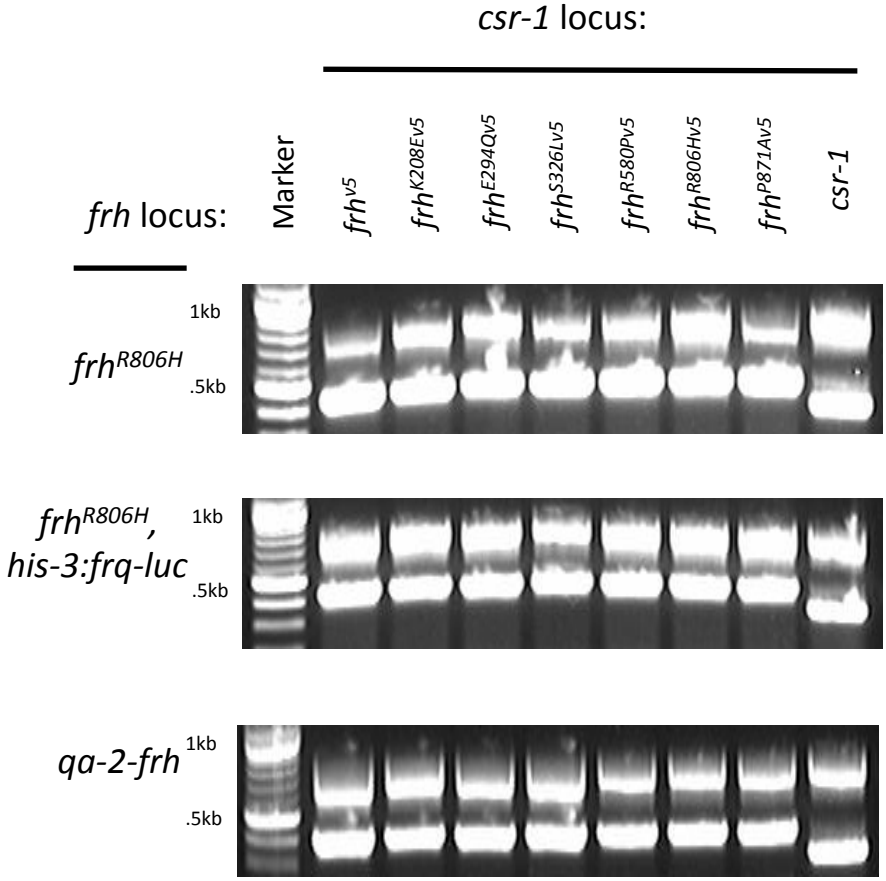
Supplemental Information

Supplemental Figures

Supplemental Figure 1) Confirmation of genotype of relevant strains. Constructs bearing the mutants described in Figure 1 were transformed into *frh*^{R806H}; *frh*^{R806H},*his-3::pfrqluc* or *qa-2-frh* strains at the *csr-1* locus. Genomic DNA samples from strains were subjected to PCR analysis to examine the *csr-1* locus; primer sequences are provided in Supplemental Table 1. Bands at 350 bp represent the presence of the intact *csr-1* locus, bands at 450 bp represent successfully transformed *frh* disrupting the *csr* locus while bands at 750 bp represent the presence of the *actin* locus (+ control).


