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A Role for Casein Kinase 2 in the Mechanism Underlying Circadian Temperature Compensation

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SUMMARY

Temperature compensation of circadian clocks is an unsolved problem with relevance to the general phenomenon of biological compensation. We identify casein kinase 2 (CK2) as a key regulator of temperature compensation of the *Neurospora* clock by determining that two long-standing clock mutants, *chrono* and *period-3*, displaying distinctive alterations in compensation encode the β1 and α subunits of CK2, respectively. Reducing the dose of these subunits, particularly β1, significantly alters temperature compensation without altering the enzyme's Q_{10}. By contrast, other kinases and phosphatases implicated in clock function do not play appreciable roles in temperature compensation. CK2 exerts its effects on the clock by directly phosphorylating FREQUENCY (FRQ), and this phosphorylation is compromised in CK2 hypomorphs. Finally, mutation of certain putative CK2 phosphosites on FRQ, shown to be phosphorylated in vivo, predictably alters temperature compensation profiles effectively phenocopying CK2 mutants.

INTRODUCTION

Most organisms employ biochemical clocks to keep daily time (Dunlap et al., 2004; Pittendrigh, 1993). Circadian clocks are oscillators defined by three key characteristics: they must free run with an approximately 24 hr period, they are entrainable by relevant environmental stimuli, e.g., light, and they exhibit temperature compensation (TC), the relative invariance of period lengths over a physiologically relevant range of temperatures. Among these defining characteristics, TC is the least well understood.

Temperature affects the clock in three ways. First, discrete temperature steps up or down can reset the clock (e.g., Glaser and Stanewsky, 2007; Liu et al., 1998). Second, temperature can impose physiological limits on rhythmicity (Kalmus, 1934; Liu et al., 1997; Njus et al., 1977). Finally, while the phase of the clock responds to discrete temperature changes, paradoxically, its period is buffered against changes in temperature. Although this relative independence of period length had been noted (Kalmus, 1935; Pittendrigh, 1954; Wahl, 1932), Hastings and Sweeney (1957) first recognized that this buffering was not due to temperature independence but rather reflected a TC mechanism. Because both the magnitude and sign of the period change with temperature varied across the physiological range, they reasoned that TC could be a network property perhaps arising from multiple reactions having opposing contributions to period length but was unlikely to be based on a single temperature-independent process as had been supposed. Notably, even homeotherms have temperature-compensated clocks (e.g., Barrett and Takahashi, 1995; Tosini and Menaker, 1998), suggesting that TC may not simply be a property appended to a temperature-dependent clock but may instead be a property of the regulatory architecture.

Information obtained from *Neurospora*, *Drosophila*, and cultured mammalian cells is consistent with a negative feedback loop of conserved architecture at the core of the clock. In this loop two proteins that interact via PAS domains comprise a transcriptional activator that drives expression of genes encoding protein(s) that, in turn, feed back to repress the activator (see Allada et al., 2001; Schibler and Sassone-Corsi, 2002; Wijnen and Young, 2006). The *Neurospora* clock provides an example of such a circuit. Briefly, WHITE COLLAR-1 (WC-1) and WC-2 interact via PAS domains to make a transcriptional activator, the white collar complex (WCC), which activates transcription of the *frequency* (*frq*) gene. FRQ protein in turn, with the help of FRQ-interacting RNA helicase (FRH), depresses the activity of the WCC, in part by promoting its phosphorylation. Over time, FRQ is progressively phosphorylated and consequently targeted by the F box and WD40 repeat-containing protein-1 (FWD-1) for degradation by the proteasome (reviewed in Brunner and Schafmeier, 2006; Dunlap and Loros, 2006; Liu and Bell-Pedersen, 2006).

frq and FRQ are implicated in a number of temperature-dependent responses (Nowrousian et al., 2003). Temperature resetting has been explained by shifts in the level of FRQ without underlying changes in *frq* expression (Liu et al., 1998), reflecting the fact that the steady-state level of FRQ rises as a function of increasing ambient temperature (Diernfellner et al., 2005; Liu

et al., 1997). Moreover, *frq* pre-mRNA is alternatively spliced to direct production of both long and short FRQ, each of which can support compensated clocks (Diernfellner et al., 2007; Dunlap et al., 2007b), and this splicing is temperature dependent (Colot et al., 2005; Diernfellner et al., 2005). Augmented levels of long FRQ at high temperatures and increased amounts of short FRQ at low temperatures extend the physiological range of rhythmic banding (Liu et al., 1997). However, changes in the ratio of long to short FRQ do not lead to altered TC, nor is this ratio relevant to TC as a general property (Diernfellner et al., 2007). These data implicate *frq*/FRQ in a number of temperature responses; however, we emphasize that not all effects of temperature on the clock contribute to TC processes.

Theory and experiment have suggested mechanisms underlying TC. The simplest model invokes a pair of opposing biochemical reactions, with normal and equal Q_{10}s having opposing effects on progress of the clock cycle (Hastings and Sweeney, 1957). As temperature changes, cycle duration is kept constant as the rates of opposing reactions change by the same magnitude. A similar solution proposed for *Arabidopsis* envisions reactions in two clock-associated feedback loops with opposing responses to temperature changes so that the composite is balanced (Gould et al., 2006). An attractive mathematical formulation for the idea of opposing reactions, the balance equation (Ruoff et al., 1997, 2005), models the rate of the circadian cycle as the sum of many reactions whose contributions to period must equal 0. A mechanistically unspecified amplitude model (Lakin-Thomas et al., 1991) having some empirical support (Lahiri et al., 2005; Liu et al., 1998) achieves TC by having the size of the circadian limit cycle increase with temperature, thereby compensating for increases in angular velocity. The possibility of competing intra- and intermolecular reactions involving *Drosophila* PER (Huang et al., 1995; Price, 1997; Sawyer et al., 1997) led to a model in which opposing effects of dimerization and nuclear entry lead to TC (Hong et al., 2007; Hong and Tyson, 1997). This and additional mathematical models (Akman et al., 2008; Kurosawa and Iwasa, 2005; Leloup and Goldbeter, 1997; Takeuchi et al., 2007) await experimental testing. Genetic screens for TC mutants have suffered from the fact that circadian period is often dependent on dosage of clock components (e.g., Smith and Konopka, 1982); thus, temperature-sensitive alleles whose effective doses drop with increasing temperature can be mistaken for TC mutants. In general, although TC is a universal aspect of circadian rhythms, mechanisms that have been generalizable (e.g., Ruoff et al., 1997) have not been reducible to mechanistic specifics, and mechanisms that are mechanistically specified (e.g., Huang et al., 1995) have not been generalizable.

Two mutant strains, however, *chrono* (*chr*) and *period-3* (*prd-3*), merited further study because they displayed the unusual characteristic of enhanced or extended TC (Gardner and Feldman, 1981). The period length of the *Neurospora* clock is slightly undercompensated between 18°C and 30°C beyond which the period shortens and TC is lost. In *chr* mutants, however, TC extends beyond 30°C, and *prd-3* strains are overcompensated such that the period increases as a function of temperature (Gardner and Feldman, 1981). Reasoning that these types of TC might be informative, we determined the molecular bases of the mutations in *chr* and *prd-3* and studied their roles in TC. The result is that these two independently derived period mutants, later found to be defective in TC, identify separate subunits of the same enzyme, casein kinase 2 (CK2). Furthermore, genetic manipulation of CK2 activity, unique among kinases and phosphatases examined, affects both period length and TC in a predictable manner. These and additional data are consistent with a role for CK2 in the mechanism underlying TC.

