Posttranslational Regulation of *Neurospora* Circadian Clock by CK1 α -dependent Phosphorylation

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Frequency (FRQ) and its transcriptional activator, the White Collar Complex (WCC), are essential components of interconnected feedback loops of the circadian clock of *Neurospora*. In a negative feedback loop, FRQ inhibits the WCC by recruiting casein kinase 1 α (CK1 α) and supporting its phosphorylation. In an interconnected positive loop, FRQ supports accumulation of high levels of WCC. Phosphorylation of clock proteins is crucial for the temporal and spatial coordination of these functions. We identified three isoforms of CK1 α generated by alternative splicing that all interact with FRQ. Furthermore, we show that WC-2 is phosphorylated by CK1 α in vitro and that WC-2 phosphorylation is inhibited in vivo by the CK1-specific inhibitor IC261. Finally, we demonstrate that CK1 α activity regulates levels of WC-2.

INTRODUCTION

Circadian clocks are timekeeping devices that organize the physiology and behavior of most eukaryotic organisms in anticipation of environmental changes associated with the daily rhythm of earth rotation. On the molecular level, circadian clocks are cell-autonomous oscillators consisting of interconnected transcriptional and translational feedback loops. The oscillations are synchronized with the exogenous day by environmental cues (“zeitgebers”) such as light and temperature. In the absence of zeitgebers, clock-driven oscillations persist with intriguingly precise periods, generating self-sustained subjective daily rhythms that usually deviate from 24 hours.

FRQ and the GATA-type zinc finger transcription factors White Collar-1 (WC-1) and WC-2 are core elements of interconnected feedback loops of the circadian clock of *Neurospora crassa* (Dunlap and Loros 2006; Liu and Bell-Pedersen 2006). WC-1 and WC-2 assemble and form the WCC that activates *frq* transcription. FRQ protein then inhibits the activity of WCC and thus feeds back on its own synthesis and regulates other genes controlled by WCC. Due to the feedback regulation, *frq* RNA and FRQ protein show robust circadian abundance rhythms. In an interconnected positive loop, FRQ supports accumulation of high levels of WCC, primarily on a posttranscriptional or posttranslational level (Lee et al. 2000; Cheng et al. 2001; Schafmeier et al. 2006). These apparently conflicting functions of FRQ are coordinated in a temporal and spatial fashion: Negative feedback is carried out by low levels of nuclear FRQ, whereas support of WCC levels requires progressive accumulation of high levels of FRQ in the cytosol (Schafmeier et al. 2006).

As a prerequisite for a robust and exactly timed circadian period, posttranscriptional and posttranslational regulation of the clock occurs on the level of mRNA processing (i.e., antisense RNA and alternative splicing), regulation of protein turnover and subcellular shuttling, as well as modulation of transcription factor activity (Diernfellner et al. 2005; Liu 2005; Brunner and Schaf-

meier 2006; Gallego and Virshup 2007). Phosphorylation is the most obvious modification of clock proteins (Schafmeier et al. 2005; Mizoguchi et al. 2006). Protein kinases and phosphatases that regulate circadian systems in a wide range of eukaryotic organisms have been described. Among them are CK1 and CK2, which are essential for a proper function of circadian systems in fungi, flies, and mammals (Kloss et al. 1998; Camacho et al. 2001; Blau 2003; Nawathean and Rosbash 2004; Eide et al. 2005; Knippschild et al. 2005; He et al. 2006; Gallego and Virshup 2007).

In the *Neurospora* genome, two genes coding for CK1 have been identified (Gorl et al. 2001). CK1 α , a homolog of *Drosophila* Doubletime, was the first clock-regulating protein kinase identified (Gorl et al. 2001; He et al. 2006). CK1 β , although important for normal growth and development, has no apparent clock-related function (Gorl et al. 2001; Yang et al. 2003). Several other kinases and phosphatases have been characterized that have distinct roles in the *Neurospora* circadian system. In particular, CK2, calcium/calmodulin-dependent kinase 1, checkpoint kinase 2, protein kinase C, and the protein phosphatases 1 and 2A functionally modulate clock proteins (Yang et al. 2001, 2002, 2004; Franchi et al. 2005; Pogue-Geile et al. 2006).

Here we summarize recent data concerning the role of CK1 in the circadian clock of *N. crassa*. Furthermore, we present evidence for a direct role of CK1 in WC-2 phosphorylation and discuss the existence of a clock-specific CK1 isoform.

