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Mechanism of the *Neurospora* Circadian Clock, a FREQUENCY-centric View

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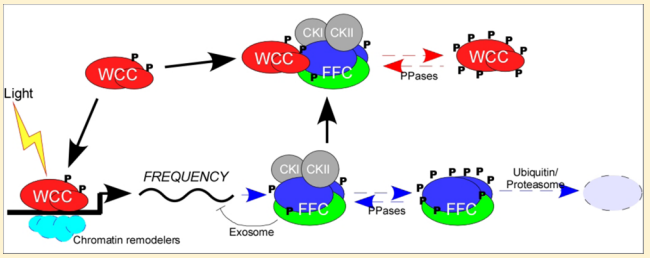
ABSTRACT: Circadian clocks are self-sustaining timekeepers found in almost all organisms on earth. The filamentous fungus *Neurospora crassa* is a preeminent model for eukaryotic circadian clocks. Investigations of the *Neurospora* circadian clock system have led to elucidation of circadian clock regulatory mechanisms that are common to all eukaryotes. In this work, we will focus on the *Neurospora* circadian oscillator mechanism with an emphasis on the regulation of the core clock component FREQUENCY.



Eukaryotic circadian oscillators are comprised of interlocked autoregulatory feedback loops that control gene expression at the levels of transcription and translation.[1,2] Despite the evolutionary distance, the molecular mechanism of the circadian oscillator of the filamentous fungus *Neurospora crassa* is remarkably similar to those of higher eukaryotes.[3−5] The asexual spore formation of *Neurospora* is governed by the circadian clock; therefore, *Neurospora* circadian rhythms are readily monitored.[6] Recently, a bioluminescence reporter has been established to monitor circadian behavior of various promoters.[7] In addition, the availability of a whole genome knockout collection of *Neurospora* has made this fungus as an outstanding model organism for research on the molecular architecture of eukaryotic circadian clocks.[8]

FREQUENCY (FRQ) is a central component of the *Neurospora* circadian oscillator like PERIOD (PER) in animals. FRQ forms FFC (FRQ-FRH complex) with its partner FRQ-interacting RNA helicase (FRH) and functions as the negative limb in the core circadian negative feedback loop.[9,10] The transcription of the *frq* gene is activated by the positive element, WHITE COLLAR complex (WCC), which is formed by the interaction of two PER-ARNT-SIM (PAS) domain-containing transcription factors WC-1 and WC-2, like CLOCK and BMAL1 in animals. WCC binds rhythmically to the promoter of *frq* to induce transcription.[11−15] To close the negative feedback loop, FFC inhibits the activity of WCC. After FRQ is synthesized, it is subjected to progressive post-translational modifications by phosphorylations that lead to its degradation by the ubiquitin/proteasome pathway.[16−22] When the amount of FRQ drops below a certain level, WCC is reactivated and a new cycle begins. As a result of this negative feedback loop, *frq* mRNA and FRQ protein exhibit robust rhythms with a period close to 24 h. The core negative feedback loop is interlocked with positive feedback loops to maintain robust rhythmicity.[23,24] This review will focus on the recent progress in understanding the molecular network governing the expression and activity of FRQ.

MOLECULAR ARCHITECTURE OF THE *NEUROSPORA* CIRCADIAN CLOCK

Identification of clock components has been central to our understanding of the clock mechanisms. Since the cloning of the *frq* gene,[25] more than 20 *Neurospora* clock components that regulate FRQ expression, function, and stability at various levels have been identified (Table 1). These components form the network that is important for the control of the clock (Figure 1).

During the early subjective day in the constant darkness, WCC activates *frq* transcription by binding to the clock box (c-box) in the *frq* promoter; this binding is essential for the circadian transcription of *frq* as well as other clock-controlled genes.[11,14,23] Transcriptional activation is followed by an increase in levels of FRQ protein, which forms a homodimer that interacts with FRH to form FFC.[26−28] The FRQ−FRH interaction is important for maintaining the steady-state levels and proper structure of FRQ.[27−31] In the absence of FRH, FRQ is unstable. FFC interacts with WCC and promotes phosphorylation of WCs. This phosphorylation inhibits WCC activity, resulting in a decrease in the level of *frq* transcription.[21,32−34] In addition, there is WC-independent transcription of *frq* that is suppressed by a mechanism that involves the RCO-1 repressor complex.[35] In addition, FFC recruits the RNA exosome complex to the *frq* mRNA to regulate its half-life.[29]

After its synthesis, FRQ is progressively phosphorylated by casein kinases (CKI and CKII) and other kinases through the subjective day and evening, leading to its degradation by the ubiquitin/proteasome pathway.[17−21,36−39] FWD-1 is the E3 ubiquitin ligase responsible for FRQ ubiquitination, and the COP9 signalosome regulates FRQ stability by modulating the

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Table 1. Components of the *Neurospora* Circadian Clock

protein (complex)	functions
FRQ (component of core loop)	forms a complex with FRH, recruits CKs to phosphorylate itself and WCC
FRH (component of core loop)	interacts with FRQ to form FFC, which stabilizes FRQ protein and recruits exosome to regulate *frq* mRNA stability
WC-1 (component of core loop)	forms WCC with WC-2; WCC activates *frq* and clock-controlled genes
WC-2 (component of core loop)	forms WCC with WC-1; WCC activates *frq* and clock-controlled genes
CKI (CK-1a)	phosphorylates FRQ and WCC
CKII	phosphorylates FRQ and WCC
PKA	phosphorylates FRQ and WCC
CAMK-1	phosphorylates FRQ
CHK2	phosphorylates FRQ
PP1	dephosphorylates (to stabilize) FRQ
PP2A	dephosphorylates WCC
PP4	dephosphorylates (to stabilize) FRQ, dephosphorylates WCC
FWD-1	part of SCF^{FWD-1}, the E3 ligase responsible for FRQ ubiquitination
COP-9 signalosome	regulates the stability of SCF^{FWD-1}
CSW-1	tegulates chromatin structure, changes DNase sensitivity of the *frq* promoter
CHD1	enhances DNA methylation on *frq* locus and regulates *frq* transcription
CATP	regulates histone occupancy of the *frq* locus and enhances WCC binding
Not1−Ccr4 complex	stabilizes WCC
RCO-1	part of a transcription repressor complex that suppresses WC-independent transcription of *frq*
SET1	histone H3K4 methyltransferase; regulates *frq* transcription