RESULTS

chrono and *period-3* Bear Mutations in the β1 and α Subunits of CK2, Respectively

First, we confirmed the phenotypes of *chr* and *prd-3* and established an epistatic relationship between these mutants. Consistent with previous data (Gardner and Feldman, 1981), *chr* shows an extended range of TC (Figure 1A, black squares versus white circles) and *prd-3* overcompensates compared to wild-type (WT). In our experiments, however, *prd-3* shows poor rhythmic banding at 30°C and above (Figure 1A, white triangles). Interestingly, *prd-3*;*chr* double mutants have longer periods than either single mutant but overcompensate and band poorly above 30°C (Figure 1A, black triangles), demonstrating additivity of period but clear epistasis of *prd-3* over *chr* with respect to TC.

chr and *prd-3* were cloned using single-nucleotide polymorphism (SNP) mapping. Genetic mapping had placed *chr* on the right arm of LG VI and *prd-3* near the centromere of LG I (Gardner and Feldman, 1981), and these locations were refined by crossing onto a WT Mauriceville background and following segregation of SNPs (Dunlap et al., 2007a; Lambreghts et al., 2009). This localized *chr* (Figure 1B) and *prd-3* (Figure 1C) on the physical map and suggested candidate genes for both mutants in different subunits of CK2 (arrows, Figures 1B and 1C).

CK2, a multifunctional kinase (Litchfield, 2003), is a tetramer composed of two identical catalytic α subunits and two, possibly different, regulatory β subunits (Figure S1 available online). Sequencing of NCU05485.3, the β1 subunit of CK2 (encoded by *ckb-1*) in the *chr* background, revealed a C→T transition, resulting in an R265C change within an extended C-terminal tail region of CK2β1 that is conserved among fungi (Figure S1, top and middle). Since the β subunit of CK2 mediates contacts between the catalytic α subunits and substrate (Litchfield, 2003), we speculate that such functions may be perturbed in *chr*. Functional complementation of *prd-3* (Figure 1C, bottom) identified NCU03124.3 on pLORIST H031 B12 as encoding the α subunit of CK2. Sequencing revealed a T→C transition resulting in a T43H mutation near the highly conserved phosphate anchor (Taylor et al., 1992) of the CK2 subdomain I (Figure S1, bottom) of CK2α (encoded by *cka*). Genetic rescue confirmed that these mutations in *ckb-1* and *cka* conferred the period length defects in *chr* and *prd-3*, respectively (see Table S1).

Thus, these decades-old mutants, unique among the dozens of known clock gene alleles as having extended or overcompensation, identify separate subunits of the same holoenzyme. Together, the simplest interpretation of the data is that CK2 plays a role in facilitating TC. Although not previously implicated in TC, CK2 has established roles in circadian clocks of *Arabidopsis* (Sugano et al., 1998), *Neurospora* (Yang et al., 2002, 2003), and *Drosophila* (Akten et al., 2003; Lin et al., 2002, 2005;

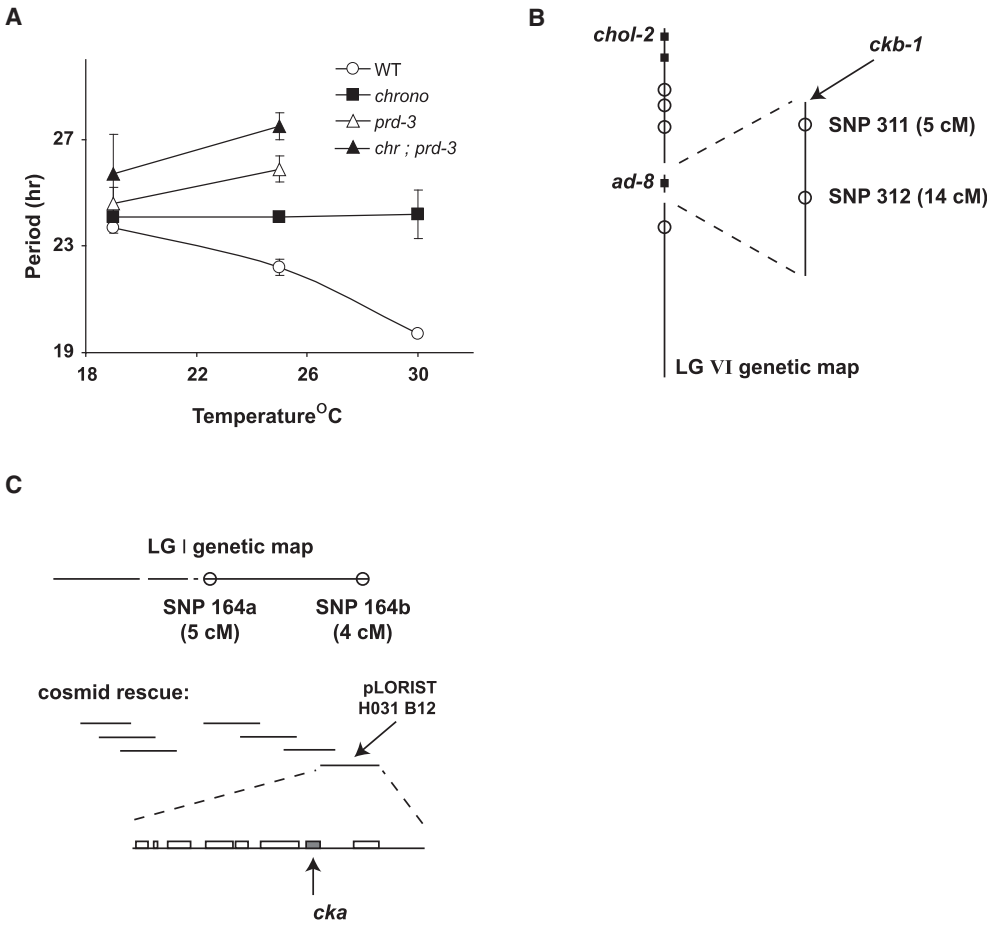

Figure 1. *chrono* and *period-3* Bear Mutations in CK2 Subunits

(A) The TC profiles of *chr* and *prd-3* are shown. Epistasis between *chr* and *prd-3* is observed with respect to TC and presence or absence of clear banding at 30°C. Values are the mean of n = 3 race tubes ± standard deviation (SD). See also Figure 2 in Gardner and Feldman (1981) for single mutant TC curves.

(B) SNP mapping of *chr* reveals linkage to a SNP on supercontig 311 (*Neurospora* genome release 3) of linkage group (LG) VI. Genetic data, shown as distance in cM between *chr* and the indicated SNP (open circles), were consistent with a location for *chr* on the end of 311, a position occupied by *ckb-1*.

(C) Mapping localizes *prd-3* approximately equidistant between SNPs 164a and 164b. This location was used to identify a series of pLORIST cosmids (horizontal lines) spanning the region. Transformation of these into *prd-3* recipients revealed rescue only by pLORIST H031 B12. Predicted ORFs within this cosmid included *cka* (gray box).

Nawathean and Rosbash, 2004) that are consistent with the increased period lengths we see in the CK2 mutants. Since a possibility was that these mutations were hypomorphic, we pursued the effect of CK2 dosage on TC by altering dosage of both subunits to see whether the independent identification of two CK2 subunits was more than a remarkable coincidence.

