Posttranslational Control of the *Neurospora* Circadian Clock

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The eukaryotic circadian clocks are composed of autoregulatory circadian negative feedback loops that include both positive and negative elements. Investigations of the *Neurospora* circadian clock system have elucidated many of the basic mechanisms that underlie circadian rhythms, including negative feedback and light and temperature entrainment common to all eukaryotic clocks. The conservation of the posttranslational regulators in divergent circadian systems suggests that the processes mediating the modification and degradation of clock proteins may be the common foundation that allows the evolution of circadian clocks in eukaryotic systems. In this chapter, we summarize recent studies of the *Neurospora* circadian clock with emphasis on posttranslational regulation in the circadian negative feedback loop.

INTRODUCTION

Circadian clocks regulate a wide variety of physiological and molecular activities in almost all eukaryotic organisms and in certain prokaryotic organisms. *Neurospora* is an excellent experimental organism for studying circadian rhythms because of its relative simplicity and because the easily monitored circadian rhythm in asexual spore development (conidiation) has proven extremely useful for measuring the effects of mutations on clock function. Furthermore, systematic knockouts of a large number of genes are available from the *Neurospora* genome project (http://www.dartmouth.edu/~neurosporagenome/). In addition to the characterization of the clock, *Neurospora* has served to understand the light input pathway, temperature entrainment, and the output pathway of the clock (Liu 2003; Diernfellner et al. 2005; Liu and Bell-Pedersen 2006).

Like the circadian oscillator in *Drosophila* and mammals, the core circadian oscillator of *Neurospora* consists of an autoregulatory negative feedback loop with four core components: FREQUENCY (FRQ), FRH (an FRQ-interacting RNA helicase), WHITE COLLAR-1 (WC-1), and WC-2 (Dunlap 1999; Dunlap and Loros 2004; Liu and Bell-Pedersen 2006; Heintzen and Liu 2007). In this negative feedback loop, a complex of FRQ and FRH (called FFC) forms the negative limb of the loop, and the PER-ARNT-SIM (PAS) domain-containing transcription factors WC-1 and WC-2 form the WC complex (WCC) and acts as the positive element. The WCC binds to two *cis* elements in the promoter of *frq* to activate its transcription. On the other hand, FFC represses *frq* transcription by inhibiting the activity of WCC. After the progressive phosphorylation and degradation of FRQ, the reactivation of WCC leads to reactivation of *frq* transcription and the start of a new cycle. Thus, this negative feedback loop generates endogenous circadian oscillations of *frq* mRNA and FRQ protein that regulate rhythmicity close to 24 hours. In addition to its essential role in the circadian negative feedback loop, WC-1 is also the blue light photoreceptor responsible for circadian entrainment and all other known light responses, emphasizing the link between light input and the circadian oscillator (Froehlich et al. 2002; He et al. 2002; Cheng et al. 2003a; He and Liu 2005).

This chapter summarizes the progress that has been made in recent years in understanding the molecular basis of the *Neurospora* circadian negative feedback loop with the emphasis on roles of posttranslational modifications.

