Functional Significance of FRH in Regulating the Phosphorylation and Stability of *Neurospora* Circadian Clock Protein FRQ*

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**FREQUENCY** (FRQ) is the central component of the *Neurospora* circadian clock. All FRQ proteins form the FFC complex with FRH (FRQ-interacting RNA helicase) that acts as the negative element in the circadian negative feedback loop by repressing *frq* mRNA levels. To understand the function of the FRQ-FRH interaction, we mapped and identified the minimal FRQ region that is required for FRQ-FRH interaction. We demonstrated that the FRQ-FRH complex formation is required for the interaction between FRQ and the White Collar Complex (WCC) and clock function. On the other hand, in the FRQ-FRH complex, FRQ is also required for the FRH-WCC interaction. Disruption of FRQ-FRH interaction or down-regulation of FRH results in hypophosphorylation, rapid degradation of FRQ, as well as low levels of WHITE COLLAR-1 and WHITE COLLAR-2. Furthermore, we showed that the rapid FRQ degradation in the absence of FRH is independent of FWD-1, the ubiquitin E3 ligase of FRQ under normal conditions, thus uncovering an alternative pathway for FRQ degradation.

The circadian clocks allow organisms to adjust their molecular, cellular, and physiological activities to the daily environmental changes. The eukaryotic circadian oscillators consist of positive elements and autoregulatory negative-feedback loops, which control the rhythmic expression of the negative elements and clock-controlled genes (1–3). In the filamentous fungus *Neurospora*, one of the best understood circadian model systems (4, 5), WHITE COLLAR-1 (WC-1) and WC-2 form the WC complex (WCC), which binds to frequency (*frq*) promoter and activates its transcription, thus serving as the positive elements in the circadian OSCILLATOR (6–12). FRQ is a core regulator modulating *Neurospora* circadian clock in a negative feedback loop (13, 14). FRQ forms homodimers, and all the FRQ protein is in complex with FRH (FRQ-interacting RNA helicase) and forms the FFC complex (FFC) (15). FFC represses the binding of WCC to *frq* gene promoter and *frq* transcription as a consequence (9, 10, 14–16). FRH is the homolog of Dob1p/Mtr4p in budding yeast, which is a cofactor of the exosome complex (15, 17). The exosome complex is involved in processing, maturation, and turnover of various RNA species (18, 19).

To close the circadian negative feedback loop, FFC represses *frq* mRNA levels by two mechanisms. On the one hand, FFC represses WCC activity on *frq* transcription by promoting WC phosphorylation, a process that requires the FFC-WCC interaction (11, 12, 14, 15, 20, 21). On the other hand, FFC promotes the degradation of *frq* RNA through the exosome complex (22). The combination of these transcriptional and post-transcriptional regulations on *frq* results in the increase and decrease in *frq* levels and a robust *frq* rhythm during a circadian cycle (23).

After its synthesis, FRQ protein is progressively phosphorylated and becomes extensively phosphorylated before its degradation, resulting in a robust rhythm of its phosphorylation profile (23–25). FRQ phosphorylation is known to promote FRQ degradation through the ubiquitin-proteasome pathway mediated by ubiquitin E3 ligase SCF{\textsuperscript{FWD-1}} (26, 27). FRQ is phosphorylated by several kinases, including CK-1α, CKII, protein kinase A, and CAMK-1 (12, 23, 28–31). FWD-1, the *Neurospora* homolog of the mammalian β-transducin repeat-containing proteins, is the substrate-recruiting subunit of the SCF E3 complex that recognizes and binds phosphorylated FRQ to mediate its ubiquitylation and degradation (26, 27). In the *fwd-1* mutant, FRQ became hyperphosphorylated, and its normal degradation pathway was blocked, resulting in high FRQ levels and arrhythmic conidiation. It is not clear whether other alternative degradation pathways are involved in FRQ degradation.