csr-1 locus:
frh locus:
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frhv5
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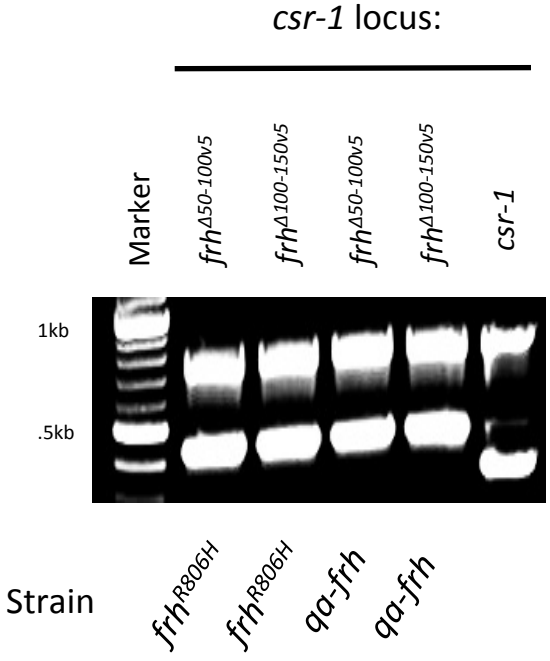
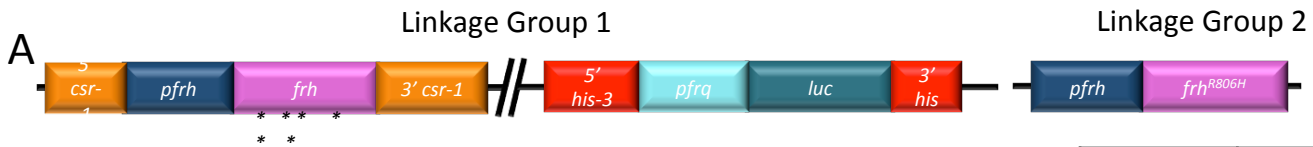
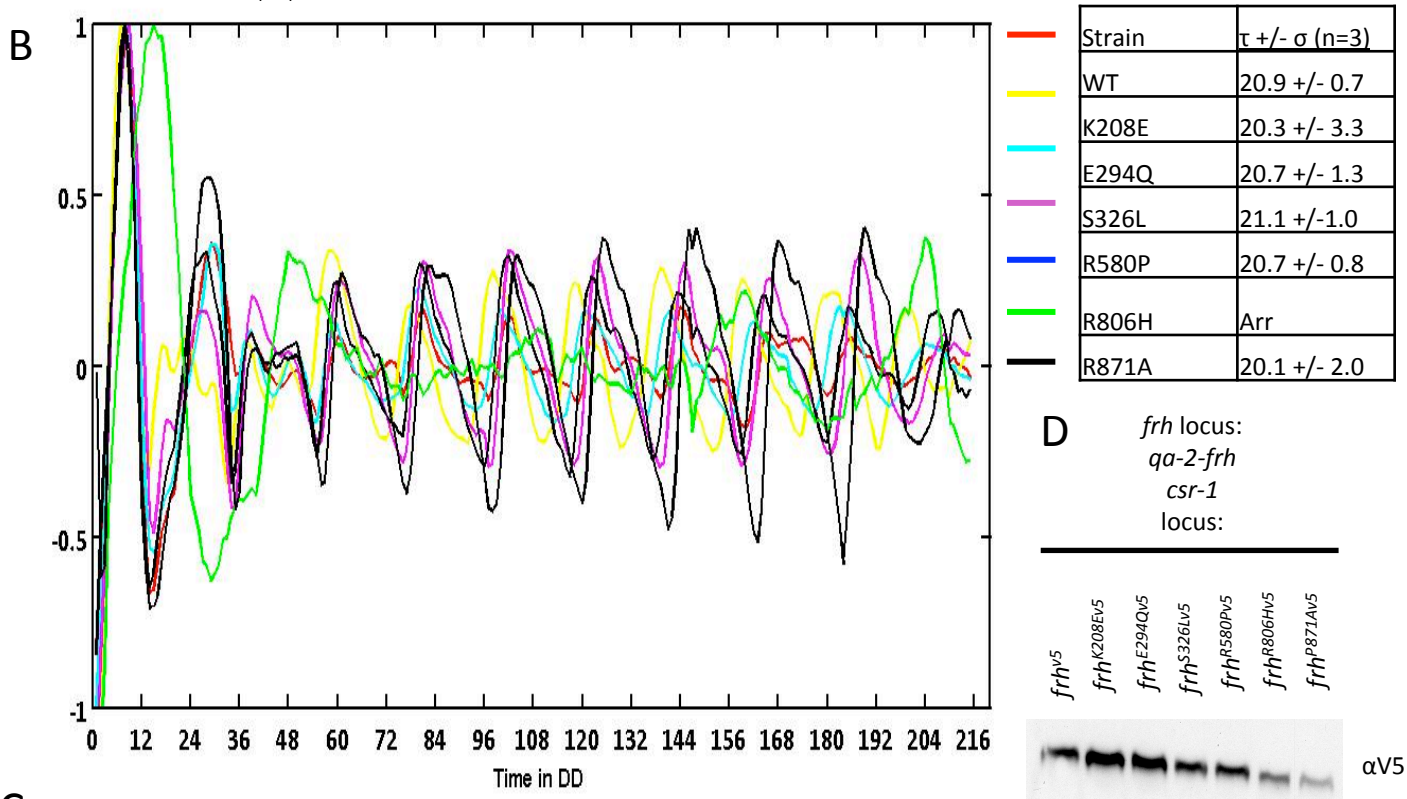
frhE294Qv5
frhS326Lv5
frhR580Pv5
frhR806Hv5
frhP871Av5
csr-1
1kb
frhR806H
.5kb
frhR806H,
his-3:frq-luc
1kb
.5kb
qa-2-frh
1kb
.5kb