THE CIRCADIAN FEEDBACK LOOPS IN *NEUROSPORA*

Our current understanding of the regulation of the *Neurospora* circadian clock is depicted in Figure 1. In constant darkness around the subjective morning, WC-1 and WC-2 form a heterodimeric complex (D-WCC) that binds to the Clock box (C box) in the *frq* promoter, leading to the activation of *frq* transcription (Crosthwaite et al. 1997; Cheng et al. 2001a; Froehlich et al. 2003; He and Liu 2005). *frq* mRNA reaches its peak in the subjective day and FRQ protein amount peaks 4–6 hours later (Aronson et al. 1994; Garceau et al. 1997). After FRQ protein is synthesized, it dimerizes through its coiled-coil domain and forms a complex with FRH (Cheng et al. 2001b, 2005). In the nucleus, FFC inhibits D-WCC activity, resulting in a decrease in *frq* mRNA levels; *frq* mRNA level reaches a trough around the subjective early evening (Aronson et al. 1994; Merrow et al. 1997; Luo et al. 1998). As soon as FRQ is synthesized, it is progressively phosphorylated by several kinases and dephosphorylated by two phosphatases (Heintzen and Liu 2007). When FRQ becomes extensively phosphorylated, it interacts with FWD-1, an F box/WD-40 repeat-containing protein and the substrate-recruiting subunit of an SCF-type ubiquitin ligase complex, resulting in the ubiquitination and degradation of FRQ by the proteasome system (He et al. 2003). When FRQ levels drop below a certain threshold around subjective late night, D-WCC is no longer inhibited by FFC and *frq* transcription is reactivated to start a new cycle. As a result of this autoregulatory negative feedback loop, *frq* mRNA and FRQ protein levels oscillate with daily rhythms. These oscillations are critical for

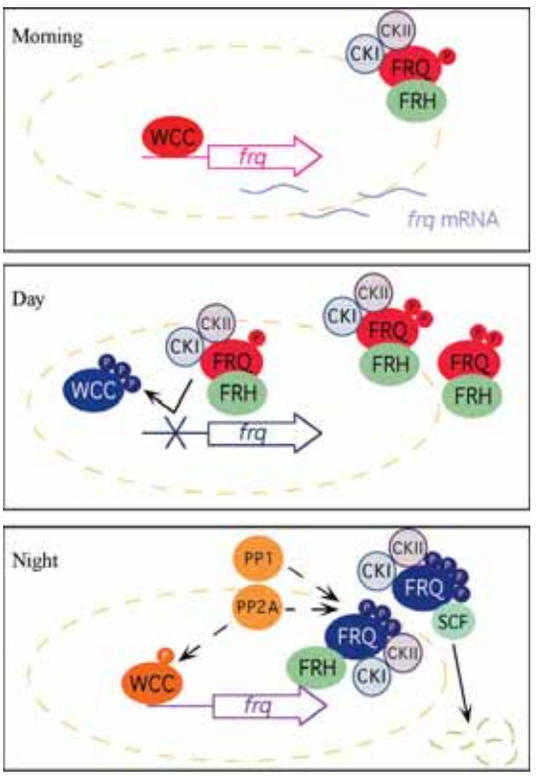

Figure 1. Current working model of *Neurospora* circadian clock. In the subjective morning, FRQ levels are low and WCs are hypophosphorylated. The hypophosphorylated WCC binds the C box to activate *frq* transcription. After FRQ protein is made, the homodimeric FRQ and FRH form FFC and the complex associates with CKI and CKII. In the subjective day, FFC interacts with WCC and recruits CKI and CKII to phosphorylate the WC proteins. As FRQ levels increase, WCC is extensively phosphorylated and therefore inactivated. This process results in the dissociation of WCC from the *frq* promoter and a decrease in *frq* transcription. At the same time, FRQ is progressively phosphorylated by CKI and CKII. The extensively phosphorylated FRQ is recognized by the SCF^{FWD-1} E3 ubiquitin ligase, leading to the ubiquitination and degradation of FRQ through the proteasome pathway. Through the night, FRQ levels decrease and the inactive hyperphosphorylated WCC is probably dephosphorylated by PP2A, which results in the reactivation of WCC and *frq* transcription in a new circadian cycle.

the normal circadian behavior of the organism (Garceau et al. 1997). The light and temperature entrainment of the *Neurospora* clock is caused by changes of FRQ expression levels after light and temperature treatments (Crosthwaite et al. 1995; Liu et al. 1998).