RESULTS

Isoforms of CK1 α in *N. crassa*

CK1s comprise a large family of evolutionarily conserved eukaryotic kinases. They consist of a highly conserved amino-terminal catalytic domain of approximately 300 amino acid residues followed by a carboxy-terminal tail that is not conserved and variable in length. The carboxy-

Figure 1. CK1a isoforms in *N. crassa*. (A) Schematic drawing of *ck1a* splice variants identified by cDNA sequencing (i-iv). In the unprocessed mRNA (top), splice donors (D) are shown as blue lines and acceptors (A) are shown as red lines. Each cDNA isoform contains three exons, referred to as E1, E2a/b, and E3a/b. (Brown lines) Stop codons used in the corresponding splice variants. For clarity, the 3' region downstream from donor D2a is not to scale. (B) Carboxyl termini of corresponding gene products. Note that two isoforms (CK1a^{short-a} and CK1a^{short-b}) are identical due to a stop codon upstream of D2b. (Orange line; isoform I) Epitope recognized by the CK1a-long antibody. (Red) Identical; (blue) similar amino acids. (C) Detection of CK1a by western blotting. (Left panel) The α CK1a-pool antiserum was raised against a fragment consisting of the 132 carboxy-terminal amino acid residues of isoform I and recognizes two bands in a *Neurospora* protein extract. Bands are labeled CK1a^{long} and CK1a^{short}, which contains CK1a^{short-a} and CK1a^{short-b}. (Right panel) α CK1a-long antiserum specifically detects CK1a^{long}. Different exposures (10 sec and 30 sec) are shown.

terminal domains are thought to confer substrate specificity (Gross and Anderson 1998; Knippschild et al. 2005). Seven *ck1* genes are present in mammals (Knippschild et al. 2005), whereas the *Neurospora* genome contains only two genes, *ck1a* and *ck1b* (Gorl et al. 2001). Ck1a, which is most homologous to mammalian CK1 ϵ/δ and *Drosophila* Doubletime (Dbt), is essential for viability and has, like its orthologs, a function in the circadian clock (Gorl et al. 2001). *ck1a* contains an intron in the 5' portion of the open reading frame (ORF). Inspection of the genomic DNA additionally suggested the presence of two alternative splice donors, D2a and D2b, followed by two alternative acceptors, A2a and A2b. (Fig. 1A). Cloning and sequencing of *ck1a* cDNAs revealed that these splice sites are used, giving rise to four alternatively spliced mRNA

species. D2a is located within the CK1 ORF immediately upstream of the stop codon in exon 2. The two mRNAs spliced at D2a encode a long (D2a/A2a) and medium size (D2a/A2b) isoform of CK1a, designated CK1a^{long} and CK1a^{short-a} (Fig. 1B). Because D2b is located downstream from the stop codon in exon 2, the two corresponding mRNA isoforms (D2b/A2a and D2b/A2b) encode the same polypeptide, CK1a^{short-b} (Fig. 1B). We generated an antibody against a fragment of CK1a comprising the carboxy-terminal 132-amino-acid residues. This antibody, α CK1a-pool, is predicted to recognize all isoforms of CK1a. The antibody detected two bands (Fig. 1C, left lanes). The upper band corresponds in size to CK1a^{long} and the lower band, to CK1a^{short-a} and CK1a^{short-b}, which are not resolved by SDS-PAGE. Thus, CK1a^{short-a} and CK1a^{short-b} will henceforth be referred to as CK1a^{short}. Furthermore, we generated a peptide antibody against the extreme carboxyl terminus of CK1a^{long} (last 13 amino acids, epitope is marked in Fig. 1B). The antiserum recognized a single band of expected size (Fig. 1C, right lanes). Thus, we have generated useful tools for further analysis of CK1a.

Biochemical Properties of CK1a

Despite its simple structure, CK1 is regulated in multiple ways, including inhibition by autophosphorylation, subcellular distribution, and probably also dimerization (Longenecker et al. 1998). We wondered whether the *Neurospora* CK1a isoforms are differentially distributed and whether they interact with each other. Subcellular fractionation of light-grown mycelia revealed that the CK1a isoforms are distributed in the same manner between cytosol and nuclei. The subcellular localization was not affected in *frq*-deficient strains (Fig. 2A).