activity and stability of the SCF^{FWD-1} complex that is critical for FRQ degradation. In contrast to the role of casein kinases in promoting the degradation of FRQ, protein kinase A (PKA) phosphorylates and stabilizes FRQ. The roles of these kinases are countered by multiple protein phosphatases, including PP1, PP2A, and PP4.[32,40,41] Thus, the phosphorylation of FRQ is fine-tuned by a series of regulators to determine its stability and the period length of the clock.[22,42] Degradation of FRQ to a certain level releases the inhibition of FRQ on WCC and results in its dephosphorylation and reactivation, a process that involves PP2A and PP4.[32,41,43] The reactivated WCC is transported to the nucleus, binds to the *frq* c-box with the help of chromatin modulating factors, and drives the next cycle of *frq* transcription.[15,41,44,45] The resulting rhythms of the abundance of *frq* mRNA and FRQ protein and the phosphorylation profile of FRQ are the hallmarks of the *Neurospora* circadian oscillation.

In addition to its role in inhibiting WCC activity, FRQ also positively regulates the expression of both WC-1 and WC-2.[23,24,46,47] The detailed mechanisms are still unknown, but FRQ regulates WC-1 expression at the post-transcriptional level, in a manner independent of WC-2. FRQ also enhances *wc-2* transcription. These positive feedback loops are interlocked with the core negative feedback loop, a feature that is shared in animal clocks.[48,49]

TRANSCRIPTIONAL REGULATION OF *FRQ*

Post-translational Regulations of the WCC: Stability, Activity, and Localization. Transcriptional regulation of *frq* is mainly achieved by post-translational regulation of its activator, WCC. Because FRQ represses its own expression, it

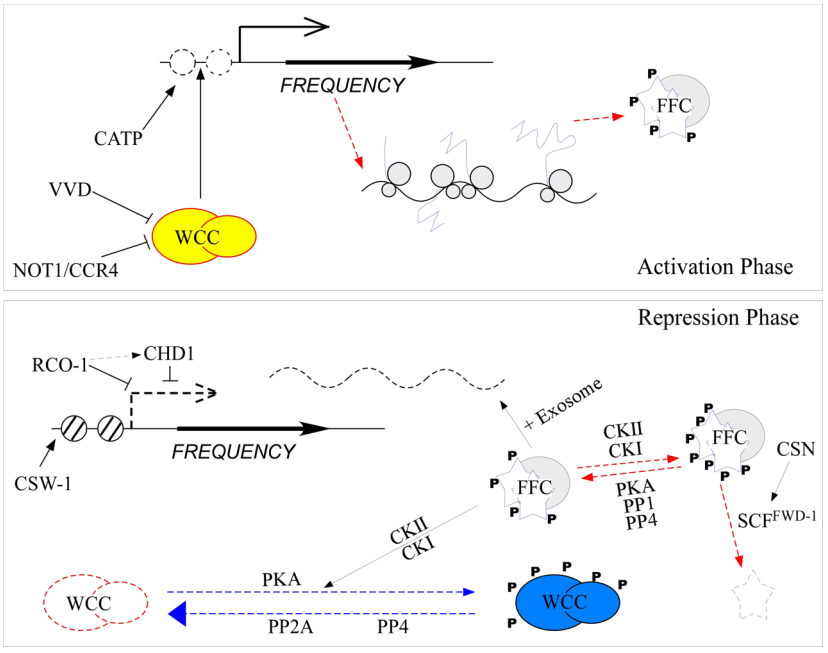

Figure 1. Model describing the core circadian negative feedback loop of *Neurospora*. In the activation phase, CATP reduces the histone occupancy on the *frq* promoter, which promotes WCC binding and activation of *frq* transcription. After transcription of *frq* mRNA, the nonoptimal codons in *frq* mRNA regulate translation speed to allow proper cotranslational folding of FRQ. The resulting FRQ protein is stabilized by its interaction with FRH to form the FFC complex. In the repression phase, FFC recruits casein kinases to phosphorylate PKA-primed WCs and promotes degradation of *frq* mRNA by the exosome. Phosphorylation of WCC inhibits its DNA binding activity and sequesters it in the cytoplasm. FRQ is progressively phosphorylated by casein kinases and degraded by the ubiquitin/proteasome system, a process that is counterbalanced by the actions of PKA, PP1, and PP4. CSW-1 relocates nucleosomes to suppress *frq* activation, whereas RCO-1 and CHD1 suppress WC-independent *frq* transcription to permit WCC-regulated *frq* transcription. Dephosphorylation of WCC by PP2A and PP4 reactivates WCC to allow reactivation of *frq* transcription.

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has been proposed that FRQ can regulate WCC activity by physical interactions alone.[26,47,50] However, the concentration of nuclear FFC is much lower than that of the nuclear WCC. Thus, the amount of nuclear FRQ is not sufficient to inhibit nuclear WCC through a direct interaction alone,[23,27,33,50−52] suggesting that FFC may behave like an enzyme to inhibit the WCC. Both biochemical purification and genetic analyses identified CKI and CKII as FRQ-interacting kinases that inhibit WCC through phosphorylation.[21] FFC acts as a substrate-recruiting subunit of CKs and mediates phosphorylation by a physical interaction with WCC. Phosphorylation of WCC occurs sequentially at multiple residues: first by PKA, which functions in a manner independent of FRQ, and then by the FRQ-recruited CKs.[21,34,40] The phosphorylation of WCs can be reversed by phosphatases PP2A and PP4 to counterbalance the actions of kinases.[32,41,43]

Phosphorylation of clock proteins is also critical to the negative feedback mechanisms in the animal circadian systems. PER-dependent rhythms of CLK phosphorylation and E-box binding were shown in *Drosophila*, and PER-DBT (*doubletime* kinase) interaction is required for the transcriptional repression process.[53,54] On the other hand, it was proposed that the 1:1 PER−CLK interaction in *Drosophila* can sequester CLK from DNA to mediate the feedback process,[55] but it is not clear whether the interaction alone is sufficient. In mammals, CK2α was shown to phosphorylate BMAL1 *in vitro* and *in vivo* to regulate its nuclear accumulation and clock function, and furthermore, hyperphosphorylated CLOCK in the repressed phase displayed less binding to E-box.[56,57] These findings suggest the conservation of the negative feedback mechanisms in eukaryotic clocks, although the exact mechanism in each system may differ.