Here, we investigated the functional importance of the FRQ-FRH interaction. By mapping and identifying the minimal FRQ region that is required for FFC formation, we demonstrated that the FRQ-FRH interaction is required for FRQ-WC interaction and clock function. In contrast to the previously known mechanism for FRQ degradation, we showed that the disruption of FRQ-FRH interaction or down-regulation of FRH results in hypophosphorylation and rapid degradation of FRQ, indicating that the FRH is required for maintaining the steady state level of FRQ. Furthermore, we showed that the degradation of FRQ in the absence of FRH is mediated by unknown pathway that is independent of FWD-1. Together, these results
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demonstrate multiple roles of FRH in the Neurospora circadian clock.

EXPERIMENTAL PROCEDURES

Constructs, Strains, and Culture Conditions—The bd, a strain was used as the wild-type strain in this work. 303-3 (bd, his3, frq10), the frq null strain, is the host strain for all FRQ mutant constructs (13). The FRH knockout strain (dsfrh) was described previously (15). In the dsfrh strain, addition of quinic acid (QA) triggers the silencing of the frh gene. frq10, qa-FRQ is a frq null strain transformed with a construct expressing frq under the control of the qa-2 promoter (8).

pkaj120 plasmids bearing different mutations (Fig. 1A) or deletions were transformed into frq10 host strain at the his-3 locus to generate FRQ6A, FRQ6B1, FRQ6B2, FRQ6B3, FRQ6B4, and FRQ6B5 strains, respectively. The deletions or mutations were generated by site-directed mutagenesis using QuickChange site-directed mutagenesis kit. The deleted sequences and the mutated amino acids are indicated in Fig. 1. Wild-type pkaj120 was also transformed to frq10 strain to generate the frq10, kaj120 strain, which was used as a control strain.

The liquid culture experiments were performed in 2% LCM media containing 1× Vogel’s medium and 0.17% arginine, with 2% glucose. Conidia were used to seed mycelial mats in 75-mm Petri dishes, and 0.5-cm disks were cut from these and used for liquid nitrogen, and ground with liquid nitrogen. For liquid culture medium containing 1× Vogel’s medium, 0.17% arginine, and 0.17% arginine (15).

Plasmids—To construct the pkaj120 plasmids comprising different mutations, the mutations were first generated in partial frq sequence cloned in PUC19 vector, by site-directed mutagenesis strategy through overlap extension using PCR with primers harboring respective mutations. Next, the fragments were subcloned into pkaj120 through several specific restriction enzyme sites.

Generation of fwd-1RIP Knockdown Strain—To generate the fwd-1RIP, dsfrh strain, the pdsfrh plasmid was transformed into the 315-13 (fwd-1RIP, his-3) strain at the his-3 locus. 315-13 (fwd-1RIP, his-3) is a strain in which the fwd-1 was disrupted. For the silencing of frh in fwd-1RIP, dsfrh is inducibly expressed by the addition of QA.

Medium for race tube assays contained 1× Vogel’s medium, 2% glucose, 50 ng of biotin/ml, and 1.5% agar. After transfer to dark, the growth fronts were marked on the race tubes at certain times every 24 h. The QA concentration for the race tube assays was 10−3 m.

Northern Blot Analysis—RNA extraction and Northern blot analyses were performed as described previously (13). Equal amounts of total RNA (20 μg) were loaded onto agarose gels for electrophoresis, and the gels were blotted and probed with frq probes.

Protein Extraction, Western Analysis, and Co-immunoprecipitation Analyses—Protein extraction, quantification, Western blot analysis, and immunoprecipitation assays were performed as described previously (30). Equal amounts of total protein (40 μg) were loaded in each protein lane of SDS-PAGE, and after electrophoresis, proteins were transferred onto polyvinylidene difluoride membrane, and Western blot analyses were performed. The immunoprecipitation assays were conducted by using specific antisera (IP) or preimmune sera (PI). Membranes stained with Amido Black (Sigma) were used as loading control for some Western blot analyses. Data are mean ± S.D., based on independent experiments.