The central role of FRQ in the *Neurospora* circadian clock was highlighted by our recent finding that FRQ is a state variable of the *Neurospora* circadian clock (Huang et al. 2006). The induction of *frq* expression at a critical phase and with appropriate strength by light pulse, temperature step-up, or inducible expression alone triggers arrythmicity (also called singularity behavior) at the physiological and molecular levels. In addition, we showed that the amplitude of FRQ rhythm reflects the amplitude of the clock and determines the sensitivity of the clock to phase-resetting stimulus. Furthermore, our results suggest that the circadian singularity behavior is a two-step process: The critical treatment first drives the circadian negative feedback loop to a steady state, after which the cell populations become desynchronized.

Beside the repression of D-WCC activity in the dark, FRQ promotes the accumulation of WC-1 and WC-2, forming positive feedback loops that are interlocked with the negative loop (Lee et al. 2000; Cheng et al. 2001a, 2003b; Merrow et al. 2001). Such interlocked positive feedback loops are also shared by animal circadian systems (Glossop et al. 1999; Shearman et al. 2000). FRQ regulates WC-1 expression posttranscriptionally, whereas it promotes *wc-2* transcription. Our experiments in which *wc-1* or *wc-2* were overexpressed from an inducible promoter to different levels demonstrated that the positive feedback loops are important for maintaining the robustness and stability of the clock (Cheng et al. 2001a). It was recently shown that the phosphorylation of the PEST-2 region of FRQ is important for the accumulation of WC-1 but not WC-2 (Schafmeier et al. 2006). We showed recently that casein kinase 1a (CK-1a) is important for maintaining the steady-state levels of WC-1 and WC-2 (He et al. 2006). Together, these results suggest that the positive role of FRQ on WC levels is at least in part mediated by protein phosphorylation.

In addition to their essential role in the circadian negative feedback loop in the dark, WC-1 and WC-2 are required for all known light responses in *Neurospora*, including the entrainment of the circadian clock (Ballario et al. 1996; Crosthwaite et al. 1997; Cheng et al. 2003b; Liu 2003). WC-1 binds to the chromophore FAD through its photosensory LOV (light, oxygen, or voltage) domain, a specialized PAS domain, and functions as the blue light photoreceptor for light responses (He et al. 2002; Cheng et al. 2003b). Light triggers the formation of a large WC complex that binds to the promoters of light-inducible genes (Froehlich et al. 2002; He and Liu 2005), resulting in light-induced transcription and light responses.

ACTIVATION OF *frq* TRANSCRIPTION

Both WC-1 and WC-2 are PAS domain-containing transcription factors (WC-1 has three PAS domains, whereas WC-2 contains only one) with GATA-type zinc finger DNA-binding domains, and they primarily reside in the nucleus (Ballario et al. 1996; Linden and Macino 1997; Talora et al. 1999; Schwerdtfeger and Linden 2000). These proteins interact through the PASC domain of WC-1 and the PAS domain of WC-2 to form the heterodimeric D-WCC (Talora et al. 1999; Cheng et al. 2002, 2003b). WC-2 is required to maintain the steady-state level of WC-1, and WC-1 levels are very low in the *wc-2* null mutant and in mutants with disrupted WC-1/WC-2 interaction (Cheng et al. 2002). It is possible that WC-1 is unstable or cannot fold properly in the absence of WC-2. D-WCC binds to the C box in the *frq* promoter and activates *frq* transcription (Froehlich et al. 2003; He and Liu 2005). Its binding to the C box is rhythmic during a circadian cycle and the deletion of the C box abrogates rhythm of *frq* mRNA accumulation, indicating that D-WCC binding is essential for the function of the clock (Froehlich et al.

2003; He et al. 2006). *frq* mRNA and FRQ protein levels are extremely low in *wc* null mutants and the induction of WC expression in *wc* mutants from an ectopic locus leads to rapid induction of *frq* transcription (Crosthwaite et al. 1997; Cheng et al. 2001a). Together, these data indicate that D-WCC is the primary activator of *frq* transcription.