On the basis of the finding that the *frq*-null strain as well as FRQ kinase mutants shows low levels of WCC, it has been proposed that the phosphorylation of WCC stabilizes the WC proteins.[21,23,24,34,40,46] Consistent with this notion, VIVID (VVD), a small photoreceptor protein, can physically interact with the light-activated WCC, inhibiting its activity and degradation.[58,59] Therefore, FFC and VVD independently and cooperatively regulate the stability of WCC by distinct mechanisms. In addition, the CCR4−Not complex, conserved among all eukaryotes, interacts with WC-1 and stabilizes it, probably by affecting its phosphorylation status.[60] These findings suggest that the phosphorylation of WCC plays a role in the stability of WCs and ensures the timely activation of the complex.

Biochemical analyses also showed that dephosphorylation of WCC enhances its DNA binding activity *in vitro*. In mutants in which the level of FFC-mediated WCC phosphorylation is reduced, higher WCC occupancy on the *frq* promoter is observed.[21,33,40,61] In contrast, hyperphosphorylated WCC and a reduced level of binding of WCC to the c-box of *frq* promoter were observed in the phosphatase mutants.[32,41,43] Therefore, the PKA-primed and FFC-mediated sequential phosphorylations of WCC can remove the complex from the *frq* promoter, resulting in the repression of *frq* transcription and, at the same time, stabilization of WCC.

Furthermore, the FFC-mediated phosphorylation of WCC also sequesters the WCs in the cytoplasm.[41,43,62] The ratio of nuclear to cytoplasmic WCC changes in a circadian manner: when WCC is hyperphosphorylated, more WCC is present in the cytoplasm; the nuclear level increases when the complex is hypophosphorylated. In strains with a defective PP2A or disrupted *pp4* gene, significantly less WCC is found in accompanying nuclei, and there are decreased levels of WC DNA binding and reduced *frq* transcription relative to wild-type levels. These studies suggest that there are multiple regulators of WC phosphorylation that control its activity, stability, and subcellular localization.

Chromatin Modulations at the *frq* Locus. The nucleosome is the basic unit of the eukaryotic chromosome. Nucleosomes are assembled from histone octamers bound to DNA strands. Post-translational modifications of histones, including H3 and H4 acetylation and methylation, are known to be the basis of epigenetic regulation that control transcription.[63] Nucleosome depletion or exchange at the promoter can modulate accessibility of DNA to transcription factors. It has been shown that many clock-controlled and core clock genes exhibit rhythmic histone modifications, which oscillate in accordance with RNA polymerase II occupancies at those loci.[64−68]

In *Neurospora*, rhythmic nucleosome occupancy and DNase hypersensitive regions are observed on the *frq* promoter.[15,44,45] Two homologues of ATP-dependent chromatin-remodeling factors, CLOCKSWITCH (CSW-1) and chromodomain helicase DNA-binding-1 (CHD1), as well as a homologue of a bromodomain-containing ATPase called Clock ATPase (CAPT) are involved in clock function through regulation of *frq* transcription. These proteins function by regulating the chromatin status of the *frq* locus through distinct mechanisms. CSW-1 and CATP affect WCC occupancy on the *frq* promoter in opposite ways: CSW-1 appears to promote nucleosome compaction because the *csw*-1-null mutant has a relatively open chromatin structure. In contrast, CATP may be involved in the histone eviction process, and the *catp* mutants have a histone occupancy on the *frq* promoter higher than that of wild-type cells. Consistent with this notion, homologues of CATP interact with histones to activate transcription.[69−72] Furthermore, rhythmic changes in H3K14 acetylation on the *frq* promoter are lost in the *catp*-null strain, suggesting that additional histone modifiers, including acetyltransferases and deacetylases, are also involved in the control of *frq* transcription.

The helicase CHD1 probably regulates *frq* transcription by modulating DNA structure at the *frq* locus. The *chd1*-null strain exhibits hypermethylation at the *frq* locus and dampened FRQ oscillation. The molecular mechanism through which DNA methylation regulates *frq* transcription is still not clear. A histone H3K4 methyltransferase SET1 is also required for normal circadian rhythms and normal WCC binding at the *frq* promoter.[73] Together, these findings emphasize the importance of epigenetic regulations in the function of the *Neurospora* circadian clock.

WC-Independent Transcription of *frq*. The rhythmic transcriptional activation by WCC is essential for rhythmic *frq* expression. Because the levels of *frq* mRNA are extremely low in *wc-1* and *wc-2* mutants,[11] WCC was thought to be the only transcriptional regulator of *frq* transcription. Recently, however, transcriptional repressor RCO-1 was shown to be an essential clock component that represses WC-independent *frq* transcription.[35] RCO-1 is a homologue of yeast Tup1p. RCO-1 interacts with Csp1 to regulate the expression of clock-controlled genes implicated in metabolism.[74,75] In the *rco-1*-null strain, *frq* mRNA and FRQ protein levels are constantly high, and conidiation and molecular rhythms are abolished. More interestingly, in the *wc rco-1* double mutants, near wild-type

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amounts of *frq* mRNA are observed, indicating that the suppression of WC-independent transcription of *frq* is essential for rhythmicity. Such an elevation of the level of *frq* transcription is also observed in the *wc-1 chd1* double mutant, suggesting that CHD1 mediates the repression of WC-independent *frq* transcription by regulating chromatin structure. The conserved RCO-1 complex is also known to participate in clock regulation in plants. In plants, the TOPLESS/TOPLESS RELATED (TPL/TPR) complex cooperates with pseudoresponse regulators, the major players of the plant circadian feedback loops, to repress the transcription of clock genes.[76]

ROLE OF *FRQ* CODON USAGE IN THE CONTROL OF FRQ EXPRESSION AND FUNCTION

There are 61 genetic codes for the 20 different translated amino acids, and thus, most amino acids (except for methionine and tryptophan) are coded by two to six synonymous codons. Different genomes have preferences for certain synonymous codons over others; this phenomenon is called codon usage bias. Codon usage bias impacts protein expression in prokaryotes and eukaryotes.[7,77−79] Codon optimization is used in research and industrial laboratories to achieve optimal protein expression.[80] Recently, codon usage has been proposed to play a role in regulating protein folding and activity;[81−83] however, its *in vivo* relevance has not been demonstrated.