Reduction of CK2 Subunit Gene Dosage Alters TC

Phosphorylation-mediated turnover of FRQ is a major determinant of period length, and reducing the activity of kinases acting on FRQ results in increased circadian period length (Liu et al., 2000). Thus, a trivial explanation for the *chr* and *prd-3* mutant phenotypes might be that period length increases with temperature simply because the mutant alleles *chr* and *prd-3* encode temperature-sensitive proteins. A more interesting result, however, would be if *chr* and *prd-3* were genuine hypomorphs having lower levels of activity but normal temperature dependence; in this case reductions of the WT allele dosage should mimic the *chr* and *prd-3* phenotypes. To achieve various gene dosages, we generated heterokaryons of either *chr* or *prd-3* bearing different proportions of knockout versus WT nuclei. In terms of protein expression these approximate an allelic series. We confirmed this directly for CK2β1 by using an antibody (Ab) against the CKB-1 protein and showed, while WT and *chr* strains have comparable levels of CKB-1, Δ*ckb-1* heterokaryons have lower levels of protein (Figure S2).

Knockout heterokaryons bearing reduced dosage of *cka* (*cka* + Δ*cka*, referred to as Δ*cka*) or *ckb-1* (*ckb-1* + Δ*ckb-1*, referred to as Δ*ckb-1*) mostly recapitulate the original homokaryotic *prd-3* and *chr* phenotypes, respectively. Like *prd-3*, many Δ*cka* heterokaryons overcompensate between 20°C and 25°C, and all show increased periods (Figure 2A). These properties are consistent with *prd-3*'s being a hypomorph. Although *prd-3* does not consistently express an overt rhythm at 30°C, the knockdown

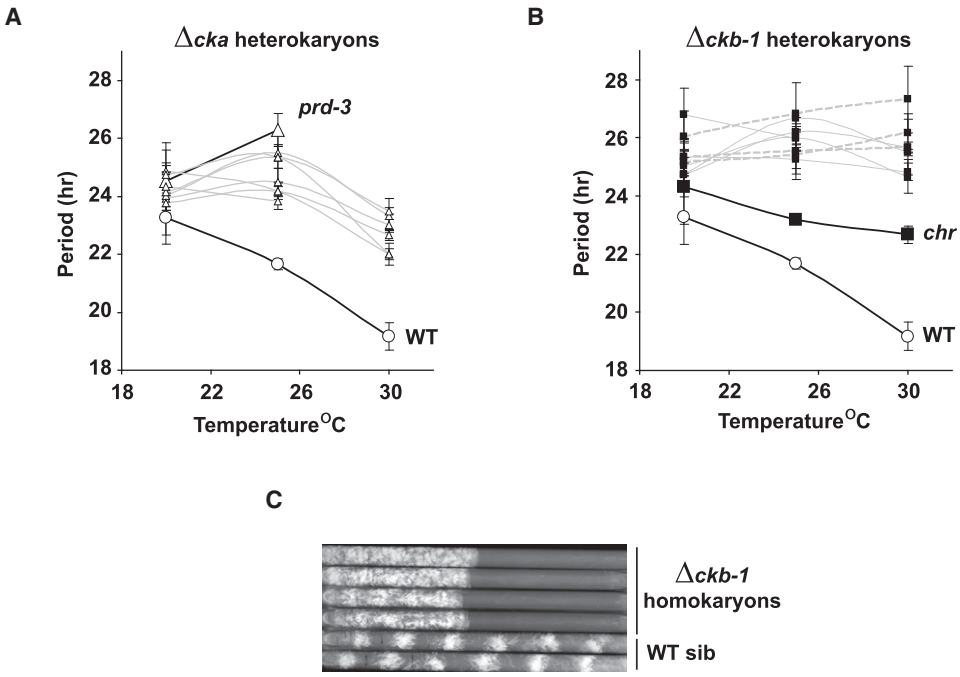

Figure 2. Heterokaryon Knockdowns of *cka* and *ckb-1* Resemble *prd-3* and *chr*, Respectively

(A) Reduction of *cka* dosage in heterokaryons increases period lengths and extends TC. TC profiles of WT (circles), *prd-3* (large triangles), and seven independent Δ*cka* heterokaryons (small triangles) are depicted ± SD, reflecting analysis of 3–6 bands in a single race tube.

(B) Reduction of *ckb-1* dosage in heterokaryons reduces period length and results in enhanced TC. TC profiles of WT (circles), *chr* (large squares), and eight independent Δ*chr* heterokaryons (small squares) are depicted ± SD, reflecting analysis of 3–6 bands in a single race tube. Heterokaryons that overcompensate across the temperature range are shown with dotted lines.

(C) Four independent Δ*ckb-1* homokaryons display an arrhythmic phenotype whereas two WT sibling controls have a normal phenotype.

strains show reduced period lengths at this temperature (Figure 2A). Heterokaryon Δ*ckb-1* knockdowns resemble the *chr* mutant, having increased period lengths with extended or overcompensation at the highest temperature (Figure 2B).

CKB-1 Dosage Qualitatively and Quantitatively Affects the Type of TC

Heterokaryon knockdowns were crossed to WT to generate homokaryotic Δ*ckb-1* knockout strains. These strains appear overtly arrhythmic (Figure 2C) and provide a context in which to test the hypothesis that CK2 plays an essential role in TC.

Because we suspected that *chr* might be hypomorphic, we hypothesized that as the level of CK2 is reduced, the TC profile of the clock should move from WT slightly undercompensated (negative slope), to extended TC (flat slope), and finally to overcompensated (positive slope). We tested this by generating a heterokaryotic strain in which the sole source of *ckb-1* mRNA is driven by the *quinic acid-2* promoter (*qa-2,* hereafter called *qa*) (Aronson et al., 1994; Dunlap and Loros, 2005) in proportion to the concentration of quinic acid (QA) in the medium (Figure 3A, top panel); the strain bears the genotype (*his-3* + *his-3*$^{+}$::*qa-ckb-1*; Δ*ckb-1*) and is referred to as *qa-ckb-1*. We confirmed that addition of QA yields increased levels of CKB-1 in a dose-dependent manner (Figure 3A, bottom panel). QA itself did not affect the TC curve of the WT (data not shown).

Inducing *ckb-1* gene expression in Δ*ckb-1* has dramatic effects on period length and TC. Higher levels of CKB-1 result in shorter periods matching WT period lengths with typical WT undercompensation (Figure 3B, squares). By contrast, reduced levels of QA, yielding lower levels of CKB-1, result in steadily longer periods progressing to overcompensation (positive slope) (Figure 3B, circles). In confirmation of our working model, we see a consistent relationship between *ckb-1* dosage and the resulting slope of the TC profile.

In summary, as CKB-1 dose increases, the TC profile changes from overcompensation to slight undercompensation. It is important to note here that although it was the characterization of TC mutants that initiated these studies, the experiment described in Figure 3B involves only WT proteins whose level of expression is altered. By controlling the amount of WT CKB-1 we can dictate the TC profile. Taken together, the data support a model in which CK2 modulates TC under physiological conditions.