WCC reconstituted in vitro binds to the *frq* promoter in gel-shift assays without any additional components (Froehlich et al. 2003; He et al. 2005b). Mutational studies show that the zinc finger DNA-binding domains of both WC proteins are necessary to activate *frq* transcription in the dark (Crosthwaite et al. 1997; Cheng et al. 2002; Collett et al. 2002). However, the zinc finger domain of WC-1 is not required for its light-signaling function, and its LOV domain is not necessary for *frq* activation in the dark (He et al. 2002; Cheng et al. 2003b). Therefore, the light and dark functions of WC-1 can be molecularly separated.

INHIBITION OF WCC ACTIVITY BY FFC

Upon synthesis, FRQ self-associates through its amino-terminal coiled-coil domain, and this association is important for its function in the negative feedback loop (Cheng et al. 2001b). All FRQ proteins form complexes with FRH, an essential RNA helicase in *Neurospora* (Cheng et al. 2005). The formation of FFC is important for maintaining the steady-state level of FRQ; this is analogous to the stabilization of WC-1 by WC-2 (Cheng et al. 2002). FRH mediates the interaction between FRQ and WCC and interacts with WCC independently of FRQ. The down-regulation of FRH using RNA interference (RNAi) led to high levels of *frq* RNA, indicating that the negative feedback loop is abolished. These data indicate that FRH is an essential component of the negative feedback loop in the *Neurospora* circadian clock. The FRH homolog in yeast, Dob1p/Mtr4p, binds to RNA and functions as an essential cofactor for the exosome, an important regulator of RNA (rRNA and mRNA) metabolism (de la Cruz et al. 1998; Mitchell and Tollervey 2000; Hilleren and Parker 2003). Although it is not clear whether FRH functions as an RNA helicase in circadian regulation, it is likely that the daily fluctuation of the FFC may mediate circadian control of RNA processing and degradation in *Neurospora*.

FRQ, WC-1, and WC-2 are regulated by phosphorylation. After its synthesis, FRQ is immediately phosphorylated and becomes progressively more phosphorylated over time. It is finally degraded through the ubiquitin-proteasome pathway mediated by FWD-1 (Garceau et al. 1997; He et al. 2003, 2005a). Thus, in the dark, both the level and the phosphorylation status of FRQ are rhythmic. CK-1a, casein kinase II (CKII), and calcium/calmodulin-dependent protein kinase I (CAMK-1) are known to phosphorylate FRQ (Gorl et al. 2001; Yang et al. 2001, 2002, 2003; He et al. 2006).

Like FRQ, WC-1 and WC-2 are phosphorylated both in the dark and in the light to regulate WCC activity (He and Liu 2005; He et al. 2005b; Schafmeier et al. 2005, 2006). We previously identified five major in vivo WC-1 phosphorylation sites, located immediately downstream from the DNA-binding domain (He et al. 2005b). Mutation of these sites showed that these light-independent sites are critical for circadian clock function and that phosphorylation negatively regulates the D-WCC activity. The importance of WC phosphorylation in the circadian clock was later confirmed by the observation that the light-independent WC phosphorylation is FRQ-dependent (Schafmeier et al. 2005). In the *frq* null strain, both WC-1 and WC-2 are hypophosphorylated. In a wild-type strain, WC-2 exhibits a robust circadian rhythm of its phosphorylation profile. Importantly, the activation of *frq* transcription correlates with the hypophosphorylation of the WCs. Furthermore, we showed that the dephosphorylation of the *Neurospora* WCC significantly enhances its binding activity to the C box (He and Liu 2005). Together, these data suggest that FFC inhibits WCC activity by promoting its phosphorylation.