Protein Stability Assay—To measure FRQ stability, the liquid cultures of Neurospora were treated with 10 μg/ml CHX and harvested at 0, 3, 6, and 9 h, respectively. Proteins were extracted and loaded (40 μg) for SDS-polyacrylamide gel running, followed by Western blots analyses of FRQ protein. The QA concentration for the protein stability assay in dsfrh and fwd-1RIP, dsfrh strains was 0.01 m.

RESULTS

Mapping of the Minimal Region Required for FRQ-FRH Interaction—We have previously shown that the region between amino acids 695 and 778 of FRQ (the region deleted in sFRQ6 mutant) is necessary and sufficient for the interaction between FRQ and FRH (Fig. 1A) (15). To map the minimal FRQ-FRH interacting region, we created two additional FRQ internal deletion mutants (frq10, FRQ6A and frq10, FRQ6B) with amino acids 695–727 and 728–778 deleted, respectively. Immunoprecipitation assays using FRH antibody showed that deletion of the FRQ6B region, but not FRQ6A, completely abolished the interaction between FRQ and FRH, indicating that a region within FRQ6B is responsible for the FFC formation (Fig. 1B).

The comparison of amino acid sequences of different FRQ homologs revealed several clusters of conserved amino acids within the FRQ6B region (Fig. 1A). We reasoned that one or some of these conserved clusters constitute the minimal region that mediates the FRQ-FRH interaction. Thus, we generated five alanine scan mutants (frq10, FRQ6B1–5), in which the conserved clusters were mutated to alanine residues (Fig. 1A). Immunoprecipitation assays showed that that the FRQ-FRH interaction was abolished in FRQ6B2 and FRQ6B5 mutants but not in FRQ6B1, FRQ6B3, and FRQ6B4 strains (Fig. 1, C and D).

FRQ levels in the FRQ6B2 and FRQ6B5 are very low. The comparison of frq mRNA levels by Northern blot analysis showed that the frq levels were comparable in these two mutants and the wild-type strains (supplemental Fig. 1, A and B), suggesting that that the FRQ-FRH interaction is important for maintaining the steady state level of FRQ at a post-translational level.

To examine whether the elimination of the FRQ-FRH interaction in the FRQ6B2 and FRQ6B5 mutants was due to low FRQ levels in these strains, we performed FRH immunoprecipitation assay in a frq null strain (frq10, qa-FRQ) with a construct that can inducibly express FRQ in the presence of QA. The level of FRQ in this strain is dependent on the concentration of QA. As shown in supplemental Fig. 1C, the levels of FRQ in frq10, qa-FRQ strain were comparable with those in the FRQ6B2 and FRQ6B5 mutants. However, the FRH immunoprecipitation assay showed that FRH was associated with FRQ in the frq10, qa-FRQ strain but not in the FRQ6B2 and FRQ6B5 mutants, indicating that the abolishment of FRQ-FRH interaction in
these two mutants was not due to their low FRQ levels. Together, these results strongly suggest that the region from 773 to 782 amino acids mediates the association between FRH and FRQ. This region of FRQ consists of mostly hydrophobic residues and is predicted to form a $\beta$-strand structure. The conservation of this region in other fungal FRQ sequences suggests it is a functionally important domain for FRQ.

FRH Mediates the Association between FRQ and WCC Complex—FFC acts as a negative element in the circadian negative feedback loop that represses the activity of the WCC. To understand the role of FRQ-FRH interaction in this process, we examined the association between FRQ and WCC in the FRQ deletion strains (FRQ6B1–5). Immunoprecipitation assays using WC-2 antibody showed that the FRQ-WCC interaction was abolished in the FRQ6B2 and FRQ6B5 mutants but was maintained in FRQ6B1, FRQ6B3, and FRQ6B4 strains (Fig. 2, A and B). These results are consistent with our previous results (15) and indicate that FRH mediates the interaction between FRQ and WCC.