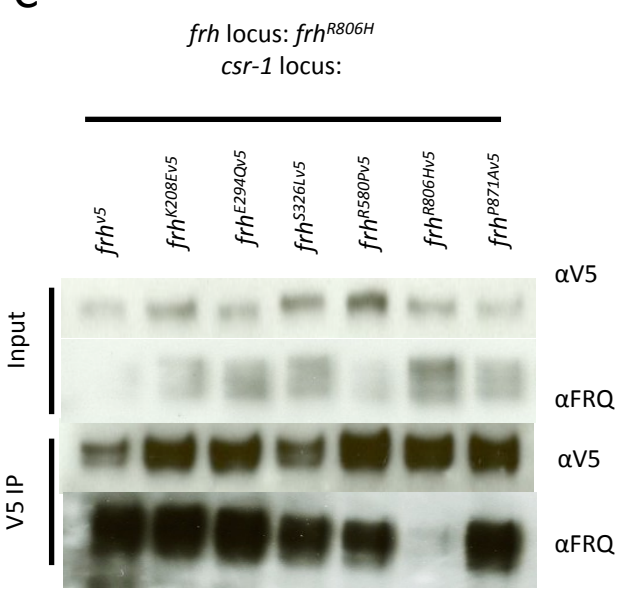

csr-1 locus:
Marker
frhΔ50-100v5
frhΔ100-150v5
frhΔ50-100v5
frhΔ100-150v5
csr-1
1kb
.5kb
Strain
frhR806H
frhR806H
qa-frh
qa-frh

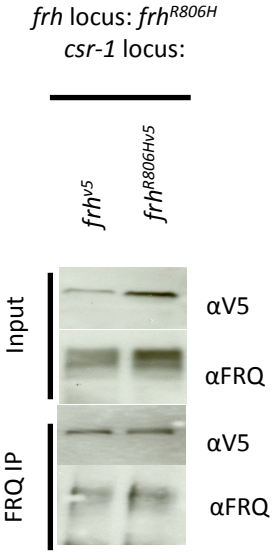
Supplemental Figure 2) Normal circadian rhythmicity in strains bearing mutations in FRH helicase function as detected by a *frq-luc* transcriptional reporter. (A) Strain genotypes: Linkage group 1; at the *csr-1* locus: mutant versions of *frh* driven by the *frh* promoter; at the *his-3* locus: luciferase driven by the *frq* promoter. Linkage group 2; at the *frh* native locus: frh^{R806H} driven by the native *frh* promoter. (B) Luciferase traces of strains described in Figure 1. Bioluminescence was sampled every 30 minutes for 9 days in darkness from race tubes of Neurospora of the genotypes shown. Luminescence as a function of time with data being normalized and background subtracted. (τ = period in hours, σ = standard deviation, n = number of race tubes). (C) Immunoprecipitation assay confirming that helicase/ATPase mutants of FRH retain stability and ability to interact with FRQ. Western blot of expression or V5 IP of mutant copies of FRH in the FRH^{R806H} background confirming expression or interaction. (D) Western blot confirming expression of mutant FRH at the *csr-1* locus in the *qa-2* driven FRH background. (E) Western blot confirming expression of FRH fragments at the *csr-1* locus in the *qa-2* driven FRH and FRH^{R806H} backgrounds. (F) Western blot of *qa-2* driven mutant FRH strains confirming expression (10^{-2}M QA added). (G) Coomassie stain confirming interaction between in vitro expressed FRQ and FRH. (PI = input, FT = flow through, IP = FRQ immunoprecipitation).