Dosage Changes in Other Kinases and Phosphatases Implicated in the *Neurospora* Clock Do Not Affect TC

The phosphorylation state of FRQ and of all clock-associated proteins is affected by other kinases and phosphatases. Given this, a simple explanation of the preceding results might be that any mutation affecting the activity of enzymes acting on clock components would affect TC. We tested this by manipulating the levels of two kinases, casein kinase 1 (CK-1a) (Gorl et al.,

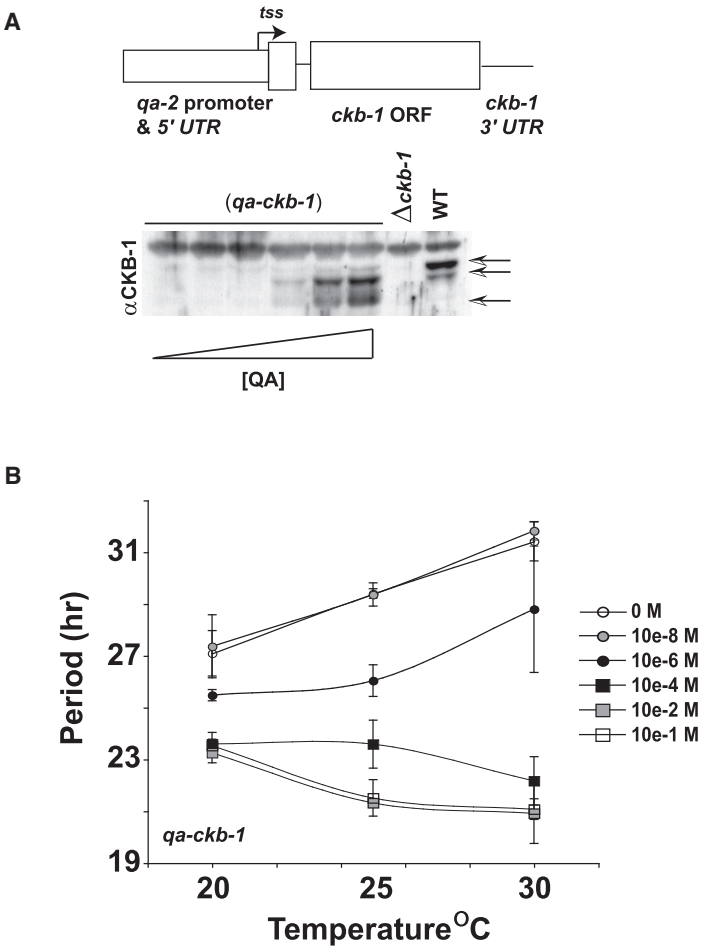

Figure 3. CKB-1 Dose Determines TC Type

(A) Quinic acid-inducible CKB-1. (Top) The schematic depicts a quinic acid (QA) inducible promoter, *qa 5′ UTR* (thin bar), and transcriptional start site (tss, arrow) driving the predicted *ckb-1* gene including ORF (thick bar), introns (line), and 3′ genomic sequences (thin line). This construct was targeted to the *his-3* locus in a *his-3*;Δ*ckb-1* homokaryon. (Bottom) Antiserum to CKB-1 was used to examine the WT, Δ*ckb-1* homokaryon, and one *qa-ckb-1* heterokaryon transformant. Tissue was collected from liquid cultures induced for ~13 hr with different QA concentrations (0 to 10^{-1} M, wedge); equal amounts of protein were loaded. Arrows depict CKB-1 isoforms similar to those seen in the WT.

(B) Dosage of WT CKB-1 dictates the form of TC ranging from undercompensation, to extended-range TC, to overcompensation. TC profiles were determined by analysis of transformed heterokaryons run on race tubes containing increasing concentrations of quinic acid (0 M to 10^{-1} M). Period lengths were determined from n = 5–6 replicate race tubes (each race tube had at least four bands) bearing one of two independent transformants (2–3 race tubes each), run at the indicated temperatures. Period lengths are mean period ± SD.

2001; He et al., 2006) and protein kinase A (PKAC-1) (Huang et al., 2007), as well as two phosphatases, protein phosphatase 1 (PPP-1) and protein phosphatase 2A (PP2A) (Yang et al., 2004).

We were particularly interested in whether changes in CK1 dosage might affect TC. Since *ck-1a* (NCU00685.3) is an essential gene in *Neurospora* (Gorl et al., 2001), we used an inducible-promoter knockin approach (Larrondo et al., 2009). Briefly, we replaced ~0.6 kb of sequence upstream of the predicted CK-1a ORF with the QA-inducible promoter similar to that described above (Figure 4A, top left). This allowed us to control and reduce CK1 levels without completely eliminating the protein, thereby allowing cell survival. We verified induction of message (data not shown) and protein expression using α-CK-1a antibodies (Figure 4A, bottom left).

Like CK2, CK1 determines period length. Unlike CK2, however, changes in CK1 levels do not significantly affect TC. In the absence of QA, the reduced dose of *ck-1a* in heterokaryons bearing the knockin construct increases period by approximately 3 hr (Figure 4B, left, top); this phenotype is rescued upon addition of QA (Figure 4B, left, bottom), consistent with previous findings (Gorl et al., 2001; He et al., 2006). To complete the analysis, a heterokaryon bearing the *qa-ck-1a* knockin was crossed to obtain a homokaryon in which the only source of CK1 is the *qa*-driven construct. When the dosage of CK1 in this strain is restricted by limiting QA, the effect on TC does not approach that seen with changing CK2 (Figure 4B, right).

The potential roles of PKA and the phosphatases were also examined by controlling their doses. Endogenous promoters were replaced with ~1.1 kb of the *qa* promoter for both *pkac-1* (NCU06240.3) and the catalytic subunit of PP2A (*pph-1,* NCU06630.3) and crossed to generate homokaryons. Changes in steady-state expression were measured by quantitative RT-PCR (Figure 4A, bottom right) on samples grown in the absence or presence of 10^{-2} M QA. Less pronounced changes in period lengths were observed in homokaryons bearing *qa-pkac-1* (Figure 4C, left) and *qa-pph-1* (Figure 4C, right) than were seen for CK1, but no significant differences were seen in the TC profiles of these strains. Finally, heterokaryon knockouts of *ppp-1* showed no significant period reduction, consistent with RIP mutants (Yang et al., 2004), and showed no effect on TC (Figure S3).

Together, these data indicate that not all regulators of clock protein phosphorylation influence TC. Rather, they suggest that CK2 plays a special role in TC.

CK2 Directly Phosphorylates FRQ

Since strains with low levels of CK2β1 show increasing periods as a function of temperature, a trivial explanation would be that CK2 activity might be compromised with increasing temperature, but only when CK2β1 levels are reduced to below normal physiological limits. To address this, we assessed endogenous CK2 activity on synthetic peptides (Kuenzel and Krebs, 1985) across a range of CK2β1 levels. Regardless of the amount of CK2β1, CK2 activity approximately doubles over the indicated ten degree temperature (representative experiment, Figure 5A); the trivial explanation does not hold. Additionally, CK2β1 levels do not vary, as assessed by western blot, between 20°C and 30°C (data not shown).

