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REVIEW

Molecular mechanism of the *Neurospora* circadian oscillator

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ABSTRACT

Circadian clocks are the internal time-keeping mechanisms for organisms to synchronize their cellular and physiological processes to the daily light/dark cycles. The molecular mechanisms underlying circadian clocks are remarkably similar in eukaryotes. *Neurospora crassa*, **a filamentous fungus, is one of the best understood model organisms for circadian research. In recent years, accumulating data have revealed complex regulation in the** *Neurospora* **circadian clock at transcriptional, post-transcriptional, post-translational and epigenetic levels. Here we review the recent progress towards our understanding of the molecular mechanism of the** *Neurospora* **circadian oscillator. These advances have provided novel insights and furthered our understanding of the mechanism of eukaryotic circadian clocks.**

KEYWORDS circadian clock, circadian oscillator, *Neurospora crassa*, eukaryotes

INTRODUCTION

Circadian clocks are intrinsic molecular devices that allow most eukaryotic and some prokaryotic organisms to adjust their molecular, cellular and behavioral activities to the daily changing environmental factors including light and temperature, caused by the self-rotation of the earth (Young and Kay, 2001; Dunlap and Loros, 2004; Foster, 2004; Heintzen and Liu, 2007). The molecular machineries that generate the endogenous daily rhythmicities are called circadian oscillators. A variety of organisms such as *Cyanobacteria*, fungi, plants (*Arabidopsis*), and animals (e.g., *Drosophila*, and mammals) (Levine, 2004; Bell-Pedersen et al., 2005; Reppert, 2006; Williams, 2007; Harmer, 2009) have been employed as model organisms to study the molecular mechanism of circadian clocks. Despite the difference in clock mechanisms, the core eukaryotic circadian oscillators consisting of negative feedback loops share many similarities in different organisms (Dunlap and Loros, 2004; Foster, 2004; Bell-Pedersen et al., 2005). In *Neurospora*, *Drosophila*, and mammals, heterodimeric complexes of two PER-ARNT-SIM (PAS) domain-containing transcription factors act as positive elements that activate the expression of negative elements. On the other hand, the negative elements repress their own transcription by inhibiting the activity of the positive elements. The cyclic activation, repression and reactivation of circadian negative elements generate the autonomous oscillation, which controls the circadian output pathway by regulating the expression of downstream clock-controlled genes (*ccgs*) (Bell-Pedersen et al., 1996; Vitalini et al., 2004; Bell-Pedersen et al., 2005; Heintzen and Liu, 2007).

The filamentous fungus *Neurospora crassa* has a distinguished history as a well-established circadian model organism (Loros and Dunlap, 2001; Dunlap and Loros, 2004). More than 40 years ago, a *band (bd) Neurospora* strain was isolated. It exhibits robust circadian rhythm of asexual spores (conidia) formation and has been widely used for circadian research because of its easily visible conidiation rhythmicity (Sargent et al., 1966; Sargent and Woodward, 1969). The mutation responsible for the *bd* phenotype was later shown to be a mutation in the *ras-1* gene (Belden et al., 2007a). Today, *Neurospora* continues to be one of the best established model organisms for circadian clock research.

Because the light entrainment and circadian output pathways of the *Neurospora* systemhave been extensively reviewed elsewhere (Liu, 2003; Liu and Bell-Pedersen, 2006; Dunlap et al., 2007; Heintzen and Liu, 2007; de Paula et al., 2008), we will focus on the recent advances of the

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molecular mechanism of the *Neurospora* circadian oscillator in this review.

THE CORE CIRCADIAN OSCILLATOR OF THE *NEUROSPORA* CIRCADIAN CLOCK

Circadian oscillators are self-sustained pacemakers that drive diverse molecular circadian rhythms (Bell-Pedersen et al., 2005). Circadian oscillators consist of positive and negative components which form negative feedback loops. The positive elements function to activate the transcription of the negative elements. On the other hand, the negative elements act to repress their own transcription by inhibiting the activity of the positive elements. In the *Neurospora* circadian clock oscillator, FREQUENCY (FRQ), FRQ-INTERACTING RNA HELICASE (FRH), WHITE COLLAR 1 (WC-1) and WHITE COLLAR (WC-2) are the core components of *Neurospora* circadian clock (Fig. 1) (Ballario et al., 1996; Linden and Macino, 1997; Talora et al., 1999; Cheng et al., 2002, 2003b, 2005). Both WC-1 and WC-2 proteins contain PAS domains and form the WC complex (WCC), which binds to *frq* promoter and activate its transcription as positive conponents (Aronson et al., 1994a, b; Crosthwaite et al., 1997; Cheng et al., 2001b, 2003b; Froehlich et al., 2003; He et al., 2005b; Liu and Bell-Pedersen, 2006).

FRQ is the key negative element in *Neurospora* circadian oscillator. The full length FRQ contains 989 amino acid residues (Aronson et al., 1994a). There are two forms of FRQ, the large FRQ (lFRQ) and small FRQ (sFRQ), which differ by 100 amino acid at the N' terminus due to alternative splicing (Liu et al., 1997; Colot et al., 2005; Diernfellner et al., 2007). The alternative splicing of *frq* functions to tune the period length and resets the clock in response to ambient temperature. The *frq* gene locus also transcribes natural antisense transcripts (NATs), which contributes to the light entrainment of the clock (Kramer et al., 2003).

