Supporting Online Material for

The *Neurospora* Checkpoint Kinase 2: A Regulatory Link Between the Circadian and Cell Cycles
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This PDF file includes
Materials and Methods
Figs. S1 and S2
References
Materials and Methods

**PRD-4 antibody.** Western blots were carried out using standard procedures [e.g. (S1)] with a rabbit antibody generated against a bacterially expressed GST::PRD-4 fusion protein. Polyclonal serum was depleted of some of the cross-reacting antibody molecules by four rounds of incubation of serum with protein extracts from a Δprd-4 strain prior to use in Western blotting.

**Construction of prd-4 knockout and homokaryotic rescued strains.** Deletion of the prd-4 coding region was obtained by homologous gene replacement (S2). Two regions of 1774 and 1053 base pairs flanking prd-4 were amplified by PCR from genomic DNA.

These flanks were then ligated to either side of an hph cassette containing the hph ORF which confers resistance to hygromycin driven by the constitutive TrpC promoter from *Aspergillus nidulans*. An aliquot of the ligation reaction was used as template for PCR to amplify the region spanning the three ligated fragments. This amplified DNA was transformed into a bd, his-3 strain by electroporation and transformants were selected on medium containing 200 µg/ml hygromycinB (Calbiochem). Initial transformants were checked for integration at and replacement of the resident prd-4 locus by PCR and Southern blot and were then backcrossed twice. The replacement removes the endogenous sequence starting 400 bp 5’ from the start codon of the prd-4 open reading frame, replacing the prd-4 gene with TrpC:hph and leaving 303 bp from the 3’ end of the ORF just downstream from the hph ORF STOP codon.

In experiments to establish that the cloned DNA would confer the prd-4 phenotypes, a construct containing the entire prd-4^m^ gene was targeted to the his-3 locus in a Δprd-4 background. This yielded heterokaryotic primary transformants in which only some nuclei of the syncytium carried the transgene; these showed weak PRD-4 expression. A cross of one such heterokaryotic primary transformant with a Δprd-4, bd strain (cross 356) yielded homokaryotic progeny, among them strain 356-16 (his-3:: prd-4^m^, Δprd-4, bd A) and strain 356-15 (Δprd-4, bd A), a nonexpressing sibling from this same cross that did not inherit the transgene.

**RACE.** 5’ RACE products were obtained from total *Neurospora* RNA using the GeneRacer kit (Invitrogen) and primer 5’GGCGATCTCGACTGTGTTGATGG3’. 3’ end clones were obtained using the GeneRacer oligo dT primer to reverse transcribe *Neurospora* poly-A RNA followed by PCR using the GeneRacer 3’ nested primer and primer 5’ GAGGAGAGGTCCAAGGACGAGG 3’. Both 5’ and 3’ RACE products were then cloned and sequenced.

**Quantitative real-time RT-PCR.** For each time point, tissue was grown in constant light for 24h at 25°C and then transferred to constant darkness at 25°C. Transfers were staggered so that all samples were collected within 11h. Total RNA was extracted using TRIZOL reagent (Invitrogen) followed by DNase-treatment with Amplification Grade DNaseI (Invitrogen). Reverse transcription was carried out using the TaqMan kit from Applied Biosystems. Quantitative PCR was performed with the ABI Prism 7700 and SYBR Green Reagents (Applied Biosystems). Briefly cDNA was amplified using primers 5’GAGGAGAGGTCCAAGGACGAGG3’ and 5’CAAGACGTTGGGTTGACTAACC3’ to yield SYBR Green-labeled products whose
fluorescence was recorded over the course of 40 cycles of PCR. Minimum threshold cycle (CT) values were taken from within the linear range of amplification according to manufacturer’s recommendations. For each sample the amount of frq and prd-4 message amplified was normalised to the amount of L6, which encodes a constitutively expressed ribosomal protein (S3), and then plotted relative to the highest value in the time series.