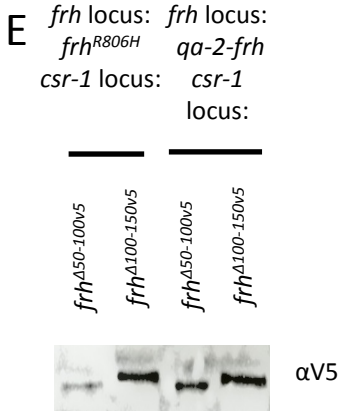


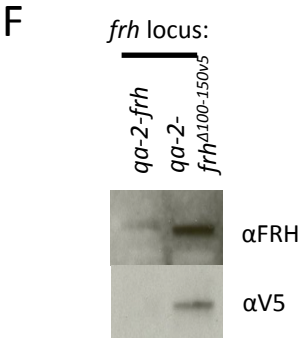


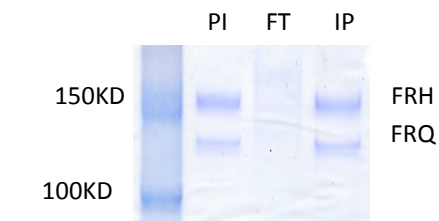
Strain	τ +/- σ (n=3)
WT	20.9 +/- 0.7
K208E	20.3 +/- 3.3
E294Q	20.7 +/- 1.3
S326L	21.1 +/-1.0
R580P	20.7 +/- 0.8
R806H	Arr
R871A	20.1 +/- 2.0