As this analysis was performed on a synthetic substrate, we wondered how CK2 might phosphorylate a bona fide substrate

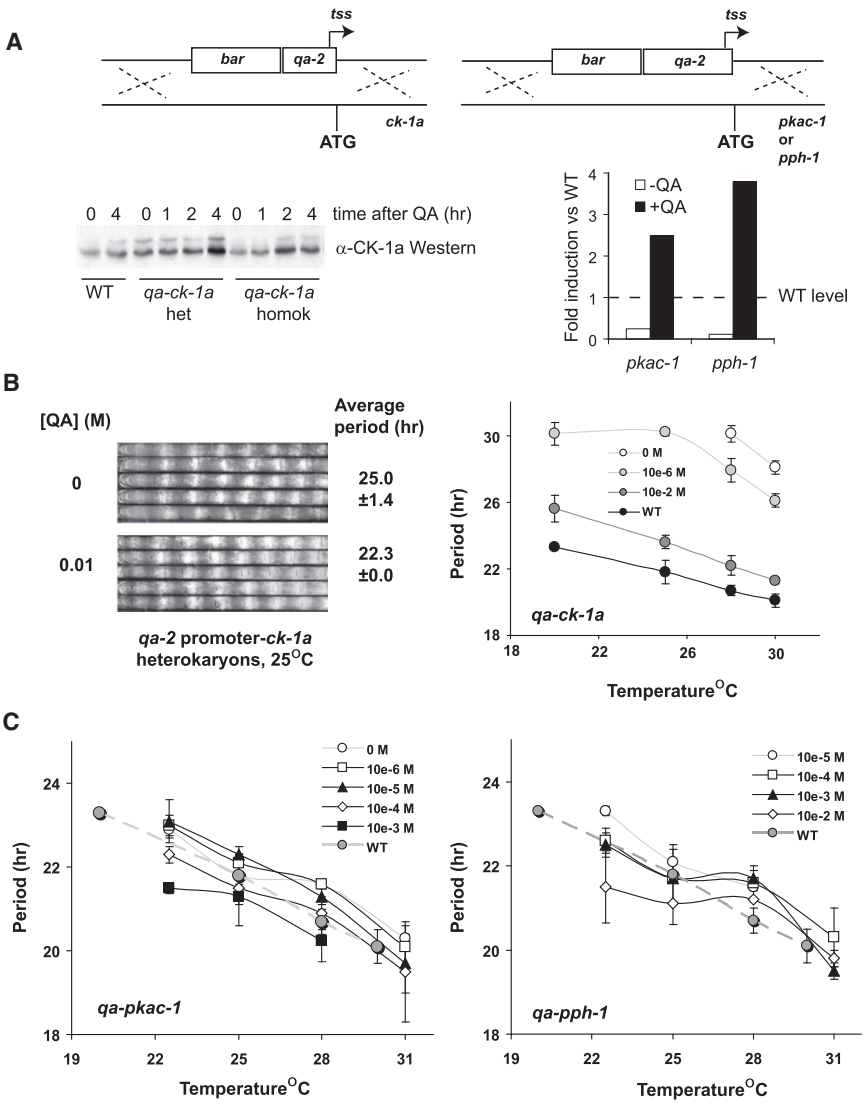

Figure 4. Reduced Dosages of Phosphatases and Kinases Previously Implicated in the *N. crassa* Clock, Other than CK2, Alter Period Length but Do Not Significantly Alter TC

(A) (Top) Schematic of the knockin strategy used to assess the effects of reduced dosage of a phosphatase and kinases other than CK2. A bialaphos resistance gene (*bar*) was fused to either ~600 bp (left panel) or ~1100 bp (right panel) of DNA upstream from the *qa* ATG, which is ~70 bp downstream of the *qa* transcriptional start site (arrow, tss). This was targeted to the endogenous promoters of various phosphatase or kinase genes by homologous recombination (dashed lines), thereby replacing the endogenous promoter and 5′ UTR with the promoter and 5′ UTR of the quinic acid-inducible *qa* gene. (Bottom left) Protein induced by addition of 10^{-2} M QA for the indicated time was checked by α-CK-1a Ab. (Bottom right) Assessment of steady-state mRNA using quantitative RT-PCR for the indicated strains—values are plotted as fold-induction versus WT controls (dotted line) from representative experiments.

(B) *ck-1a* dramatically affects period length but not TC. (Left) Independent lines of endogenous *qa-ck-1a* heterokaryons have increased period lengths in the absence of QA. Addition of 10^{-2} M QA causes these to revert to the WT period length of 22.3 ± 0.0 hr, n = 6 race tubes. (Right) Dosage of *ck-1a* does not strongly influence the TC profile. Endogenous *qa-ck-1a* homokaryons show increased period lengths at all temperatures as a function of QA but TC profiles remain similar to WT. Data are plotted as the mean period length determined from n = 6 race tubes ± SD (two independent lines).

(C) Changing *pkac-1* or *pph-1* levels has subtle effects on period length but no effect on TC. Neither *qa-pkac-1* homokaryons (left panel) nor *qa-pph-1* homokaryons (right panel) show significant deviations from a WT TC profile. A representative WT is replotted from (B) for reference. Data are plotted as the mean period length determined from n = 6 (for WT), n = 3 replicate race tubes ± SD for the other genotypes and n = 2 replicates in a few cases.

as a function of temperature. While CK2 has many targets in *Neurospora*, one potential target likely to affect TC is the central clock component FRQ. FRQ has a number of putative CK2 phosphosites, and calmodulin kinase-free, partially purified lysate fractions containing CK2 subunits phosphorylate FRQ in vitro while cka^{RIP} and $ckb\text{-}1^{RIP}$ mutant extracts show reduced levels of FRQ phosphorylation (Yang et al., 2002).

To confirm direct activity of CK2 on FRQ, we performed in vitro kinase assays with full-length GST-FRQ. CK2 is unique among kinases in that it can efficiently use GTP as a co-substrate in phosphotransfer reactions; thus, phosphorylation by GTP is considered strong evidence that a substrate is a direct CK2 target (Sugano et al., 1998; Yde et al., 2005). WT lysates incubated with full-length GST-FRQ and GTP yielded efficient phosphorylation (Figure 5B), indicating that CK2 can directly phosphorylate FRQ. However, since in vitro kinase assays can be promiscuous, we also assayed for phosphorylation of endogenous levels of FRQ by CK2. Briefly, whole-cell extracts from cells grown at 30°C were incubated at 30°C with radioactive GTP and were subsequently immunoprecipitated with antiserum against FRH, a protein that interacts strongly and stoichiometrically with FRQ (Cheng et al., 2005). Immunoprecipitates revealed bands the size of FRQ in WT, but not in frq^{10} or in *qa-ckb-1* under noninducing conditions (Figure 5B, lower panels). FRQ is directly phosphorylated in a time-dependent manner; however, as lysates contain both kinases and phosphatases and only a limited pool of radioisotope, phosphorylation peaks at 30 min and then declines, presumably reflecting phosphatase activity and depletion of radioisotope. In this assay, there appears to be no detectable phosphorylation of FRQ when CK2β1 levels are low, so we could not assess Q_{10}.