Selection for codons that ensure efficient translation is thought to be the major cause of codon usage bias in different organisms. Highly expressed genes are encoded predominantly by codons that correspond to highly abundant tRNAs. This codon usage bias is thought to allow highly expressed genes to be rapidly translated with high fidelity.[84,85] In contrast, the core clock genes such as *frq* in *Neurospora* and *kaiBC* in cyanobacteria have nonoptimal codons in their open reading frames.[86,87]

We showed recently that N-terminal codon optimization of *frq* in *Neurospora* not only results in higher FRQ expression levels but also abolishes circadian clock function.[86] Several lines of biochemical evidence suggested that codon optimization impairs FRQ function in both circadian negative and positive feedback loops and alters FRQ structure. The effect of nonoptimal codon usage was partially reversed by growing *Neurospora* at low temperatures, suggesting that the codon optimization accelerates translation and affects the cotranslational folding of the FRQ protein. Furthermore, codon optimization of the central region of FRQ, containing the CKI-interacting domains, alters the FRQ phosphorylation profile and stabilizes the protein. These results suggest that the nonoptimal codons in *frq* are evolutionarily selected to ensure that the pace of translation in these regions allows proper folding.

Nonoptimal codon usage of clock components also plays an important role in cyanobacteria. The nonoptimal codon usage in the *kaiBC* gene in cyanobacteria enhances organismal fitness at cool temperatures.[87] This is an example of how natural selection resulted in the use of nonoptimal codons so that the organism can properly grow under different environmental conditions. These *in vivo* studies in *Neurospora* and cyanobacteria suggest that codon usage is an important mechanism that can regulate both protein expression and protein structures, previously thought to be controlled mainly by transcriptional and post-translational mechanisms, respectively.

POST-TRANSLATIONAL REGULATION OF FRQ

Similar to its animal homologue PER, FRQ is progressively phosphorylated as soon as it is made and becomes extensively phosphorylated before degradation.[16] Mass spectrometry (MS) analyses of recombinant peptides phosphorylated *in vitro* by the casein kinases and of FRQ protein purified from *Neurospora* led to the identification of more than 100 phosphorylation sites in the 989-amino acid protein.[22,42] The comparison of *in vitro* and *in vivo* sites confirmed that CKI and CKII are responsible for most of the phosphorylation events. FRQ phosphorylation is known to promote its degradation through the ubiquitin/proteasome pathway mediated by the ubiquitin E3 ligase-containing complex SCF^{FWD-1}.[19,37,38] FWD-1 acts as the substrate-recruiting subunit of the E3 ligase that recognizes and binds phosphorylated FRQ through its WD-40 domain. It is known that the mammalian FWD-1 homologue β-TRCP recognizes a DpSGϕXpS (ϕ is any hydrophobic residue) motif in the substrates.[88] The substrate-recognizing domain of β-TRCP is conserved in FWD-1, but the target motif is not found in any of the phosphorylated regions of FRQ. This suggests that FWD-1 may not recognize phosphorylated FRQ through a single high-affinity binding site. Instead, it may sense a conformational change of FRQ that results from extensive phosphorylations. The presence of multiple low-affinity binding sites in FRQ may result in high-affinity binding to FWD-1. This model predicts that multiple FRQ phosphorylation events contribute to FRQ stability. Indeed, systematic mutagenesis of identified phosphorylation sites showed that mutation of many phosphorylation sites affects FRQ stability.[22,42] In addition, although most FRQ phosphorylation events promote its degradation, phosphorylation of the C-terminal region of FRQ results in its stabilization, suggesting the importance of interdomain interactions to FRQ stability.

Unlike most kinase substrates, FRQ forms a stable complex with CK-1a through two separate motifs on FRQ.[18,21,89] Both motifs are required for the FRQ−CK1-a interaction and CK-1a-dependent phosphorylation of FRQ and WCs. Biochemical analyses suggest that these two motifs may interact with each other and mediate the CK-1a interaction and the subsequent phosphorylation may trigger a change in FRQ conformation that facilitates the degradation of FRQ by the ubiquitin/proteasome pathway.[89]

Although FRQ nuclear localization is essential for its function in the circadian clock, most FRQ is found in the cytoplasm.[23,27,33,51] An SV-40-like nuclear localization signal located downstream of the coiled-coil domain is required and sufficient for the nuclear localization of FRQ, suggesting that the FRQ cellular distribution profile is regulated by an active nuclear export process. A study using a FRQ−FRB fusion protein showed that there is rapid nuclear−cytoplasmic shuttling of FRQ, suggesting that the phosphorylation of FRQ inhibits its nuclear import.[90] However, we showed in a later analysis that the nuclear to cytoplasmic ratio of the FRQ protein does not significantly change during the circadian cycle despite differences in phosphorylation status.[91] In addition, mutations in FRQ kinases, phosphatases, and FWD-1, which all have severe effects on FRQ phosphorylation profiles, do not significantly alter the nuclear to cytoplasmic ratios of FRQ relative to that of the wild-type strain. Furthermore, period-changing mutations of FRQ-phosphorylated residues do not significantly alter the subcellular localization. These results

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argue against the role of FRQ phosphorylation in the cellular localization of FRQ.

What then is the function of FRQ phosphorylation in addition to its effect on FRQ stability? Biochemical analyses suggest that the ability of FRQ to interact with its partners changes during a circadian cycle.[42,91] When FRQ is hypophosphorylated in the subjective early day, it interacts with WCs and CK-1a. When FRQ becomes hyperphosphorylated in the subjective evening, however, its interaction with WC-2 is weakened. High levels of hyperphosphorylated FRQ accumulate in the *fwd-1*-null strain because of the inability of the phosphorylated FRQ to be ubiquitinated and degraded through the proteasome pathway. This hyperphosphorylated FRQ is not functional in the negative feedback loop: It does not repress the transcription of *frq*.[91] Furthermore, the levels of FRQ–WC and FRQ–CK-1a interaction are dramatically reduced in the *fwd-1*-null mutant. These results indicate that the phosphorylation of FRQ regulates FRQ activity in the negative feedback loop by affecting its ability to interact with WCs and CK-1a. Thus, the rhythmic FRQ phosphorylation profile alone can lead to rhythmic FRQ activity, so rhythm generation may not be totally dependent on the amount of FRQ.