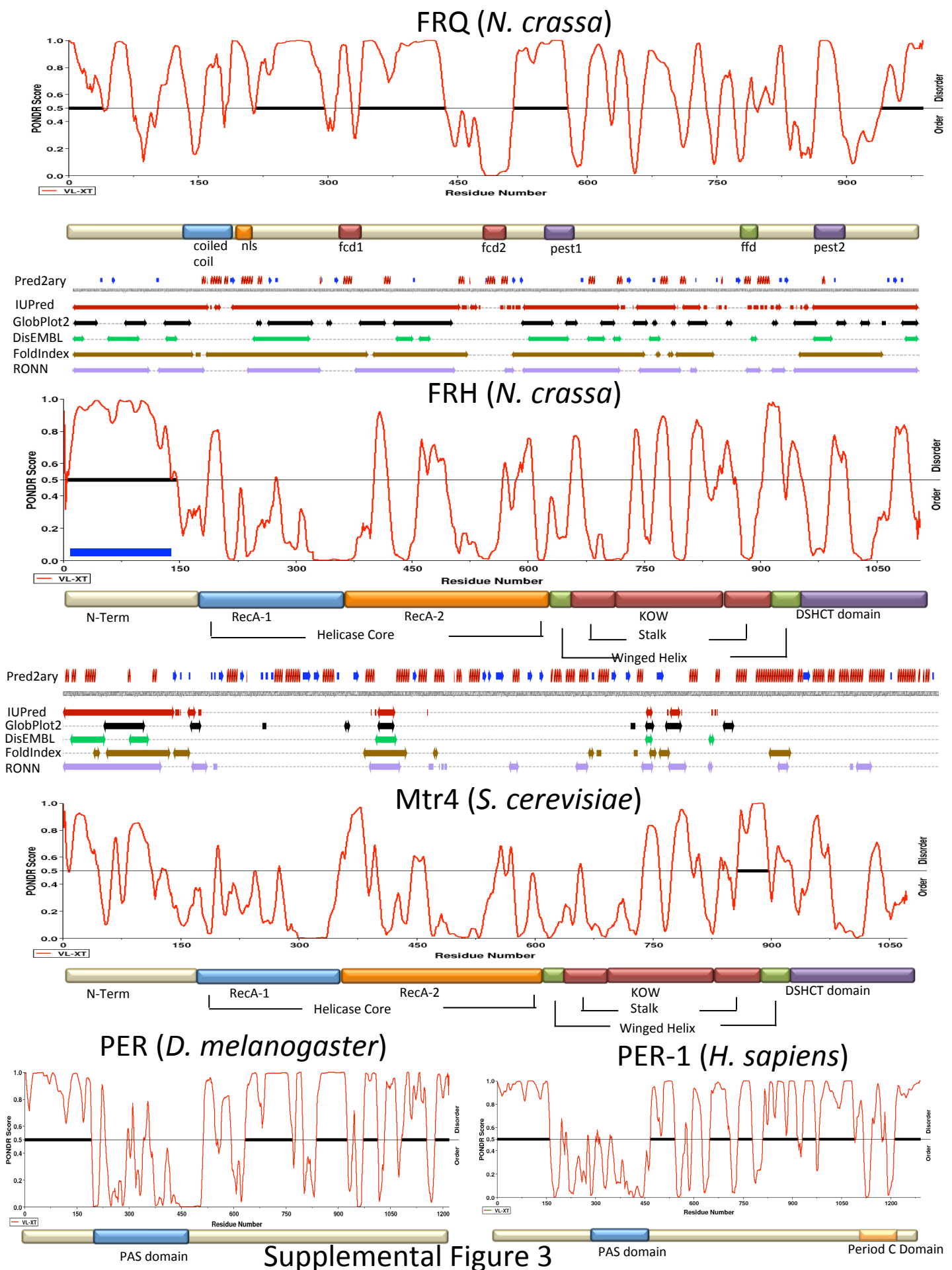








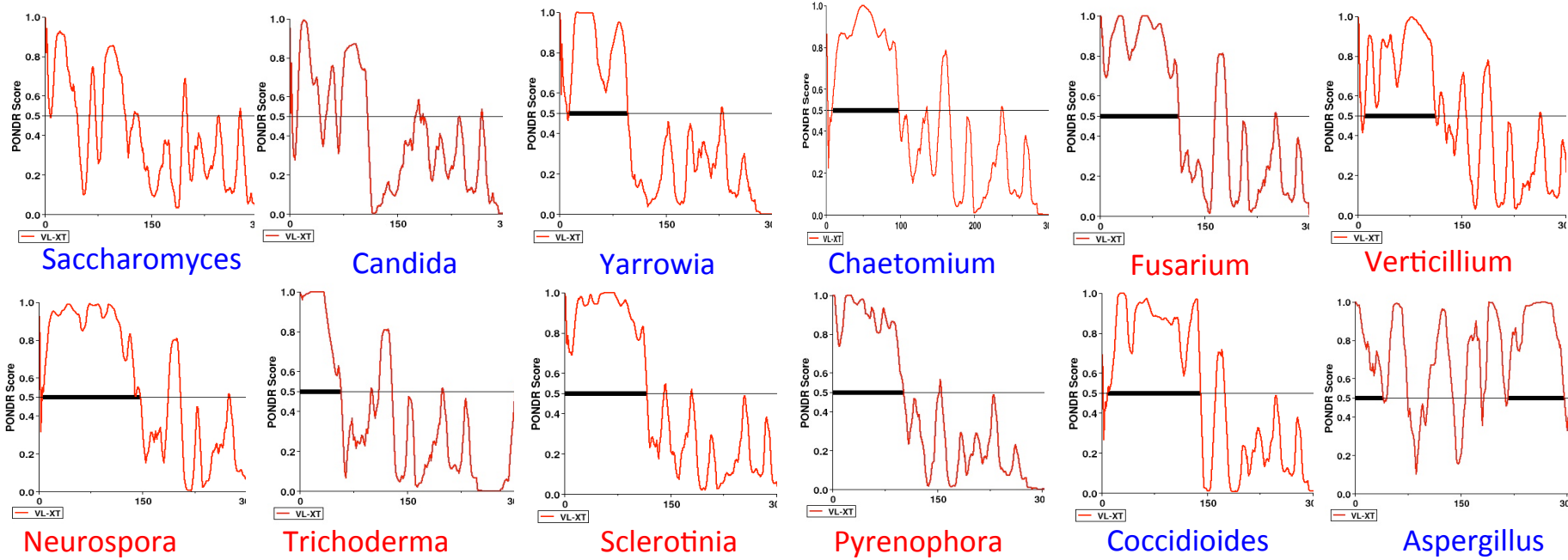
Supplemental Figure 3) Computational analysis of the structural propensity of FRQ, FRH, Mtr4p and clock proteins from eukaryotes using PONDR and additional structural prediction programs. Graphs show PONDR analysis of the full length FRH, Mtr4p, FRQ, PER from *D. melanogaster* and PER-1 from *H. sapiens*. Basic structural domains of each are shown as predicted. Black lines represent distinct and probable regions of low structural complexity (notably absent in first 150 amino acids of Mtr4p). In the cases of FRH and FRQ, structural prediction was also preformed by MeDor (Lieutaud et al., 2008) and is aligned with each PONDR graph. Secondary structural prediction (StrBioLib/Pred2ary) is the top line of each and denotes predicted alpha helices (red bars) and beta sheets (blue arrows). IUPred predicted unstructured regions are shown in red; GlobPlot2 predicted unstructured regions are shown in black; DisEMBL predicted unstructured regions are shown in green; FoldIndex predicted unstructured regions are shown in brown; and RONN predicted unstructured regions are shown in purple.