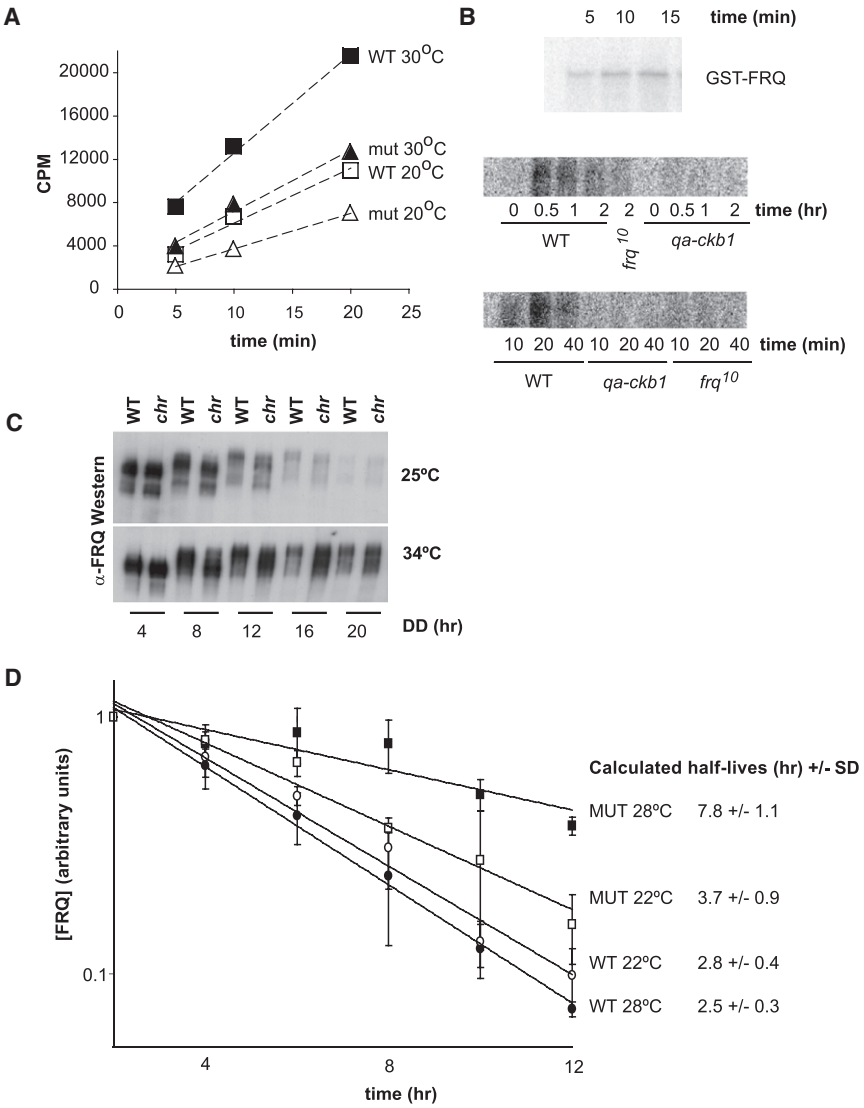

Figure 5. CK2 Directly Phosphorylates FRQ in a CKB-1-Dependent Manner and Exhibits Normal Temperature Sensitivity across a Range of *ckb-1* Expression

(A) The Q_{10} of the kinase acting on a synthetic peptide substrate does not change when CK2β1 is expressed at a low dose. Plotted are scintillation counts from kinase reactions (WT extracts, squares or *qa-ckb-1* extracts, "mut" triangles) grown at indicated temperatures (30°C, filled symbols or 20°C, open symbols). The slopes of the lines reflect the rate of phosphorylation.

(B) Direct phosphorylation of FRQ by CK2. (Top) GST-FRQ is directly phosphorylated by CK2. Phosphorimaging of in vitro kinase reactions from WT whole-cell extracts on purified GST-FRQ with radioactive GTP reveals radiolabeled bands at the predicted size of GST-FRQ. (Bottom) Endogenous FRQ is phosphorylated by CK2. Whole-cell extracts from the indicated strains were incubated at 30°C for the indicated times with radioactive GTP. Phosphorimaging of gels bearing α-FRH immunoprecipitations reveals bands at the predicted size of FRQ only from the WT reactions. The two images are from two different experiments varying the total protein and time of incubation.

(C) α-FRQ western blots in WT and *chrono* (*chr*) mutant strains. Cultures were shifted from 4°C to the temperatures indicated on the right. FRQ phosphorylation and degradation are observed as a function of time in DD (hours after transfer from cold to warm).

(D) (Left) Degradation profiles of FRQ protein in WT (circles) and the *ckb-1* inducible strain (MUT, squares). Cultures were transferred from 6 hr in constant light to dark at the indicated temperatures and were collected at the indicated times. NIH Image densitometry profiles of nonsaturated α-FRQ autoradiographs for samples between DD 2–12 hr were plotted on a log-linear scale and fitted to exponential decay functions. Data points represent the Coomassie-normalized, western blot densitometric means of n = 2–3 replicates ± SD. (Right) Calculated FRQ half-lives ± SD in the indicated strain and temperature. The half-life for WT FRQ is consistent with previously published estimates (Ruoff et al., 2005).

chrono and Strains with Reduced CKB-1 Exhibit a Temperature-Dependent Decrease in FRQ Phosphorylation In Vivo

Since the rate of FRQ degradation is a key factor in determining period length (Liu et al., 2000; Ruoff et al., 2005), we assessed the rate of FRQ degradation as a function of reduced CK2β1 activity and temperature. We found FRQ to be degraded slightly more slowly in *chr* than in WT at 25°C (Figure 5C, top panel), an effect that is exacerbated at higher temperature (Figure 5C, bottom panel). An artificially high temperature of 34°C was chosen to exaggerate the subtle difference in FRQ stability seen between WT and *chr.* FRQ is similarly phosphorylated more slowly in a *chr;frq*^{7} background compared to *frq*^{7} (data not shown). On one level, these data are simply consistent with expectations based on prior work, in that increased periods are due to increased FRQ stability. However, Figure 5A shows that even with low CK2β1 activity, consistent with *chr*, the rate of in vitro kinase activity appears to increase with temperature. Thus, WT levels of CK2 appear critical for modulating FRQ stability *specifically at higher temperatures*.

To assess this directly, we tested FRQ stability in a strain expressing low levels of CK2β1. For this, we used a standard light to dark transfer assay: steady-state levels of *frq*/FRQ are high in light and *frq* mRNA is rapidly degraded after the transfer to darkness, approximating the chase of a pulse-chase. In WT, FRQ degradation does not vary between the two temperatures (Figure 5D). In contrast, FRQ is roughly twice as stable at higher temperature relative to lower temperature in the *qa-ckb-1* strain in the absence of QA (Figure 5D, right). Taken together with the *chr* data, this suggests that FRQ is degraded more slowly as temperature increases when CK2β1 activity is reduced. This is consistent with a model in which over- and extended TC in strains containing hypomorphic CK2 or low dosage of WT CK2 results in a temperature-dependent misregulation of FRQ turnover. As