CONCLUSION

During the past two decades, *Neurospora* has been an excellent model organism for studying the molecular mechanisms of eukaryotic circadian clocks. Studies of *Neurospora* have uncovered mechanisms that are critical for circadian clock functions. These mechanisms operate at the transcriptional, post-transcriptional, cotranslational, and post-translational levels to regulate *frq* expression, FRQ activity, and stability. These analyses not only established the FRQ-based negative feedback loop as the core of the circadian machinery but also provided an explanation of how circadian period length and clock entrainment are determined at the molecular level.

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Notes

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REFERENCES

(1) Dunlap, J. C. (1999) Molecular bases for circadian clocks. *Cell 96*, 271–290.

(2) Young, M. W., and Kay, S. A. (2001) Time zones: A comparative genetics of circadian clocks. *Nat. Rev. Genet. 2*, 702–715.

(3) Heintzen, C., and Liu, Y. (2007) The *Neurospora crassa* circadian clock. *Adv. Genet. 58*, 25–66.

(4) Cha, J., Huang, G., Guo, J., and Liu, Y. (2007) Posttranslational control of the *Neurospora* circadian clock. *Cold Spring Harbor Symp. Quant. Biol. 72*, 185–191.

(5) Lakin-Thomas, P. L., Bell-Pedersen, D., and Brody, S. (2011) The genetics of circadian rhythms in *Neurospora*. *Adv. Genet. 74*, 55–103.

(6) Sargent, M. L., Briggs, W. R., and Woodward, D. O. (1966) Circadian nature of a rhythm expressed by an invertaseless strain of *Neurospora crassa*. *Plant Physiol. 41*, 1343–1349.

(7) Gooch, V. D., Mehra, A., Larrondo, L. F., Fox, J., Touroutoutoudis, M., Loros, J. J., and Dunlap, J. C. (2008) Fully codon-optimized luciferase uncovers novel temperature characteristics of the *Neurospora* clock. *Eukaryotic Cell 7*, 28–37.

(8) Colot, H. V., Park, G., Turner, G. E., Ringelberg, C., Crew, C. M., Litvinkova, L., Weiss, R. L., Borkovich, K. A., and Dunlap, J. C. (2006) A high-throughput gene knockout procedure for *Neurospora* reveals functions for multiple transcription factors. *Proc. Natl. Acad. Sci. U.S.A. 103*, 10352–10357.

(9) Aronson, B. D., Johnson, K. A., Loros, J. J., and Dunlap, J. C. (1994) Negative feedback defining a circadian clock: Autoregulation of the clock gene frequency. *Science 263*, 1578–1584.

(10) Cheng, P., He, Q., Wang, L., and Liu, Y. (2005) Regulation of the *Neurospora* circadian clock by an RNA helicase. *Genes Dev. 19*, 234–241.

(11) Crosthwaite, S. K., Dunlap, J. C., and Loros, J. J. (1997) *Neurospora* wc-1 and wc-2: Transcription, photoresponses, and the origins of circadian rhythmicity. *Science 276*, 763–769.

(12) Cheng, P., Yang, Y., Gardner, K. H., and Liu, Y. (2002) PAS domain-mediated WC-1/WC-2 interaction is essential for maintaining the steady-state level of WC-1 and the function of both proteins in circadian clock and light responses of *Neurospora*. *Mol. Cell. Biol. 22*, 517–524.

(13) Cheng, P., Yang, Y., Wang, L., He, Q., and Liu, Y. (2003) WHITE COLLAR-1, a multifunctional neurospora protein involved in the circadian feedback loops, light sensing, and transcription repression of wc-2. *J. Biol. Chem. 278*, 3801–3808.

(14) Froehlich, A. C., Loros, J. J., and Dunlap, J. C. (2003) Rhythmic binding of a WHITE COLLAR-containing complex to the frequency promoter is inhibited by FREQUENCY. *Proc. Natl. Acad. Sci. U.S.A. 100*, 5914–5919.

(15) Belden, W. J., Loros, J. J., and Dunlap, J. C. (2007) Execution of the circadian negative feedback loop in *Neurospora* requires the ATP-dependent chromatin-remodeling enzyme CLOCKSWITCH. *Mol. Cell 25*, 587–600.

(16) Garceau, N. Y., Liu, Y., Loros, J. J., and Dunlap, J. C. (1997) Alternative initiation of translation and time-specific phosphorylation yield multiple forms of the essential clock protein FREQUENCY. *Cell 89*, 469–476.

(17) Liu, Y., Loros, J., and Dunlap, J. C. (2000) Phosphorylation of the *Neurospora* clock protein FREQUENCY determines its degradation rate and strongly influences the period length of the circadian clock. *Proc. Natl. Acad. Sci. U.S.A. 97*, 234–239.

(18) Gorl, M., Merrow, M., Huttner, B., Johnson, J., Roenneberg, T., and Brunner, M. (2001) A PEST-like element in FREQUENCY determines the length of the circadian period in *Neurospora crassa*. *EMBO J. 20*, 7074–7084.

(19) He, Q., Cheng, P., Yang, Y., He, Q., Yu, H., and Liu, Y. (2003) FWD1-mediated degradation of FREQUENCY in *Neurospora* establishes a conserved mechanism for circadian clock regulation. *EMBO J. 22*, 4421–4430.

(20) Yang, Y., Cheng, P., He, Q., Wang, L., and Liu, Y. (2003) Phosphorylation of FREQUENCY protein by casein kinase II is necessary for the function of the *Neurospora* circadian clock. *Mol. Cell. Biol. 23*, 6221–6228.