Consistent with the molecular results, race tube assays showed that the circadian conidiation rhythms were completely abolished in the FRQ6B2 and FRQ6B5 strains. In contrast, clear but low amplitude circadian conidiation rhythms could be observed in the FRQ6B3 and FRQ6B4 mutants (Fig. 2C). These results indicate that the FRQ-FRH interaction is required for clock function. Interestingly, the FRQ6B1 strain also exhibited arrhythmic conidiation, suggesting that the five conserved amino acids immediately upstream of the minimal FRQ-FRH interaction domain may contribute to FRQ function although not FFC formation. Interestingly, FRQ6A also exhibited arrhythmic conidiation on race tube, indicating that another functionally important domain within the region deleted in FRQ6A.

Both FRQ and FRH Contribute to the FFC-WCC Interaction—We have previously shown that FRH associates with WCC in the frq null strain (frq<sup>−</sup>), indicating that FRH can interact with WCC independent of FRQ (15). However, it is unclear whether FRQ also contributes to FFC-WCC interaction. To understand the role of FRQ in this interaction, we conducted immunoprecipitation assays using WC-2 antibody in a series of FRQ internal deletion mutants (sFRQ1–8) (11), which covers most of the FRQ open reading frame (Fig. 3A). As expected, FRH-WCC was abolished in the sFRQ6 mutant. Although FRH associated with WCC in the sFRQ3, sFRQ5a, and sFRQ8 strains, surprisingly,
the interaction was completely eliminated in the sFRQ1, sFRQ2, sFRQ4, sFRQ5b, and sFRQ7 mutants. These results demonstrate that although FRH can interact with WCC in the total absence of FRQ, FRQ also contributes to the interaction between FFC and WCC. Thus, in a wild-type strain, FFC interacts with WCC as a complex but not as individual proteins. It is likely that the presence of FRQ can interfere or block the interaction surface of FRH that can associate with WCC in the frq null strain.

Consistent with our previous results (15), we found that in FRQ mutants lacking the FRH-WCC interaction (sFRQ1, sFRQ2, sFRQ4, sFRQ5b, sFRQ6, and sFRQ7), the association between FRQ and WCC was also abolished. In contrast, the FRQ-WCC interaction was maintained in the sFRQ3, sFRQ5a, and sFRQ8 strains (Fig. 3A). These results provide further evidence supporting the importance of both FRQ and FRH in mediating the FFC-WCC interaction and strongly suggest that both FRQ and FRH are required for the
FFC-WCC interaction, and multiple domains of FRQ are involved in this process.

We previously showed that FRQ forms homodimer through its coiled-coil domain (32). To determine whether FRQ interacts with FRH independent of its homodimer formation, we examined the FRQ-FRH interaction in the frq9, Myc-FRQ165RR strain. In this strain, two point-mutations in the coiled-coil domain abolished the FRQ-FRQ interaction (32). The immunoprecipitation assay showed that FRQ-FRH was maintained in this strain (Fig. 3B), indicating that the FRQ-FRH interaction is not dependent on FRQ dimerization.

Disruption of FRQ-FRH Interaction or Down-regulation of FRH Results in Hypophosphorylated FRQ—FRQ levels are low in strains that either have the FRQ-FRH interaction abolished or FRH expression silenced (Fig. 1D) (15). Because FRQ phosphorylation promotes FRQ degradation, we examined the FRQ phosphorylation profiles in these strains (Fig. 4A). Because of low levels of FRQ in these mutants, the total proteins for the Western blot analysis were adjusted so that similar amounts of FRQ were present in each sample. Surprisingly, we found that FRQ was hypophosphorylated in the FRQ6B2 and FRQ6B5 strains. Similarly, hypophosphorylated FRQ was found in the presence of quinic acid in the dsfrh strain (Fig. 4, A and B). The dsfrh strain contains a construct that can inducibly express a hairpin RNA specific for frh mRNA with the addition of QA (15). As control, QA had no effect on FRQ level or FRQ phosphorylation profile in a wild-type strain. Note that for the gel in Fig. 4A, more total protein was loaded for the dsfrh sample with QA so that the FRQ levels were similar. These results indicate that the normal FRQ phosphorylation process requires FRH and the FRQ-FRH interaction.