**Immunoprecipitation.** 1 mg of total protein extract was incubated with antiserum to PRD-4 or with preimmune serum (1:250) and 30 μL of 50% v/v Protein A Agarose Beads (Invitrogen) in IP buffer (50 mM Hepes, 137 mM KCL, 5% Glycerol, 0.05% Triton-X 100, 1 mM EDTA, 1 μg/mL pepstatin A, 1 μg/mL Leupeptin) for 3 hours rotating at 4 °C. Beads were washed 3 times before a final wash in water, resuspended in 40 μL 1X SDS loading buffer, boiled for 5 minutes, and analysed by 7.5% SDS PAGE. For these experiments, Western blots for FRQ detection were incubated with a monoclonal antibody directed against FRQ (S4).

**Methyl methane sulfonate (MMS) treatments.** Unless otherwise specified, strains were grown at 30°C for a day. Tissue was collected at the indicated times, and treated or not for 2 hours with MMS at a final concentration of 0.1%. Samples were then collected and processed for Western Blotting. In Figure 2B an aliquot of each MMS-treated protein extract was also incubated with lambda protein phosphatase for 1 hour at 30°C (labeled 2PP).

**Yeast rescue experiments.** Rationale: Rad53 is required to down regulate the ribonucleotide reductase (RNR) protein inhibitor Sml1 (S5–S8); in the absence of Rad53, Sml1 constitutively inhibits RNR (S6, S7), and mutations that interfere with Sml1 binding to RNR suppress the lethality of rad53 loss-of-function alleles (S6, S9). Without the Rad53 checkpoint however, these double mutant strains, while alive, remain sensitive to DNA damaging agents (S10) so such strains can be used to examine rad53 complementation.

All four strains were grown in liquid SC-leu medium with 2% raffinose overnight at 30°C, and induced with 2% galactose for 4 hours before plating (S11). The different cultures were diluted to the same concentration, plated in 3-fold serial dilutions on leucine dropout plates with galactose, containing 0.014% MMS or no MMS. The plates were incubated at 30°C for 4 days.

**Analysis of circadian rhythm data via race tubes.** Race tubes were run as previously described [e.g.(S12)] and period length values estimated from densitometric analyses of race tubes using the CHRONO program (S13). Phase shifts in Fig. 4B were determined by comparing the steady-state phase of treated cultures with controls.
References
Figure S1. PRD-4 is similar to checkpoint kinase-2 proteins from many organisms. On the top for orientation is shown the intron/exon structure of the prd-4 transcript with untranslated regions (light blocks), the ORF (dark blocks), and introns (diagonal lines). Variation in the polyadenylation sites is depicted by differences in light block height. Below this are the locations of the FHA and kinase domains in the PRD-4 protein. The sequences of the (top) FHA and (bottom) kinase domains of PRD-4 are aligned with the corresponding domains of human Chk2 and seven other known homologues in other species. The small orange arrow in the kinase domain points to D414 mutated to alanine to create the kinase dead mutant, and the large red arrowhead at the bottom marks serine 493 which is mutated to leucine in the canonical prd-4 mutant.
Figure S2. Changes in FRQ phosphorylation as a function of time and MMS treatment in wt, ∆prd-4, and prd-4(S493L) strains. To better allow comparison of samples across the time series, the samples collected and analyzed for Fig. 3B and C were loaded in a different order and electrophoresed in parallel. A, The kinetics of FRQ phosphorylation in wt versus the canonical prd-4 mutant strain prd-4(S493L). The dashed line marks the position of the unphosphorylated newly synthesized FRQ protein. As soon as FRQ appears in the prd-4(S493L) strain it is already partially phosphorylated. B, MMS induces phosphorylation of FRQ, but only in the presence of functional PRD-4 protein. As in panel A, the dashed line is provided for reference to mark the mobility of unphosphorylated FRQ. Loading controls (protein stained with Coomassie Blue) appear at the bottom.