(21) He, Q., Cha, J., Lee, H. C., Yang, Y., and Liu, Y. (2006) CKI and CKII mediate the FREQUENCY-dependent phosphorylation of the WHITE COLLAR complex to close the *Neurospora* circadian negative feedback loop. *Genes Dev. 20*, 2552–2565.

(22) Tang, C. T., Li, S., Long, C., Cha, J., Huang, G., Li, L., Chen, S., and Liu, Y. (2009) Setting the pace of the *Neurospora* circadian clock by multiple independent FRQ phosphorylation events. *Proc. Natl. Acad. Sci. U.S.A. 106*, 10722–10727.

(23) Cheng, P., Yang, Y., and Liu, Y. (2001) Interlocked feedback loops contribute to the robustness of the *Neurospora* circadian clock. *Proc. Natl. Acad. Sci. U.S.A. 98*, 7408–7413.

DOI: 10.1021/bi5005624
Biochemistry 2015, 54, 150–156

(24) Schafmeier, T., Kaldi, K., Diernfellner, A., Mohr, C., and Brunner, M. (2006) Phosphorylation-dependent maturation of *Neurospora* circadian clock protein from a nuclear repressor toward a cytoplasmic activator. *Genes Dev. 20*, 297−306.
(25) McClung, C. R., Fox, B. A., and Dunlap, J. C. (1989) The *Neurospora* clock gene frequency shares a sequence element with the *Drosophila* clock gene period. *Nature 339*, 558−562.
(26) Cheng, P., Yang, Y., Heintzen, C., and Liu, Y. (2001) Coiled-coil domain-mediated FRQ-FRQ interaction is essential for its circadian clock function in *Neurospora*. *EMBO J. 20*, 101−108.
(27) Cheng, P., He, Q., He, Q., Wang, L., and Liu, Y. (2005) Regulation of the *Neurospora* circadian clock by an RNA helicase. *Genes Dev. 19*, 234−241.
(28) Guo, J., Cheng, P., and Liu, Y. (2010) Functional significance of FRH in regulating the phosphorylation and stability of *Neurospora* circadian clock protein FRQ. *J. Biol. Chem. 285*, 11508−11515.
(29) Guo, J., Cheng, P., Yuan, H., and Liu, Y. (2009) The exosome regulates circadian gene expression in a posttranscriptional negative feedback loop. *Cell 138*, 1236−1246.
(30) Shi, M., Collett, M., Loros, J. J., and Dunlap, J. C. (2010) FRQ-interacting RNA helicase mediates negative and positive feedback in the *Neurospora* circadian clock. *Genetics 184*, 351−361.
(31) Hurley, J. M., Larrondo, L. F., Loros, J. J., and Dunlap, J. C. (2013) Conserved RNA helicase FRH acts nonenzymatically to support the intrinsically disordered *Neurospora* clock protein FRQ. *Mol. Cell 52*, 832−843.
(32) Yang, Y., He, Q., Cheng, P., Wrage, P., Yarden, O., and Liu, Y. (2004) Distinct roles for PP1 and PP2A in the *Neurospora* circadian clock. *Genes Dev. 18*, 255−260.
(33) Schafmeier, T., Haase, A., Kaldi, K., Scholz, J., Fuchs, M., and Brunner, M. (2005) Transcriptional feedback of *Neurospora* circadian clock gene by phosphorylation-dependent inactivation of its transcription factor. *Cell 122*, 235−246.
(34) He, Q., Shu, H., Cheng, P., Chen, S., Wang, L., and Liu, Y. (2005) Light-independent phosphorylation of WHITE COLLAR-1 regulates its function in the *Neurospora* circadian negative feedback loop. *J. Biol. Chem. 280*, 17526−17532.
(35) Zhou, Z., Liu, X., Hu, Q., Zhang, N., Sun, G., Cha, J., Wang, Y., Liu, Y., and He, Q. (2013) Suppression of WC-independent frequency transcription by RCO-1 is essential for *Neurospora* circadian clock. *Proc. Natl. Acad. Sci. U.S.A. 110*, E4867−E4874.
(36) Yang, Y., Cheng, P., Zhi, G., and Liu, Y. (2001) Identification of a calcium/calmodulin-dependent protein kinase that phosphorylates the *Neurospora* circadian clock protein FREQUENCY. *J. Biol. Chem. 276*, 41064−41072.
(37) He, Q., Cheng, P., He, Q., and Liu, Y. (2005) The COP9 signalosome regulates the *Neurospora* circadian clock by controlling the stability of the SCFFWD-1 complex. *Genes Dev. 19*, 1518−1531.
(38) He, Q., and Liu, Y. (2005) Degradation of the *Neurospora* circadian clock protein FREQUENCY through the ubiquitin-proteasome pathway. *Biochem. Soc. Trans. 33*, 953−956.
(39) Pregueiro, A. M., Liu, Q., Baker, C. L., Dunlap, J. C., and Loros, J. J. (2006) The *Neurospora* checkpoint kinase 2: A regulatory link between the circadian and cell cycles. *Science 313*, 644−649.
(40) Huang, G., Chen, S., Li, S., Cha, J., Long, C., Li, L., He, Q., and Liu, Y. (2007) Protein kinase A and casein kinases mediate sequential phosphorylation events in the circadian negative feedback loop. *Genes Dev. 21*, 3283−3295.