To understand the mechanism of how FFC inhibits WCC activity, we recently identified two kinases that can mediate the FRQ-dependent WC phosphorylation (He et al. 2006). CK-1a is the *Neurospora* homolog of the *Drosophila* DBT and it can phosphorylate FRQ in vitro (Gorl et al. 2001). More importantly, CK-1a was found to associate with FRQ (Gorl et al. 2001; Cheng et al. 2005), suggesting that it may phosphorylate FRQ in vivo. In addition, the association of FRQ and CK-1a raises the possibility that FRQ may recruit CK-1a to phosphorylate WCC. However, in vivo evidence for the involvement of CK-1a in the clock was previously not available because it is essential for cell survival in *Neurospora*. By mapping and mutating the FRQ–CK-1a interaction domain on FRQ, we showed that the FRQ–CK-1a interaction is essential for clock function (He et al. 2006). Importantly, both FRQ and WCs are hypophosphorylated in mutants with disrupted an FRQ–CK-1a interaction, indicating that CK-1a mediates the phosphorylation of FRQ and WCs. In addition, we found that WCC associates with CK-1a in an FRQ-dependent manner. To obtain direct genetic evidence for the role of CK-1a in the clock, we created a *ck-1a* knockin mutant, *ck-1a*^{L}, that carries a mutation equivalent to that of the *Drosophila dbt*^{L} mutation. In this mutant, FRQ and WCs are hypophosphorylated and the circadian rhythms exhibit long period (~32 hr) due to significant delay of FRQ progressive phosphorylation. These results indicate that CK-1a is a major kinase for both FRQ and WCs. The levels of WC-1 and WC-2 are low in the *ck-1a*^{L} strain, indicating that CK-1a is important for the circadian-positive feedback loops. Despite the low WC levels in the *ck-1a*^{L} strain, chromatin immunoprecipitation (ChIP) assays indicated that the hypophosphorylated WCC efficiently binds to the C box within the *frq* promoter and WCC cannot be efficiently inactivated by FRQ.

CKII is another kinase that we previously identified as a major kinase that phosphorylates FRQ in vivo (Yang et al. 2002, 2003). In addition to its role in promoting FRQ degradation as CK-1a, CKII is required for the repressor activity of FRQ, as indicated by high *frq* RNA levels in the CKII mutants despite their high FRQ protein levels. Like in the *ck-1a*^{L} strain, we found that WC-1 and WC-2 are hypophosphorylated in WCC in the *cka* mutant strain in which the only catalytic subunit of CKII is disrupted

(He et al. 2006). In addition, WCC in this mutant constantly binds to *frq* promoter at higher levels despite high FRQ levels (He et al. 2006). Taken together, our results strongly suggest that FRQ closes the circadian negative feedback loop by mediating CK-1a and CKII phosphorylation of WCC. In this model, FRQ acts as the substrate-recruiting subunit of CK1 and CKII; the amount of FRQ determines the amount of kinases that can be recruited to phosphorylate WCC and thus the extent of WCC phosphorylation and its activity. A similar negative feedback process was also suggested in *Drosophila* (Kim and Edery 2006; Yu et al. 2006; Kim et al. 2007).

The *Neurospora* circadian negative feedback also involves phosphatase activities. We previously reported that the serine/threonine protein phosphatase 2A (PP2A) has an important role in the circadian negative feedback loop (Yang et al. 2004). The null mutation of one of the regulatory subunits of PP2A, *rgb-1*, leads to low *frq* mRNA and FRQ protein levels and a low-amplitude long-period rhythm. Such a conclusion was later confirmed by Schafmeier et al. (2005) showing that RGB-1 regulates the phosphorylation and activity of WC proteins. Thus, PP2A may regulate the circadian negative feedback process by dephosphorylating and reactivating WCC. Interestingly, the fly homolog RGB-1 is also an important clock component (Sathyanarayanan et al. 2004).