Because CK1 is supposed to form dimers, interaction of the different isoforms could be a possible reason for the equal distribution. To analyze whether CK1a forms heterodimers, we immunoprecipitated CK1a^{long} with an antibody specific to the carboxy-terminal tail and performed western analysis with α CK1a-pool. As shown in Figure 2B, CK1a^{long} was immunodepleted from the protein extract by affinity-purified α CK1a-long antiserum. In contrast, CK1a^{short} remained entirely in the supernatant. These data suggest that the CK1a^{long} does not form stable heterodimers with the medium-size and short isoforms.

To analyze whether CK1a forms dimers at all, we constructed a version of the *ck1a* gene that contains an amino-terminal double-FLAG tag. The splice sites giving rise to the CK1a isoforms were present in this recombinant gene. The tagged gene was expressed in a wild-type background, and immunoprecipitation was performed with α FLAG agarose. As shown in Figure 2C, the α FLAG agarose efficiently immunoprecipitated the FLAG-tagged isoforms of CK1a. In addition, endogenous CK1a isoforms were coprecipitated, indicating dimerization of CK1a. Together, the data suggest that CK1a has the potential to form homodimers, whereas heterodimers may not form or are less stable.

Figure 2. (A) Subcellular distribution of CK1a. (Upper panel) Western blot of subcellular fractions of *frq*⁺ and *frq*⁰ mycelia decorated with αCK1a-pool antiserum. (Middle panel) Analysis of the same fractions with αCK1a-long antiserum. (Lower panel) Blots are redecorated with α-tubulin antiserum to control the fractionation. (T) Total; (C) cytosol; (N) nuclei. (B) Immunoprecipitation of total protein extracts with αCK1a-long antiserum. Western blots were decorated with αCK1a-pool antibody. Immunoprecipitation with preimmune serum was performed for control. (L) Load; (IP) immunoprecipitation; (SN) supernatant; (PI) preimmune serum. (C) Immunoprecipitation with αFLAG agarose of extracts from a strain expressing FLAG-tagged CK1a isoforms in addition to endogenous CK1a. Western blots are immunodecorated with αCK1a-pool (upper panel) and αCK1a-long antiserum (lower panel).

Interaction of CK1a with Clock Proteins

As reported previously, CK1a is in complex with FRQ, a central component of the circadian clock (Gorl et al. 2001). To identify the region of FRQ that interacts with CK1a, we analyzed strains expressing full-size FRQ, the carboxy-terminally truncated FRQ⁹ protein and an amino-terminal fragment of FRQ consisting of the first 366 amino acid residues (Fig. 3A). Extracts from light-grown mycelia were prepared and subjected to immunoprecipitation with αCK1a-pool antiserum (Fig. 3B). FRQ and FRQ⁹ copurified with αCK1a-pool antiserum, whereas the amino-terminal fragment of FRQ was not coprecipitated. These findings demonstrate that CK1a interacts with the central region of FRQ, in accordance with recent data showing that residues 488–496 of FRQ are crucial for CK1a binding (He et al. 2006).

To analyze whether the CK1a isoforms interact differently with FRQ, we used a strain that expresses a functional His-tagged version of FRQ under control of the *qa-2* promoter (Schafmeier et al. 2005). Extract prepared from light-grown mycelia was incubated with Ni-NTA

Figure 3. Interaction of CK1a with FRQ. (A) Scheme of FRQ constructs expressed for analysis of CK1a interaction. (CC) Coiled-coil domain; (NLS) nuclear localization sequence; (PEST) proline-, glutamate-, serine-, threonine-rich region. (B) Immunoprecipitation of total protein extracts with αCK1a-pool antiserum followed by Western analysis. Immunodecoration was performed with monoclonal αFRQ antibody. (L) Load; (IP) immunoprecipitation; (SN) supernatant. (C) Ni-NTA purification of total protein extracts. (Upper and middle panel) Extract obtained from a strain expressing His₆-tagged FRQ; (lower panel) control experiment with wild-type extract. Western blots were decorated with αFRQ and αCK1a-pool antibody as indicated. (L) Load; (FT) flowthrough; (E) elution. (D) Same experiment as in C; Western analysis with αCK1a-long antiserum. Asterisk indicates cross-reacting band enriched in the elution fraction. (E) The central domain of FRQ carries determinants for rapid protein turnover. FLAG-tagged GFP-fusion proteins with the amino-terminal, the middle, and the carboxy-terminal domain of FRQ are schematically outlined. FRQ-deficient strains expressing the fusion proteins were grown in constant light (LL) and transferred to medium containing 10 μg/ml cycloheximide (CHX), and samples were harvested after the indicated time points. Kinetics of degradation of GFP-FRQ-fusion proteins were determined by quantification of Western blots. (Circles) Amino-terminal domain (2–412); (squares) central domain (413–683); (triangles) carboxy-terminal domain. (F) Exchange of S513 to alanine stabilizes the central portion of FRQ. Degradation kinetics of GFP-fusion proteins with the central portion of FRQ. (Upper panel) Wild type; (lower panel) S513 to alanine mutation. Western analysis and immunodecoration with αFLAG antibody are shown.