Disruption of FRQ-FRH Interaction or Down-regulation of FRH Regulates FRQ Phosphorylation and Stability—The low FRQ levels in strains lacking either the FRQ-FRH interaction or FRH expression suggest that FRQ may be unstable in these strains. To test this hypothesis, we compared the FRQ stability after the addition of the protein synthesis inhibitor CHX. Upon addition of CHX, FRQ was degraded much faster in the FRQ6B2 and FRQ6B5 strains than the strain carrying a wild-type frq construct (kaj120) (Fig. 5A). Despite the low levels of the hypophosphorylated FRQ in the mutant strains at time 0, FRQ was rapidly degraded after the CHX treatment. Interestingly, unlike the KAJ120 strain, in which FRQ first became hyperphosphorylated before its degradation, most of FRQ in the mutant strains was degraded within the first 3 h after the addition of CHX. This result suggests that the wild-type and mutant FRQ were degraded by different mechanisms.

Similarly, rapid degradation of FRQ was also observed in the dsfrh strain when frh was silenced by the QA treatment (Fig. 5B). In contrast, the FRQ stability was not affected by the QA treatment in the wild-type strain. Together, these results demonstrate that FRH and the FRQ-FRH interaction stabilize FRQ. The rapid degradation of FRQ in the absence of FRH provides a molecular explanation to the low FRQ protein levels when FRH is silenced.
Disruption of FRQ-FRH Interaction or Down-regulation of FRH Results in Reduction of WC Proteins—We also compared the WC protein levels between the wild-type and the FRQ6B2 and FRQ6B5 strains. As shown in Fig. 6, A and B, both WC-1 and WC-2 levels were significantly reduced in FRQ6B2 and FRQ6B5 strains. In the wt,dsfrh strain, the silencing of FRH also resulted in the reduction of WC levels (Fig. 6, C). The comparison of WC stability between the wild-type and the mutant strains showed that WC stability was not significantly changed in these mutant strains (supplemental Fig. 2). Thus, the low levels of WCs in these strains indicate the loss of positive feedback loops that maintain the WC levels (8, 33). Therefore, as FRQ and FRH also plays an important role in the positive feedback loops of the clock (33).

**FRH Mediates the Degradation of FRQ Protein Independent of SCF FWD-1**—FWD-1, the substrate-recruiting subunit of an SCF complex, mediates the phosphorylation-dependent ubiquitination and degradation of FRQ (26, 27, 34). The hypophosphorylation and rapid degradation of FRQ in the mutants shown above suggest an alternative FRQ degradation pathway. To test this hypothesis and to examine the role of FWD-1 in the degradation of FRQ in the absence of FRH, we transformed the dsfrh construct into a fwd-1RIP,dsfrh strain (fwd-1RIP,dsfrh). As shown by the race tube assays in Fig. 7A, the addition of QA resulted in dramatic inhibition of its growth on race tube, indicating that frh expres-

![Figure 6](image_url)

**FIGURE 6.** Abolishment of FRH-FRQ association resulted in decrease of WC proteins. A–F, Western blot analysis results showing the levels of WCs in the indicated strains. Amido Black-stained membranes were used as loading control. WT, wild type.

![Figure 7](image_url)

**FIGURE 7.** FRH regulates FRQ stability independent of FWD-1. A, race tube tests of fwd-1RIP,dsfrh. The growth was inhibited in the presence of QA (10^{-2} M). B, Western blot analysis showing that FRH was silenced in fwd-1RIP,dsfrh strain by QA treatment (10^{-2} M). Non-specific bands served as control. C, FRQ protein was hypophosphorylated and dramatically reduced in fwd-1RIP,dsfrh strain in the presence of QA (10^{-2} M). D, FRQ protein exhibited rapid degradation in the fwd-1RIP,dsfrh strain after QA treatment (10^{-2} M). Membranes stained with Amido Black were used as loading control. Cycloheximide was added to inhibit the protein translation (10 μg/ml final concentration). Data are mean ± S.D., n = 3. Asterisks indicate p < 0.05. WT, wild type; mem, membrane.
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sion was silenced. In agreement with this result, FRH level was dramatically reduced in the presence of QA (Fig. 7B).