FRQ PHOSPHORYLATION AND DEGRADATION PATHWAY

The degradation and posttranslational modifications of FRQ have an essential role in period length determination and the function of the circadian negative feedback loop (Liu 2005; Liu and Bell-Pedersen 2006; Heintzen and Liu 2007). FRQ is progressively phosphorylated by several kinases, and its phosphorylation triggers its degradation through the ubiquitin-proteasome pathway (He et al. 2003). Mutation of FRQ phosphorylation sites was found to lengthen the period of the clock, suggesting that phosphorylation of FRQ promotes its turnover (Liu et al. 2000; Gorl et al. 2001; Yang et al. 2003; He et al. 2006). FRQ is phosphorylated by CK-1a, CKII, and CAMK-1 (Gorl et al. 2001; Yang et al. 2001, 2002, 2003; He et al. 2006). Like mutation of its *Drosophila* homolog, *ck-1a*^{L}, a hypomorphic mutation in *ck-1a* results in hypophosphorylation of FRQ, decreased rate of degradation, and long period of rhythm in the constant darkness (He et al. 2006). Disruption of the CKII catalytic subunit (*cka*) or regulatory subunit (*ckb-1*) in *Neurospora* also leads to hypophosphorylated FRQ and increased FRQ stability and results in abolishment of circadian rhythmicity or low-amplitude long-period rhythms, respectively (Yang et al. 2002, 2003). Interestingly, the phosphorylation events of FRQ appear to be independent of each other, suggesting that FRQ is phosphorylated by multiple kinases at multiple independent sites and each contributes to FRQ stability.

In addition to the kinases, protein phosphatases PP1 and PP2A also have important roles in regulating FRQ phosphorylation (Yang et al. 2004). Unlike PP2A, which appears to function mostly in the circadian negative feedback loop, PP1 regulates FRQ stability. A hypomorphic mutation of *ppp-1*, the catalytic subunit of PP1, leads to increased FRQ degradation rate, which results in rhythms of a short period and advanced phase. Thus, these phosphatases counteract kinases to regulate the phosphorylation status of FRQ. Taken together, these data suggest that the progressive phosphorylation of FRQ, regulated by multiple kinases and phosphatases at multiple independent sites, fine tunes the stability of FRQ and is a major determinant of the period length of the clock.

The phosphorylation-dependent degradation of FRQ is mediated by FWD-1, an F box/WD-40 repeat-containing protein and the *Neurospora* homolog of the *Drosophila* protein Slimb and mammalian β-TRCPs (Grima et al. 2002; He et al. 2003; Eide et al. 2005). FWD-1 physically interacts with phosphorylated forms of FRQ transiently and functions as the substrate recruiting subunit of the SCF-type ubiquitin (E3) ligase SCF^{FWD-1} to mediate FRQ ubiquitination (He et al. 2003, 2005a). In an *fwd-1* mutant strain, circadian rhythms are abolished and FRQ protein accumulates to high levels in its hyperphosphorylated forms. FWD-1 without its F box forms a stable complex with FRQ in vivo, suggesting that FWD-1 is a major component in the FRQ ubiquitination and degradation pathway.

The COP9 signalosome, a conserved multisubunit deneddylation complex in all eukaryotes, regulates the stability of the SCF^{FWD-1} complex in *Neurospora* and is thus critical to clock function (He et al. 2005a). The disruption of CSN leads to hyperneddylation of CULLIN-1 (a component of SCF complexes), which results in autoubiquitination and destruction of the SCF^{FWD-1} complex. This leads to low levels of SCF^{FWD-1} complex and impaired FRQ degradation and clock function.