resin. The His-tagged FRQ was depleted from the protein extract (Fig. 3C). A portion of CK1a specifically bound to the Ni-NTA resin when extract containing His-tagged FRQ was used (Fig. 3C,D). This confirms that CK1a is in a complex with FRQ. Both CK1a^{long} and CK1a^{short} were recovered at a ratio similar to that present in total extracts, suggesting that the isoforms interact equally well with

FRQ. The majority of CK1 α did not bind to the Ni-NTA resin, indicating that it was not in complex with His-tagged FRQ. Because about 30% of FRQ coprecipitated when pull-down assays were performed with α CK1 α -pool antiserum (Gorl et al. 2001), the data suggest that expression levels of CK1 α are much higher than those of FRQ. CK1 α has essential functions that are not related to the circadian clock.

In the course of a circadian day, FRQ is progressively phosphorylated and degraded with a half-time of 3–4 hours. The turnover kinetics is regulated by phosphorylation. In particular, putative phosphorylation sites in the central portion of FRQ have been reported to be crucial for FRQ stability. Thus, mutation of serine residue 513 (S513) to isoleucine (Liu et al. 2000) and deletion of the PEST-1 region (residues 540–566), which contains several putative phosphorylation sites (Gorl et al. 2001), led to a substantial stabilization of FRQ. We asked whether the central domain of FRQ carries the determinants that are sufficient to destabilize heterologous proteins and mediate their rapid turnover. Therefore, three fragments corresponding to the amino-terminal, carboxy-terminal, and the central portion of FRQ were fused to green fluorescent protein (GFP) and expressed in a *frq*-null background under control of the *frq* promoter. To assess the kinetics of turnover of these GFP-fusion proteins, cultures were incubated with cycloheximide (CHX) to block protein synthesis and samples were analyzed over a time course of 10 hours (Fig. 3E). The GFP fused with the central portion of FRQ was rapidly degraded with a half-life time of approximately 2 hours, whereas fusion proteins with the amino- and carboxy-terminal portion of FRQ were stable. When S513 was exchanged with alanine to abolish phosphorylation, the corresponding fusion protein with the central portion of FRQ was stable (Fig. 3F). In summary, the results demonstrate that the central portion of FRQ carries the determinants for rapid regulated protein turnover.

FRQ inhibits the activity of the WCC by mediating its phosphorylation (Schafmeier et al. 2005) and CK1 α is, at least partially, responsible for the FRQ-dependent phosphorylation of WCC (He et al. 2006). We asked whether CK1 α directly interacts with the WCC. WC-1 and WC-2 are GATA-type zinc finger proteins that bind efficiently to Ni-NTA resin. As shown in Figure 4A, both proteins were fully depleted from a *Neurospora* protein extract passed over a Ni-NTA column. However, CK1 α did not bind to the affinity matrix, indicating that it is not in a stable complex with the WC proteins.

FRQ was expressed under control of the inducible *qa-2* promoter in a *frq*-null background. FRQ was efficiently induced over a time course of 8 hours (Fig. 4B). The phosphorylation status of WC-2 was analyzed by two-dimensional gel electrophoresis. WC-2, which was hypophosphorylated before induction of FRQ, was efficiently phosphorylated 4 hours after FRQ induction. This demonstrates that the WCC is phosphorylated in response to FRQ expression. CK1 α is essential for the viability of *Neurospora* (Gorl et al. 2001). To access whether FRQ recruits CK1 α to phosphorylate the WCC, cultures were treated with the CK1 inhibitor IC261 (Behrend et al. 2000).