Consistent with our previous results (26), FRQ was hyperphosphorylated in this strain in the absence of QA (Fig. 7C). In the presence of QA, however, FRQ level was dramatically reduced, and FRQ became hypophosphorylated. The comparison of FRQ stability showed that similar to the frq6B2 and FRQ6B5 strains, FRQ was rapidly degraded when frh was silenced (Fig. 7D). Together, these results demonstrate that in the absence of FRH, FRQ was degraded independent of FWD-1 through an alternative pathway.

DISCUSSION

In addition to FRQ and the WCs, FRH is a core component of the Neurospora circadian oscillator. In Neurospora, all FRQ protein binds to FRH to form FFC, which acts as a negative element in the circadian negative feedback loops. In addition to its role as a transcriptional inhibitor repressing frq transcription, FFC promotes the degradation of frq mRNA through the exosome complex and is part of the post-transcriptional negative feedback loop critical for clock function (22). In this study, we established a new function of FRH in the Neurospora circadian clock, provided molecular details on how FFC complexes are formed and interact with WCC and uncovered a new pathway for FRQ degradation.

By identifying the minimal domain on FRQ that is required for the FFC formation and by creating point mutation FRQ mutants, we showed that the FRQ-FRH interaction is required for the interaction between FRQ and WCC and is essential for clock function as a consequence. Although FRH is able to interact with WCC independent of FRQ, we showed here that in the FFC, FRQ is also required for the FFC-WCC interaction. In several FRQ deletion mutants, the association between FRH and WCC is abolished although the interaction between FRQ and FRH is maintained. These results indicate that in a wild-type strain, FRQ and FRH interact with WCC as a complex, and both FRQ and FRH contribute to such an interaction.

Silencing of FRH expression results in high levels of frq mRNA (15, 22). We have shown that the high levels of frq mRNA are due to the loss of inhibition of WCC activity by the FFC and the impaired frq mRNA degradation through the exosome (22). Contrary to the high mRNA levels, FRQ protein levels are low when FRH is silenced or when the FRQ-FRH interaction was abolished. We now showed that FRQ protein is hypophosphorylated and rapidly degraded in the absence of FRH. Furthermore, we showed that FRQ binds to FRQ independent of the FRQ-FRH interaction. Together, these data suggest that FRH is critical for the maintenance of FRQ phosphorylation profile and stability. In addition, we showed that FRH and the FRH-FRQ interactions are also important for the maintenance of the levels of WC proteins in the positive feedback loops.

In contrast to the known FRQ degradation pathway, which is promoted by phosphorylation and mediated by the ubiquitin E3 ligase FWD-1, we found that FRQ is hypophosphorylated when the FRQ-FRH interaction is disrupted or when FRH is silenced. In addition, the rapid degradation of FRQ in the absence of FRH is independent of FWD-1. These results reveal an alternative FRQ degradation pathway (26, 34). It is also possible that this alternative FRQ degradation process also contributes to the overall FRQ stability under normal conditions. Consistent with this hypothesis, significant amounts of FRQ degradation were previously observed in the fwd-1 mutant strain after a light-dark transition (26). Although the molecular nature of this degradation process is currently not known, it is likely that in the absence FRH, FRQ may be adopt an alternative conformation that can be rapidly degraded in the cell (31, 32).

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Note Added in Proof—Since the submission of this manuscript, a study was published describing the roles of FRH in both negative and positive feedback loops (Shi, M., Collett, M., Loros, J. J., and Dunlap, J. C. (2010) Genetics 184, 351–361).

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