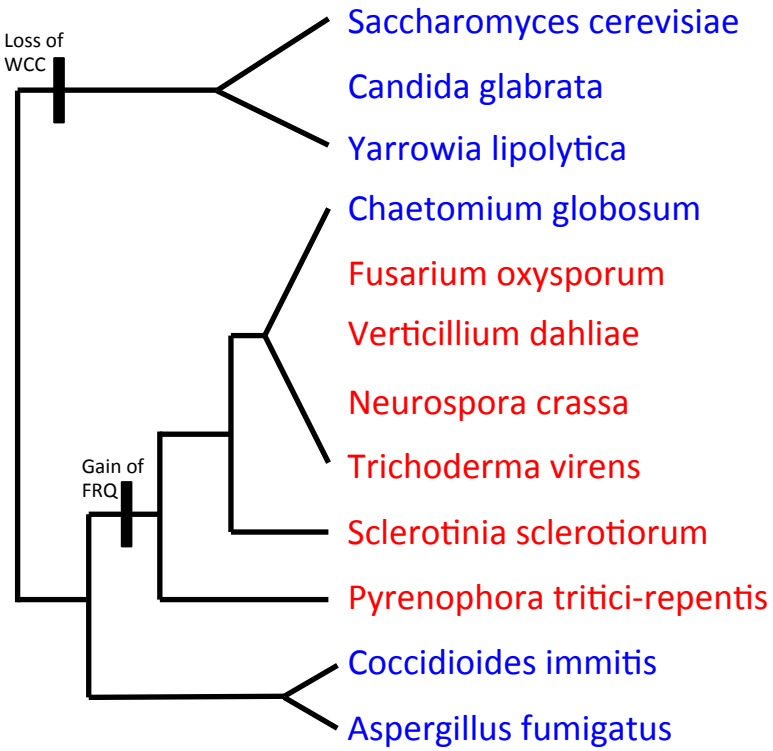
Supplemental Figure 3

Supplemental Figure 4) PONDR analysis of FRH orthologues from fungi. (A) PONDR analysis of the first three hundred amino acids of a variety of fungi that have been shown to either have (Red) or not have (blue) *frq* in their genome sequence. (B) Phylogenetic tree of FRH adapted from (Salichos and Rokas, 2010) showing species used in part (A).

A


PONDR Score
VL-XT
Saccharomyces
Candida
Yarrowia
Chaetomium
Fusarium
Verticillium
Neurospora
Trichoderma
Sclerotinia
Pyrenophora
Coccidioides
Aspergillus

B


Loss of WCC
Gain of FRQ
Saccharomyces cerevisiae
Candida glabrata
Yarrowia lipolytica
Chaetomium globosum
Fusarium oxysporum
Verticillium dahliae
Neurospora crassa
Trichoderma virens
Sclerotinia sclerotiorum
Pyrenophora tritici-repentis
Coccidioides immitis
Aspergillus fumigatus

Supplemental Materials and Methods

Strains, plasmids and reagents

Transformations were performed as described (Bardiya and Shiu, 2007; Colot et al., 2006) and screened by allele-specific nested PCR (Supplemental Figure 1) (primers in Supplemental Data Table 1). Strains are listed in supplemental data table 2. Strains have been deposited into the FGSC.

Race tube assays, circadian liquid culture experiments, luciferase trace experiments, and FRQ degradation experiments were performed as described with slight modifications (Garceau et al., 1997; Gooch et al., 2008; Hong et al., 2008; Loros et al., 1989; Ruoff et al., 2005). For Neurospora culture, conidia were inoculated into Bird medium (Metzenberg, 2004) containing 1.8% glucose.