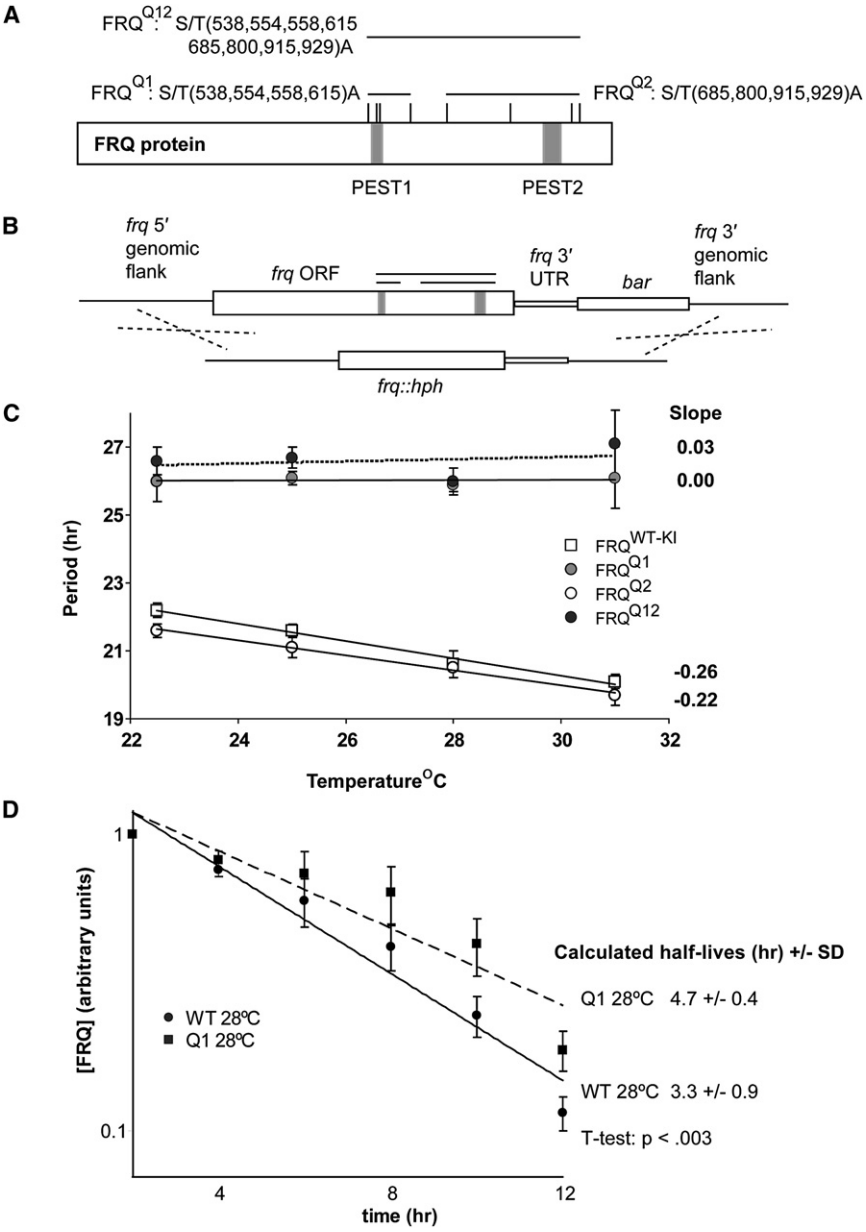

Figure 6. Mutations in FRQ Can Phenocopy CK2 Hypomorphs

(A) A schematic representation (to scale) of FRQ depicting the positions of two groups of serines that have been altered to alanines (vertical lines). FRQ^{Q1} (short horizontal line) bears a tight cluster of mutations around PEST1 (Aronson et al., 1994; Gorl et al., 2001). FRQ^{Q2} has a broader distribution of mutations in the C terminus of the protein (intermediate horizontal line). FRQ^{Q12} (long horizontal line) bears all eight changes.

(B) A schematic representation (roughly to scale) of the *frq* replacement strategy. The transforming construct (top) reintroduces *frq* into a *hygromycin phosphotransferase* (*hph*)-bearing KO strain at the endogenous *frq* locus.

(C) A class of putative CK2 phosphosite mutants of FRQ can phenocopy the TC defect of *chr*. Strains bearing FRQ^{Q1} (gray circles) or FRQ^{Q12} (black circles) show increased period lengths and extended TC. $FRQ^{WT\text{-}KI}$ and FRQ^{Q2} (squares and white circles, respectively) show WT-like TC and period lengths. Values are the mean of n = 6 race tubes ± SD. Slopes of a best linear fit are indicated to the right of each series.

(D) (Left) Degradation profiles of FRQ protein in $frq^{WT\text{-}KI}$ (WT, circles) and the frq^{Q1} strain (Q1, squares). Cultures were transferred from 6 hr in constant light to dark at the indicated temperatures and were collected at the indicated times. NIH image densitometry profiles of nonsaturated α-FRQ autoradiographs were plotted on a log-linear scale and fitted to exponential decay functions between DD 2–12 hr. Data points represent the Coomassie-normalized, western blot densitometric means of n = 3 replicates ± SD. (Right) Calculated FRQ half-lives ± SD in the indicated strain and temperature. A two-tailed t test indicates a significant difference between mean half-lives.

noted above, other kinases known to phosphorylate FRQ, including CK1, do not show this effect. These data implicate CK2 in a special and perhaps unique role in maintaining the rate of FRQ turnover specifically at higher temperatures and thereby sculpting the TC profile.

A Putative CK2 Phosphosite Mutant in FRQ Phenocopies *chrono*

Extended TC may arise at least in part due to misregulated CK2-mediated phosphorylation of FRQ at high temperatures. Extended TC might thus also result from eliminating those CK2 target sites on FRQ that are less effectively phosphorylated at high temperature by low CK2 doses. Hence, we analyzed FRQ strains bearing mutations in multiple putative CK2 phosphosites, which increased period length (m4, m5, and m6 from Yang et al., 2003, generous gift of Y. Liu). However, these strains did not show TC defects (data not shown). We then constructed a set of S/T→A mutations in FRQ using three criteria to identify candidate sites; sites chosen were predicted to be CK2 targets (score > 0.5 as assessed by NetPhosK 1.0 http://www.cbs.dtu.dk/services/NetPhosK/), conserved in at least 3/5 related fungal species, and known to be phosphorylated in vivo as assessed by mass spectrometry (Baker et al., 2009). We made multiple rather than individual mutations as CK2 hypomorphs likely result in the coordinated hypophosphorylation of several sites. Thus, we settled on eight possible CK2 sites and three cluster mutants were made, frq^{Q1}, frq^{Q2}, and frq^{Q12}, as well as a knockin version of the WT, $frq^{WT\text{-}KI}$ (Figure 6A).

To assess the effects of these mutant *frq* alleles on TC, we used each to replace the endogenous locus (Figure 6B). Homokaryotic strains bearing frq^{Q1} and frq^{Q12}, but not $frq^{wt\text{-}KI}$ or frq^{Q2}, show defects in TC (Figure 6C). frq^{Q1} strains have a longer

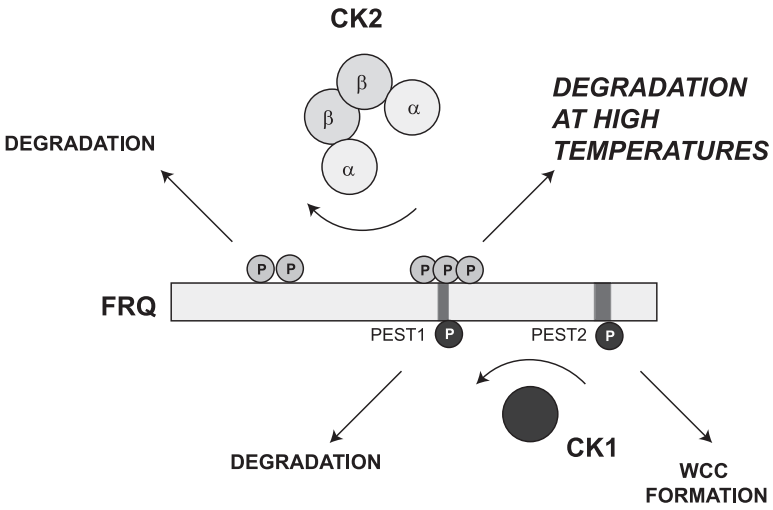

Figure 7. Model of FRQ Phosphosite Heterogeneity

Multiple classes of phosphorylation on FRQ are depicted. One type, CK1 target(s), leads to temperature-independent association of degradation cofactors (not depicted). Additionally, other CK1 phosphosite(s) promote WCC formation. Meanwhile, while some CK2 phosphosite phosphorylation promotes degradation at all temperatures, other sites (near PEST1) facilitate degradation of FRQ preferentially at higher temperatures.

period, which persists as temperature increases. Moreover, FRQ^{Q1} has a longer half-life than $FRQ^{WT\text{-}KI}$ in vivo at 28°C (Figure 6D). This pattern is reminiscent of the *chr* mutant, albeit with a longer period. By contrast, frq^{Q2} strains show no significant difference from WT. Thus, we predicted and found phosphorylation sites on FRQ that, when mutated, phenocopy the extended TC characteristic of *chr*. The enhanced compensation characteristic of these strains is unique among existing *frq* missense mutants.