Figure 4. (A) CK1 α is not associated with the WC proteins. The GATA-type zinc finger proteins WC-1 and WC-2 were affinity-purified with Ni-NTA agarose. Total extracts loaded on the Ni-NTA column (L), the flowthrough fraction (FT), and the bound proteins eluted with imidazole (E) were analyzed by western blotting with the indicated antisera. Copurification of CK1 α with the WC proteins was not observed. (B) Inhibition of FRQ-dependent phosphorylation of WC-2 by the CK1 inhibitor IC261. Dark-grown *frq*¹⁰ *qa-2frq* cultures were shifted to medium containing 0.3% quinic acid (QA) in the presence of 10 μ M IC261 and DMSO (dimethylsulfoxide) for control. Total extracts were subjected to SDS-PAGE and immunoblotting with monoclonal α FRQ (left) or two-dimensional electrophoresis and analysis with α WC-2 (right). (Black arrowheads) Hyperphosphorylated WC-2 appearing in response to FRQ induction; (red arrowheads) expected positions of hyperphosphorylated WC-2 forms. (C) Phosphorylation of immunopurified WC-2 by recombinant CK1 α in vitro. Hypophosphorylated WC-2 was immunoprecipitated from *frq*¹⁰ extracts and incubated with ATP and recombinant CK1 α ^{long}. Samples were analyzed by two-dimensional gel electrophoresis. Arrowheads indicate hyperphosphorylated forms of WC-2.

FRQ expression, as well as the overall phosphorylation pattern of FRQ, was not affected by IC261. This suggests that CK1 α was not efficiently inhibited by IC261 and/or that other kinases are able to phosphorylate FRQ (Fig. 4B, left). However, IC261 interfered with FRQ-dependent phosphorylation of WC-2 (Fig. 4B, right). Hyperphosphorylated species of WC-2, which appeared 4 hours after FRQ induction in the control, were absent in the sample with IC261. This suggests that FRQ recruits CK1 and promotes WC-2 phosphorylation.

In an additional approach, we investigated whether purified recombinant CK1 α is able to phosphorylate WC-2 in vitro. We purified hypophosphorylated WC-2 from a *frq*-null strain by immunoprecipitation. When the immunoprecipitate was incubated with recombinant CK1-His₆ and ATP, WC-2 was efficiently phosphory-

lated at multiple sites. Phosphorylation of WC-2 in vitro was more efficient than in vivo, suggesting that in a living cell, phosphorylation is regulated and/or antagonized by phosphatases (Fig. 4C). Taken together, our data present evidence for a direct phosphorylation of WC-2 by CK1a.

Effects of Constitutive Active and Dominant-negative Casein Kinase

For a further investigation of the function of CK1a in the *Neurospora* clock, we generated a constitutive active (CA) and a dominant-negative (DN) *Ck1a* allele, which can be expressed under control of the inducible *qa-2* promoter in a wild-type background. To obtain a CA allele, we inserted a stop codon (Q299^{TER}) to delete the regulatory carboxy-terminal domain. The DN form was generated by exchange of a conserved aspartic acid with asparagine (D131N) in accordance to a DN-CKI from *Xenopus* (Peters et al. 1999). To distinguish mutant and endogenous CK1a forms, we inserted an amino-terminal double-FLAG tag as shown in Figure 5A.

Figure 5. Effect of CA and DN versions of CK1a on expression levels and phosphorylation status of clock proteins. (A) Schematic drawing of FLAG-tagged *ck1a* constructs under control of the inducible *qa-2* promoter. The indicated point mutations were introduced to generate CA and DN CK1a. (B) Induction (6 hours) of CA-CK1a and DN-CK1a expression with quinic acid (QA). (Upper panels) Western blot of total protein extracts decorated with α CK1a-pool antiserum; (middle panels) same extracts were analyzed with monoclonal α FLAG antibody; (lower panels) unspecific band recognized by the α FLAG antibody confirms equal loading of the gel. (C) Expression of clock proteins in DN and CA mutants. Same extracts as in B were analyzed with α FRQ, α WC-2, or α WC-1 antibodies. (D) Two-dimensional analysis of extracts obtained from DN and CA mutants with α WC-2 antiserum.

After quinic acid (QA) induction, both mutant forms were detected in *Neurospora* protein extracts with an α FLAG antibody. Before induction, low levels were detected probably due to a weak leaky expression. After induction, expression levels increased (Fig. 5B). As expected, one form of the carboxy-terminally truncated CA-CK1a was detected and several splice isoforms of DN-CK1a were expressed. The DN-CK1a isoforms together with the endogenous CK1a were also detected by the α CK1a-pool antiserum, which was raised against the carboxy-terminal fragment of CK1a (132 amino acid residues). However, the short and medium splice isoforms of FLAG-tagged DN-CK1a comigrate with the long isoform of endogenous CK1a. Thus, only the long isoform of FLAG-tagged DN-CK1a can be clearly distinguished. Because the carboxyl terminus is lacking in the CA-CK1a, the protein cannot be detected with our α CK1a antisera.

