PKA can efficiently phosphorylate the C-terminal part of RCM-1 *in vitro* (Fig. 5C). Third, mass spectrometry analyses of RCM-1 purified from *Neurospora* led to the identification of eight *in vivo* RCM-1 phosphorylation sites at the C terminus of RCM-1, including two PKA consensus sites S718 and S733 and other noncanonical PKA sites (Fig. 6B). Fourth, Western blot analyses using PKA substrate-specific antibody showed that the level of RCM-1 phosphorylation is altered when PKA activity is altered in *Neurospora*, and the mutation of S718 and S733 of RCM-1, two PKA consensus phosphorylation sites, abolishes most of RCM-1 phosphorylation (Fig. 5E and 6D). Together, these results indicate that PKA is the major kinase that phosphorylates RCM-1 *in vivo*.

Our results suggest that the phosphorylation of RCM-1 inhibits its activity that suppresses WC-independent *frq* transcription. A knock-in strain containing five mutated PKA phosphorylation sites of RCM-1 that mimic constant phosphorylation exhibited severely dampened circadian conidiation rhythms and WC-independent *frq* transcription (Fig. 7), indicating that the proper phosphorylation of RCM-1 is critical for circadian clock function. This is consistent with the loss of circadian rhythm and upregulation of WC-independent *frq* transcription in the *mcb* mutant (Fig. 1), which has elevated PKA activity, further indicating the role of PKA in regulating WC-independent *frq* transcription by phosphorylating RCM-1. Although the circadian rhythms are abolished in *rcm-1^{RIP}* and *mcb* mutants, it should be noted that despite reduced amplitude, the clock function was not completely abolished in the *rcm-1^{5E}* strain. This is likely due to the existence of other unidentified functional PKA sites on RCM-1.

How RCO-1 represses *frq* gene transcription was previously unclear. It has been shown that there is no detectable association between RCO-1 and chromatin (29). Surprisingly, we showed here that RCM-1 associates with chromatin at the *frq* locus (Fig. 8A), providing a mechanism for how RCO-1-RCM-1 complex regulates the chromatin status at the *frq* locus. Thus, RCM-1 may recruit RCO-1 to the chromatin, which may explain the lack of detectable RCO-1/chromatin association by ChIP assays at the *frq* locus. However, neither RCO-1/Tup1 nor RCM-1/Ssn6 has a known DNA binding domain, and thus they may interact with DNA through another partner capable of binding to specific DNA sequences (30, 31). The identity of such a factor (indicated as TF in Fig. 9E), if it exists, is still unknown. In addition, we showed that the RCM-1/chromatin association was severely impaired in the *mcb* mutant (Fig. 8B). We also observed that the enrichment of RCM-1 was low when it was hyperphosphorylated, while the enrichment of RCM-1 was high when it was hypophosphorylated (Fig. 8C). These results suggest that PKA-dependent phosphorylation of RCM-1 inhibits its association with chromatin, thus affecting its role in regulating chromatin structure.

How does WC-independent *frq* transcription affect circadian *frq* transcription? A previous study suggests that PKA regulates *frq* expression by acting as a priming kinase for WC phosphorylation that leads to their phosphorylation by CKI and CKII (32). The increase in PKA activity in the *mcb* mutant drastically reduces the WCC binding to the *frq* C-box. Here, we showed that the *mcb* mutation also results in WC-independent *frq* transcription and increased RCM-1 phosphorylation, indicating that PKA has two distinct roles in regulating *frq* transcription. In the *rcm-1^{RIP}* mutants, in which WC-independent *frq* transcription cannot be repressed, both WC-1 and WC-2 are hyperphosphorylated and WCC binding to C-box is constantly low (Fig. 9A and B). These results indicated that the WC-independent FRQ expression leads to WC phosphorylation by FRQ-dependent kinases and inhibition of WC activity. As a result, the circadian negative-feedback loop is disrupted.

cAMP is implicated in the regulation of entrainment or maintenance of clock functions and clock output in molluscs, birds, and the mammalian SCN (52, 53). In mammals, cAMP-dependent signaling oscillates in a circadian manner, which in turn regulates core circadian oscillators (54–57). In *Neurospora*, the level of cAMP is controlled by the circadian clock (58). The elevated intracellular levels of cAMP result in the activation of PKA (59). Thus, the daily rhythm of cAMP may regulate PKA activity rhythmically. In the present study, we showed that the phosphorylation profile of RCM-1 and the enrichment of RCM-1 at the *frq* C-box were rhythmic (Fig. 8B and C). Our results suggest that PKA may play a role in the on-off switch of WC-independent *frq* repression mediated by the RCO-1/RCM-1 complex. Low PKA activity controlled by low levels of cAMP results in hypophosphorylation of RCM-1 and helps to maintain the repressed state of WC-independent *frq* transcription, allowing the activation of *frq* transcription by the WC complex. High levels of cAMP in cells, on the other hand, lead to elevated activity of PKA and phosphorylation of RCM-1, resulting in WC-independent *frq* transcription. The accumulation of WC-independent and WC-dependent FRQ mediates WC phosphorylation and results in dissociation of WCC from *frq* promoter and inactivation of WC-dependent *frq* transcription (Fig. 9E). Thus, the control of RCM-1 phosphorylation by PKA should contribute to the daily rhythm of *frq* transcription.

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