Finally, we wanted to ensure that CK2 was capable of directly phosphorylating those residues that we had mutated in frq^{Q1}. Thus, we made GST and GST-fusions of peptides bearing the S/Ts of interest (Figure S4A). These were purified (Figure S4B) and radioactive kinase assays were performed with either recombinant human CK2α/β (Figure S4C, left side) or recombinant *N. crassa* CKA (Figure S4C, right side). All GST-peptide fusions were phosphorylated to varying degrees (see bands at the level of the asterisks) while GST alone was not. The reduction in phosphorylation intensity between GST-538 and GST-538A indicates that serine 538 is indeed phosphorylated, while the other serines appear also to be phosphorylated. In the lane with GST-554/558 either or both S/Ts are phosphorylated, and we cannot distinguish between these possibilities. Under these conditions, bands corresponding to the sizes of various CK2 proteins (arrows) are seen indicating the anticipated autophosphorylation of this kinase (Pagano et al., 2005). Thus, this together with our previous data strongly suggests that CK2 directly phosphorylates these residues in vivo.

DISCUSSION

We suggest a mechanistic entrée to the cryptic processes underlying TC, a canonical characteristic of circadian rhythms. Two classically isolated mutants were chosen because they showed unusual characteristics, extended TC and overcompensation. Analysis of these mutants identified CK2 as a key regulator of TC. The identities of *chr* and *prd-3* are consistent with mounting evidence for the role of CK2 in circadian clocks of many model organisms (for a recent review see Mizoguchi et al., 2006). Moreover, the epistatic relationship we observed between *chr* and *prd-3* is consistent with their identities: any activity of the β1 kinase regulatory subunit requires a functioning α catalytic subunit. The perhaps remarkable coincidence that two strains selected for defects in period length, and only later shown to have defects in TC, both bear mutations in subunits of the same kinase emphasizes that CK2 plays a key role in establishing TC.

Importantly, these analyses have defined a role for CK2 in TC without solely relying on the use of missense mutant alleles. While many mutants affecting TC exist (e.g., frq^{7}, per^{S}, per^{L}, etc.), it could not be excluded that their phenotypes were due to temperature-sensitive defects, irrelevant to their WT function in TC control. The use here of controlled-expression strains with only WT proteins has ruled out this caveat. Moreover, these results demonstrate that not all mutations that change period lead to defects in TC, consistent with other alleles, e.g., $timeless^{UltraLong}$ (Rothenfluh et al., 2000) and frq^{1} and frq^{2} (Gardner and Feldman, 1981).

The data can be thought about in terms of a simple model (Figure 7). CK2 and CK1 are essential for FRQ phosphorylation and period length determination; however, CK1 dose has little effect on TC. We show that FRQ is a direct target of CK2 and that FRQ is poorly phosphorylated by CK2 when CK2β1 is low. FRQ is degraded more slowly as temperature increases when CK2β1 is at low levels. This is consistent with mutants in which putative CK2 sites are deleted (frq^{Q1}, frq^{Q12}), thereby phenocopying CK2 hypomorphs. Thus, it appears that certain CK2 target sites are needed on FRQ for efficient FRQ degradation preferentially (though not exclusively) at high temperature. Since FRQ steady-state levels increase with temperature (Liu et al., 1997), either CK2′s differential phosphorylation of these sites on FRQ at high temperature or differing effects on degradation (e.g., FRQ might bind FWD-1 more efficiently at high temperature when these sites are phosphorylated) would counteract the accumulation of excess FRQ as temperature increases. In this way, we imagine that the TC curve is sculpted or, to reiterate the conclusion of Hastings and Sweeney (1957), that the effect of ambient temperature on period length is actively managed by the cell. By contrast, CK1 phosphorylation of FRQ is not required differentially at different temperatures, as if CK1 is necessary but not sufficient for timely FRQ turnover at elevated temperatures.

Finally, the data suggest that TC is an independently evolved layer that is not intrinsic to a core oscillator. In this scenario, through evolution, proto-oscillators that may not have been initially compensated may have co-opted the functions of utility kinases (such as CK2) to effect TC, and perhaps for other

advantages as well (see Allada and Meissner, 2005). Since the Q_{10} of CK2 activity seems to be within a thermodynamically predicted range, it seems that evolution has not tinkered much with the catalytic arm of this enzyme to achieve TC. This is reasonable given the many roles of CK2 in the cell; rather, evolution may have used the necessities arising from the global involvement of CK2 to an advantage in developing a capacity for TC. Interestingly, an observation in *Arabidopsis* is consistent with this view (Edwards et al., 2005). It seems plausible that effective CK2 activity is held within limits to service the many roles of the enzyme and that this homeostasis aids TC. We also note that TC is robust as regards CK2 levels: frank overcompensation was seen only at inducer levels four log orders below those that gave WT period lengths, levels that drove biochemically undetectable amounts of CK2β1 synthesis. It is apparent that in this system or in similarly constructed oscillators, 2- or even 4-fold changes (as from loss of an allele in a diploid) would not affect TC. Instead, we suspect that subtle changes in target clock proteins—e.g., in a constellation of CK2 sites in FRQ—may allow the clock to fine-tune TC. This is akin to the idea of a region in *Drosophila* PER that is polymorphic across latitudinal clines (Sawyer et al., 1997). A definitive catalog and time history of FRQ phosphorylation would provide further insight into this problem.

EXPERIMENTAL PROCEDURES

Strain Constructions and Culture Conditions

WT *Neurospora crassa* strains used include 328-4 (*ras-1^{bd} A*) and 87-74 (*his-3; ras-1^{bd} a*). Knockouts (Δ*cka*, Δ*ckb-1*, and Δ*ppp-1*) were generated by using *hph* to replace the entire ORF using the *Neurospora* Knockout Consortium's methods (Colot et al., 2006). Culture conditions, including race tubes and handling of *Neurospora*, were as previously described (Dunlap and Loros, 2005).

Antibodies and Western Blotting

For α-CKB-1, a polyclonal Ab was raised in rabbits against the synthetic peptide LRSKPDDLSVLDEARRY-amide by 21st Century Biochemicals, LLC (Marlboro, MA, USA). Westerns were performed as previously described (Garceau et al., 1997). The α-FRH Ab was made as previously described (Cheng et al., 2005). The α-CK-1a Ab was a gift of M. Brunner.

In Vitro Kinase Assays

Kinase assays with the synthetic peptide were performed with a modified protocol using a commercial CK2 Assay Kit (Upstate, Lake Placid, NY, USA).

FRQ Degradation Profiles

FRQ degradation was assessed by western blot after transfer from light to dark and FRQ half-lives were calculated by the following equation: $t_{1/2} = \ln(2)/m$ where m is determined from an exponential fit in the form $y = A\, e^{-mx}$. Half-lives were averaged from three independent curves and standard deviations were determined.

For more details, see the Supplemental Experimental Procedures.

SUPPLEMENTAL DATA

Supplemental Data include Supplemental Experimental Procedures, one table, and four figures and can be found with this article online at http://www.cell.com/supplemental/S0092-8674(09)00318-3.

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