Expression of CA-CK1a caused a slight reduction of FRQ and a pronounced reduction of WC-2 levels, suggesting a role of CK1a-dependent phosphorylation in the regulation of FRQ and WC-2 turnover (Fig. 5C). The DN form of the CK1a did not affect expression levels of FRQ and WC-2 (Fig. 5C).

To investigate the influence of CA-CK1a and DN-CK1a on the phosphorylation status of WC-2, we performed two-dimensional gel electrophoresis. Before induction of CA-CK1a and DN-CK1a, WC-2 was highly phosphorylated at multiple sites (Fig. 5D). When expression of CA-CK1a was induced by supplementing the growth medium with 0.3% QA for 6 hours, WC-2 protein levels decreased and mainly hyperphosphorylated species were detected. In particular, unphosphorylated and hypophosphorylated forms were completely absent (Fig. 5D, upper). Expression of DN-CK1a did not significantly affect expression and phosphorylation of WC-2 (Fig. 5D, lower), suggesting that it may not efficiently compete with the endogenous CK1a.

In summary, we show that expression of a CA-CK1a form results in hyperphosphorylation of WC-2 and a reduction of WC-2 protein levels. FRQ expression may also be affected by CA-CK1a, although to a much lesser extent.

CONCLUSIONS

We addressed the role of CK1a in the *Neurospora* circadian clock and identified four splice isoforms of *ck1a* mRNA. These mRNAs encode three polypeptides differing in length and primary structure of their extreme carboxyl termini. Carboxy-terminal variations were reported to determine substrate specificity of casein kinase isoforms (Knippschild et al. 2005). We show that the clock protein FRQ interacts with an affinity similar to that of the long and short isoforms of CK1a. Because CK1a has at least two known substrates among the clock proteins of *Neurospora*, i.e., FRQ and WCC, it is tempting to speculate whether the isoforms differ in specificity for phosphorylation of FRQ and WCC.

The CK1a-binding site in FRQ was recently mapped (He et al. 2006). Our data confirm that CK1a binds to the

central portion of FRQ. Furthermore, our data show that the central domain of FRQ carries the determinants for rapid phosphorylation-dependent protein turnover. Thus, a fusion of the central domain to GFP is sufficient to promote rapid, regulated turnover. The central domain harbors, in addition to the CK1a interaction site, the PEST-1 domain and S513, both crucial elements for phosphorylation-dependent degradation (Liu et al. 2000; Gorl et al. 2001). The GFP-fusion protein with the central domain of FRQ was stabilized when S513 was exchanged to an alanyl residue. This demonstrates that the central portion of FRQ is a functional domain that regulates turnover via phosphorylation.

The phosphorylation of WCC depends on recruitment of CK1a by FRQ (He et al. 2006). This suggests that CK1a directly phosphorylates the WCC and implies a transient ternary complex of FRQ, WCC, and the kinase (Fig. 6).

A stable interaction of CK1a with the WCC was not detected. However, we provide evidence that WC-2 is a direct substrate of CK1a. Thus, WC-2 is phosphorylated by recombinant CK1a in vitro, demonstrating that it contains phosphorylation sites recognized by the kinase. In addition, IC261, a CK1-specific inhibitor, reduces phosphorylation of WC-2 in vivo. The apparent phosphorylation status of FRQ was not affected by IC261, suggesting that CK1a was still partially active and/or that other kinases substitute for CK1a.

Expression of a CA form of CK1a substantially reduced WC-2 expression. This observation suggests that

both WCC activity (Schafmeier et al. 2005) and protein turnover are regulated via phosphorylation by CK1a. This is reminiscent to distinct CK1a phosphorylations of FRQ at PEST-1 and PEST-2 that regulate turnover and function, respectively (Schafmeier et al. 2006). Similarly, *Drosophila* PER and CLOCK proteins are also differentially regulated by phosphorylation, suggesting that similar functional aspects are regulated by phosphorylation of clock proteins (Kim et al. 2002; Kim and Edery 2006; Gallego and Virshup 2007). Whether the function and stability of clock proteins are regulated by distinct CK1a isoforms remains to be investigated.

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Figure 6. Model of CK1a functions in the *Neurospora* clock. For details, see text.

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