The *E. coli* strain BL21(DE3) (F^- *omp*T hsdS_β(r_β-m_β) *dcm gal* (DE3) tonA) (Invitrogen) was used for all protein expression. The *E. coli* strain TOP10 (F-*mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) φ80*lac*ZΔM15 Δ*lac*X74 *rec*A1 *ara*D139 Δ(*ara-leu*) 7697 *gal*U *gal*K *rps*L (Str^R) *end*A1 *nup*G λ-) (Invitrogen) was used for all cloning experiments. Short-FRQ and FRH, codon optimized as designed by the authors for expression in *E. coli* were created by Genscript and cloned into pCOLD1 (a generous gift from the Masayori Inouye lab). All bacterial liquid cultures were grown in LB media (M9) at 17 °C, unless otherwise noted. The working concentration of ampicillin was 100g/ml. The accuracy of the DNA sequences of PCR products used for cloning was confirmed by automated DNA sequence analysis.

Western blots, protein preparation and immunoprecipitation

Protein lysates were prepared on a small scale with a protease inhibitor mixture (P9599; Sigma). For Western blot analysis, 10 µg of total protein was loaded per lane. Anti-V5 antibody (Invitrogen) was diluted 1:5,000. Anti-FLAG antibody was diluted 1:1000 (F3165, Sigma). Anti-FRQ antibody was diluted 1:250 (Garceau et al., 1997). Anti-FRH antibody was diluted 1:10, 000 (Shi et al., 2010). SuperSignal West Femto ECL (Pierce) was used for signal development.

To perform the co-IP assay, 1 mg of total protein extract was incubated with 30 µL of anti-V5 antibody-coated agarose (A7345; Sigma), ANTI-FLAG M2 Magnetic beads (A2220, Sigma) or Protein G Mag Sepharose beads (28-9440-08, GE Healthcare) with appropriate antibody (overnight at 4 °C). The agarose/beads were washed with protein extraction buffer four times before elution with Laemmle buffer (65 °C for 15 min) before loading on the gel.

Luciferase assays

Assays were preformed and analyzed as described in (Gooch et al., 2008; Larrondo et al., 2012).

Protein structural prediction

Proteins were subjected to PONDR and MeDor analysis as described (Li et al., 1999; Lieutaud et al., 2008; Romero et al., 1997; Romero et al., 2001) As part of the MeDor analysis, structural predictions from StrBioLib/Pred2ary (Chandonia, 2007), IUPred (Dosztanyi et al., 2005), Globplot2 (Linding et al.,

2003b), DisEMBL (Linding et al., 2003a), FoldIndex (Prilusky et al., 2005) and RONN (Yang et al., 2005) were analyzed.

Protein stability assay

Whole cell protein extracts from *Neurospora crassa* were diluted to 10mg/ml. 200ul aliquots were treated with proteinase K (0.2 ug/ml final concentration) and placed at 37°C. A 20-µl sample was taken from the reaction at each time point (0, 15, 30, 45 and 60 mins) after addition of proteinase K. Protein samples were mixed with protein loading buffer and resolved by SDS–PAGE and subjected to western blot analysis.

Recombinant FRQ and FRH

Protein from short-frq(His)6-pCOLD1 and frh(His)6-pCOLD1 constructs were transformed into BL21(DE3) cells and induced with 1mM isopropyl 1-thio-β-D-galactopyranoside for 6 h. Cells were disrupted using sonication; the protein extracts were applied to nickel-nitrilotriacetic acid resin (Qiagen), and the His6-tagged proteins were purified as recommended by Qiagen, followed by passage over a ZEBA desalting column (Thermo Scientific). The protein content of each eluted fraction was visualized by SDS-PAGE followed by Coomassie staining.

Circular Dichroism

CD spectra were recorded at 20°C on a Jasco J-815 CD spectrometer equipped with a Peltier temperature control system and the data was collected using the Jasco Spectra Manager II software package. The proteins were

prepared in 10 mM potassium phosphate at a concentration of 2.5 mg/ml and 0.85 mg/ml for FRQ and FRH respectively. CD spectra were measured between 200 and 250 nm in a 1 mm cuvette and averaged from three scans. The contribution of buffer was subtracted from experimental spectra. The mean residue weight (MRW) was calculated by MRW = molecular weight [Da]/(number of residues – 1). The mean ellipticity values [θ] were calculated by [θ] = (millidegrees value × MRW)/(pathlength [mm] × concentration [mg/ml]).

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