(41) Cha, J., Chang, S. S., Huang, G., Cheng, P., and Liu, Y. (2008) Control of WHITE COLLAR localization by phosphorylation is a critical step in the circadian negative feedback process. *EMBO J. 27*, 3246−3255.
(42) Baker, C. L., Kettenbach, A. N., Loros, J. J., Gerber, S. A., and Dunlap, J. C. (2009) Quantitative proteomics reveals a dynamic interactome and phase-specific phosphorylation in the *Neurospora* circadian clock. *Mol. Cell 34*, 354−363.
(43) Schafmeier, T., Diernfellner, A., Schafer, A., Dintsis, O., Neiss, A., and Brunner, M. (2008) Circadian activity and abundance rhythms of the *Neurospora* clock transcription factor WCC associated with rapid nucleo-cytoplasmic shuttling. *Genes Dev. 22*, 3397−3402.
(44) Belden, W. J., Lewis, Z. A., Selker, E. U., Loros, J. J., and Dunlap, J. C. (2011) CHD1 remodels chromatin and influences transient DNA methylation at the clock gene frequency. *PLoS Genet. 7*, e1002166.
(45) Cha, J., Zhou, M., and Liu, Y. (2013) CATP is a critical component of the *Neurospora* circadian clock by regulating the nucleosome occupancy rhythm at the frequency locus. *EMBO Rep. 14*, 923−930.
(46) Lee, K., Loros, J. J., and Dunlap, J. C. (2000) Interconnected feedback loops in the *Neurospora* circadian system. *Science 289*, 107−110.
(47) Merrow, M., Franchi, L., Dragovic, Z., Gorl, M., Johnson, J., Brunner, M., Macino, G., and Roenneberg, T. (2001) Circadian regulation of the light input pathway in *Neurospora crassa*. *EMBO J. 20*, 307−315.
(48) Glossop, N. R., Lyons, L. C., and Hardin, P. E. (1999) Interlocked feedback loops within the *Drosophila* circadian oscillator. *Science 286*, 766−768.
(49) Shearman, L. P., Sriram, S., Weaver, D. R., Maywood, E. S., Chaves, I., Zheng, B., Kume, K., Lee, C. C., van der Horst, G. T., Hastings, M. H., and Reppert, S. M. (2000) Interacting molecular loops in the mammalian circadian clock. *Science 288*, 1013−1019.
(50) Denault, D. L., Loros, J. J., and Dunlap, J. C. (2001) WC-2 mediates WC-1-FRQ interaction within the PAS protein-linked circadian feedback loop of *Neurospora*. *EMBO J. 20*, 109−117.
(51) Luo, C., Loros, J. J., and Dunlap, J. C. (1998) Nuclear localization is required for function of the essential clock protein FRQ. *EMBO J. 17*, 1228−1235.
(52) He, Q., Cheng, P., Yang, Y., Wang, L., Gardner, K. H., and Liu, Y. (2002) White collar-1, a DNA binding transcription factor and a light sensor. *Science 297*, 840−843.
(53) Yu, W., Zheng, H., Houl, J. H., Dauwalder, B., and Hardin, P. E. (2006) PER-dependent rhythms in CLK phosphorylation and E-box binding regulate circadian transcription. *Genes Dev. 20*, 723−733.
(54) Kim, E. Y., Ko, H. W., Yu, W., Hardin, P. E., and Edery, I. (2007) A DOUBLETIME kinase binding domain on the *Drosophila* PERIOD protein is essential for its hyperphosphorylation, transcriptional repression, and circadian clock function. *Mol. Cell. Biol. 27*, 5014−5028.
(55) Menet, J. S., Abruzzi, K. C., Desrochers, J., Rodriguez, J., and Rosbash, M. (2010) Dynamic PER repression mechanisms in the *Drosophila* circadian clock: From on-DNA to off-DNA. *Genes Dev. 24*, 358−367.
(56) Yoshitane, H., Takao, T., Satomi, Y., Du, N. H., Okano, T., and Fukada, Y. (2009) Roles of CLOCK phosphorylation in suppression of E-box-dependent transcription. *Mol. Cell. Biol. 29*, 3675−3686.
(57) Tamaru, T., Hirayama, J., Isojima, Y., Nagai, K., Norioka, S., Takamatsu, K., and Sassone-Corsi, P. (2009) CK2α phosphorylates BMAL1 to regulate the mammalian clock. *Nat. Struct. Mol. Biol. 16*, 446−448.
(58) Hunt, S. M., Thompson, S., Elvin, M., and Heintzen, C. (2010) VIVID interacts with the WHITE COLLAR complex and FREQUENCY-interacting RNA helicase to alter light and clock responses in *Neurospora*. *Proc. Natl. Acad. Sci. U.S.A. 107*, 16709−16714.
(59) Chen, C. H., DeMay, B. S., Gladfelter, A. S., Dunlap, J. C., and Loros, J. J. (2010) Physical interaction between VIVID and white collar complex regulates photoadaptation in *Neurospora*. *Proc. Natl. Acad. Sci. U.S.A. 107*, 16715−16720.
(60) Huang, G., He, Q., Guo, J., Cha, J., and Liu, Y. (2013) The Ccr4-not protein complex regulates the phase of the *Neurospora* circadian clock by controlling white collar protein stability and activity. *J. Biol. Chem. 288*, 31002−31009.
(61) He, Q., and Liu, Y. (2005) Molecular mechanism of light responses in *Neurospora*: From light-induced transcription to photoadaptation. *Genes Dev. 19*, 2888−2899.