CONSERVATION OF POSTTRANSLATIONAL REGULATION IN THE EUKARYOTIC CIRCADIAN SYSTEMS

The conservation of the posttranslational mechanisms is remarkable among different eukaryotic circadian systems from fungi to humans (Gallego and Virshup 2007; Heintzen and Liu 2007). Like FRQ and WCs in *Neurospora*, the animal and plant core clock components are also regulated by phosphorylation (Young and Kay 2001). FRQ and the animal PER proteins are all phosphorylated by CKI and CKII, dephosphorylated by the same phosphatases, and degraded by the ubiquitin/proteasome system using a conserved E3 ubiquitin ligase (Liu and Bell-Pedersen 2006). As in *Neurospora*, CKI is tightly associated with PER proteins in *Drosophila* and mammals (Kloss et al. 1998; Lee et al. 2001, 2004). Recently, the PER-DBT interaction domain was shown to be critical for PER phosphorylation, transcriptional repression, and circadian clock function (Kim et al. 2007). Together with the observations of the *Neurospora ck-1a*^{L} mutant strain, these results demonstrate the highly conserved role of FRQ and PER in recruitment of kinases. In mouse, the PER/CKI interaction appears to be critical for regulation of mammalian PER phosphorylation and the function of the clock (Lee et al. 2004). In humans, two types of famil-

ial advanced sleep phase syndromes (FASPS) are due to mutations of the human CKIδ or its phosphorylation sites on human PER2 (Toh et al. 2001; Xu et al. 2005). CKII in *Drosophila*, as in *Neurospora*, is required for the PER repressor function (Nawathean and Rosbash 2004), although how CKII functions to promote the repressor activity of PER is not clear. In *Arabidopsis*, where CKII was first implicated in clock function, CKII phosphorylates CCA1 and regulates its DNA-binding activity (Sugano et al. 1998).

The phosphorylation-dependent degradation of negative elements is also well conserved throughout the evolution. *Drosophila* PER proteins are phosphorylated by CKI and associate with Slimb (Grima et al. 2002; Ko et al. 2002; Eide et al. 2005). Another *Drosophila* locus, *jetlag*, contains an open reading frame (ORF) encoding an F-box protein with leucine-rich repeats and is important for light-induced degradation of TIM in the light entrainment pathway (Koh et al. 2006). Both biochemical and genetic approaches revealed that mammalian CRY and PER proteins are also regulated by an F-box protein, FBXL3 (Busino et al. 2007; Godinho et al. 2007; Siepka et al. 2007).

Like the WC proteins, the PAS-domain containing transcriptional factors that act as the positive components of the animal circadian negative feedback loops are also regulated by phosphorylation. The mammalian Bmal1 and CLK and the *Drosophila* CLK also exhibit robust rhythms in their phosphorylation profiles (Lee et al. 2001; Kim and Edery 2006; Yu et al. 2006; Kim et al. 2007). Similar to the case in *Neurospora*, the phosphorylation of CLK in *Drosophila* was recently found to be dependent on PER, DBT, and the PER-DBT inteaction. In addition, the CLK-CYC binding to the *per* E box correlates with the accumulation of hypophosphorylation of CLK and the hyperphosphorylation of CLK correlates with the peak of *per* mRNA repression. Furthermore, PP2A was also shown to be involved in the regulation of CLK phosphorylation. Together, these results strongly suggest that mechanisms of the circadian negative feedback process are highly conserved from *Neurospora* to animals.

CONCLUSION

Autonomous oscillations of clock components are generally mediated by posttranslational modifications, and overall, these modifications and the proteins involved are well conserved from fungi to humans. The identification of kinases and phosphatases of clock proteins and the studies of protein degradation pathway and the circadian negative feedback process have established the essential roles of protein phosphorylation in these processes and in clock functions. Furthermore, the identification of FRH as a core component of the circadian negative feedback loop raises the possibility of clock-controlled RNA metabolism. These recent advances in our understanding of circadian regulation in *Neurospora* have made its circadian oscillator one of the best understood among those in eukaryotes, and research in this organism has benefited parallel research in higher eukaryotes. The in vitro reconstitution of the cyanobacterial circadian clock indicated the fundamental importance of protein phosphorylation in clock regulation (Nakajima et al. 2005). Similar efforts in *Neurospora* and other eukaryotic organisms in the future should significantly enhance our current understanding of the mechanisms of eukaryotic circadian clocks.

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