DOI: 10.1021/bi5005624
Biochemistry 2015, 54, 150−156

(62) Hong, C. I., Ruoff, P., Loros, J. J., and Dunlap, J. C. (2008) Closing the circadian negative feedback loop: FRQ-dependent clearance of WC-1 from the nucleus. *Genes Dev. 22*, 3196−3204.
(63) Berger, S. L. (2007) The complex language of chromatin regulation during transcription. *Nature 447*, 407−412.
(64) Etchegaray, J. P., Lee, C., Wade, P. A., and Reppert, S. M. (2003) Rhythmic histone acetylation underlies transcription in the mammalian circadian clock. *Nature 421*, 177−182.
(65) Ripperger, J. A., and Schibler, U. (2006) Rhythmic CLOCK-BMAL1 binding to multiple E-box motifs drives circadian Dbp transcription and chromatin transitions. *Nat. Genet. 38*, 369−374.
(66) Katada, S., and Sassone-Corsi, P. (2010) The histone methyltransferase MLL1 permits the oscillation of circadian gene expression. *Nat. Struct. Mol. Biol. 17*, 1414−1421.
(67) DiTacchio, L., Le, H. D., Vollmers, C., Hatori, M., Witcher, M., Secombe, J., and Panda, S. (2011) Histone lysine demethylase JARID1a activates CLOCK-BMAL1 and influences the circadian clock. *Science 333*, 1881−1885.
(68) Koike, N., Yoo, S. H., Huang, H. C., Kumar, V., Lee, C., Kim, T. K., and Takahashi, J. S. (2012) Transcriptional architecture and chromatin landscape of the core circadian clock in mammals. *Science 338*, 349−354.
(69) Zou, J. X., Revenko, A. S., Li, L. B., Gemo, A. T., and Chen, H. W. (2007) ANCCA, an estrogen-regulated AAA+ ATPase coactivator for ERα, is required for coregulator occupancy and chromatin modification. *Proc. Natl. Acad. Sci. U.S.A. 104*, 18067−18072.
(70) Gradolatto, A., Smart, S. K., Byrum, S., Blair, L. P., Rogers, R. S., Kolar, E. A., Lavender, H., Larson, S. K., Aitchison, J. D., Taverna, S. D., and Tackett, A. J. (2009) A noncanonical bromodomain in the AAA ATPase protein Yta7 directs chromosomal positioning and barrier chromatin activity. *Mol. Cell. Biol. 29*, 4604−4611.
(71) Revenko, A. S., Kalashnikova, E. V., Gemo, A. T., Zou, J. X., and Chen, H. W. (2010) Chromatin loading of E2F-MLL complex by cancer-associated coregulator ANCCA via reading a specific histone mark. *Mol. Cell. Biol. 30*, 5260−5272.
(72) Lombardi, L. M., Ellahi, A., and Rine, J. (2011) Direct regulation of nucleosome density by the conserved AAA-ATPase Yta7. *Proc. Natl. Acad. Sci. U.S.A. 108*, E1302−E1311.
(73) Raduwan, H., Isola, A. L., and Belden, W. J. (2013) Methylation of histone H3 on lysine 4 by the lysine methyltransferase SET1 protein is needed for normal clock gene expression. *J. Biol. Chem. 288*, 8380−8390.
(74) Bobrowicz, P., Pawlak, R., Correa, A., Bell-Pedersen, D., and Ebbole, D. J. (2002) The *Neurospora crassa* pheromone precursor genes are regulated by the mating type locus and the circadian clock. *Mol. Microbiol. 45*, 795−804.
(75) Sancar, G., Sancar, C., Brugger, B., Ha, N., Sachsenheimer, T., Gin, E., Wdowik, S., Lohmann, I., Wieland, F., Hofer, T., Diernfellner, A., and Brunner, M. (2011) A global circadian repressor controls antiphasic expression of metabolic genes in *Neurospora*. *Mol. Cell 44*, 687−697.
(76) Wang, L., Kim, J., and Somers, D. E. (2013) Transcriptional corepressor TOPLESS complexes with pseudoresponse regulator proteins and histone deacetylases to regulate circadian transcription. *Proc. Natl. Acad. Sci. U.S.A. 110*, 761−766.
(77) Sharp, P. M., Tuohy, T. M. F., and Mosurski, K. R. (1986) Codon usage in yeast: Cluster analysis clearly differentiates highly and lowly expressed genes. *Nucleic Acids Res. 14*, 5125−5143.
(78) Sørensen, M. A., Kurland, C. G., and Pedersen, S. (1989) Codon usage determines translation rate in *Escherichia coli*. *J. Mol. Biol. 207*, 365−377.
(79) Lavner, Y., and Kotlar, D. (2005) Codon bias as a factor in regulating expression via translation rate in the human genome. *Gene 345*, 127−138.
(80) Brocca, S., Schmidt-Dannert, C., Schmid, R. D., Lotti, M., and Alberghina, L. (1998) Design, total synthesis, and functional overexpression of the *Candida rugosa* lipl gene coding for a major industrial lipase. *Protein Sci. 7*, 1415−1422.
(81) Zhang, G., Hubalewska, M., and Ignatova, Z. (2009) Transient ribosomal attenuation coordinates protein synthesis and co-translational folding. *Nat. Struct. Mol. Biol. 16*, 274−280.
(82) Siller, E., DeZwaan, D. C., Anderson, J. F., Freeman, B. C., and Barral, J. M. (2010) Slowing Bacterial Translation Speed Enhances Eukaryotic Protein Folding Efficiency. *J. Mol. Biol. 396*, 1310−1318.
(83) Spencer, P. S., Siller, E., Anderson, J. F., and Barral, J. M. (2012) Silent Substitutions Predictably Alter Translation Elongation Rates and Protein Folding Efficiencies. *J. Mol. Biol. 422*, 328−335.
(84) Akashi, H. (1994) Synonymous codon usage in *Drosophila melanogaster*: Natural selection and translational accuracy. *Genetics 136*, 927−935.
(85) Drummond, D. A., and Wilke, C. O. (2008) Mistranslation-induced protein misfolding as a dominant constraint on coding-sequence evolution. *Cell 134*, 341−352.
(86) Zhou, M., Guo, J., Cha, J., Chae, M., Chen, S., Barral, J. M., Sachs, M. S., and Liu, Y. (2013) Non-optimal codon usage affects expression, structure and function of clock protein FRQ. *Nature 495*, 111−115.
(87) Xu, Y., Ma, P., Shah, P., Rokas, A., Liu, Y., and Johnson, C. H. (2013) Non-optimal codon usage is a mechanism to achieve circadian clock conditionality. *Nature 495*, 116−120.
(88) Wu, G., Xu, G., Schulman, B. A., Jeffrey, P. D., Harper, J. W., and Pavletich, N. P. (2003) Structure of a β-TrCP1-Skp1-β-catenin complex: Destruction motif binding and lysine specificity of the SCF(β-TrCP1) ubiquitin ligase. *Mol. Cell 11*, 1445−1456.
(89) Querfurth, C., Diernfellner, A. C., Gin, E., Malzahn, E., Hofer, T., and Brunner, M. (2011) Circadian conformational change of the *Neurospora* clock protein FREQUENCY triggered by clustered hyperphosphorylation of a basic domain. *Mol. Cell 43*, 713−722.
(90) Diernfellner, A. C., Querfurth, C., Salazar, C., Hofer, T., and Brunner, M. (2009) Phosphorylation modulates rapid nucleocytoplasmic shuttling and cytoplasmic accumulation of *Neurospora* clock protein FRQ on a circadian time scale. *Genes Dev. 23*, 2192−2200.
(91) Cha, J., Yuan, H., and Liu, Y. (2011) Regulation of the activity and cellular localization of the circadian clock protein FRQ. *J. Biol. Chem. 286*, 11469−11478.

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Biochemistry 2015, 54, 150−156