FRQ protein self-associates via the coil-coil region near the N' terminus (Cheng et al., 2001a), and all FRQ protein is in

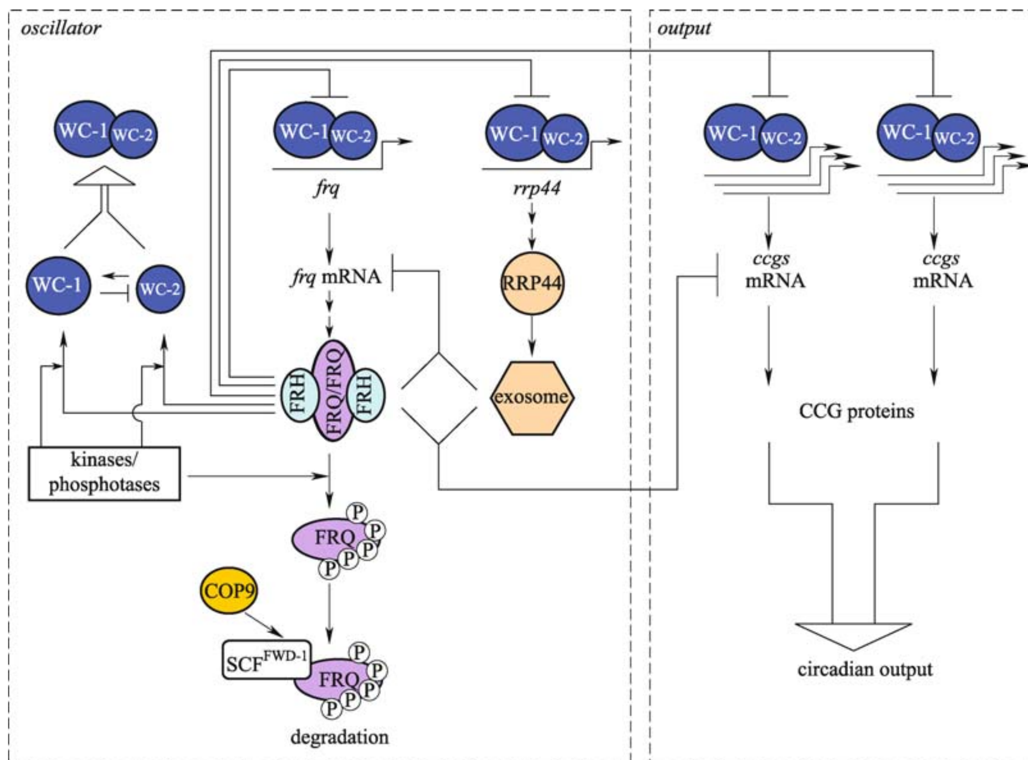

Figure 1. Molecular network of the *Neurospora* circadian clock. WC-1 and WC-2, two PAS-domain containing proteins form the White Collar complex (WCC) which binds the *frq* gene promoter and activate *frq* transcription. FRQ protein associates with FRH to form the FRQ-FRH complex (FFC). FFC recruits kinases to phosphorylate WC proteins which leading to dissociation of WCC from the *frq* promoter and inhibition of *frq* transcription. In the meantime, FRQ promotes the steady-state levels of WC-1 and WC-2, forming the positive feedback loop. Upon progressive phosphorylation events, hyperphosphorylated FRQ is degraded by 26S proteasome either through the pathway involving FWD-1, or an unknown pathway independent of FWD-1. The signalosome COP9 regulates circadian clock by promoting the stability of SCF^{FWD-1}. The expression of *rrp44*, which encoding the catalytic subunit of exosome, is rhythmically regulated by FFC. Exosome and FFC function to facilitate the decay of *frq* mRNA and a subset of *ccg* mRNAs, forming a post-transcriptional negative feedback loop. CSW-1 (Clock-Switch 1) regulates the accessibility of *frq* promoter to the transcription factors at the epigenetic level.

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complex with FRH to form the FRQ-FRH complex (FFC). In addition, FRQ binds to FRH over the entire circadian cycles (Baker et al., 2009). More importantly, downregulation of FRH completely abolishes circadian rhythmicity (Cheng et al., 2005). FFC enters the nucleus and dissociates WCC from the *frq* promoter by promoting phosphorylation of WCC through the recruitment of the casein kinases, resulting in the suppression of *frq* transcription and closing of the transcription-based negative feedback loop (Cheng et al., 2005; He et al., 2005b; Schafmeier et al., 2005; He et al., 2006; Guo et al., 2009). These results indicate that FRH is also an essential component of the circadian negative feedback loop.

After its synthesis, FRQ protein undergoes progressive phosphorylation and become extensively phosphorylated before its degradation mediated by ubiquitin/proteasome (He et al., 2003; He and Liu, 2005a). Elimination of FRQ in turn liberates WCC and its reactivation of *frq* transcription. The cyclic activation, repression and reactivation of *frq* expression generate the endogenous circadian rhythmicity, which then controls the rhythmic expression of clock-controlled genes.

WC-1 and WC-2, the positive elements in *Neurospora* circadian negative feedback loop, form heteromeric WC complexes mediated by the PASC domain of WC-1 and the single PAS domain of WC-2 (Talora et al., 1999; Cheng et al., 2002; Cheng et al., 2003a). *wc-1* gene comprises three promoters: Pdist, Pprox and Pint (Káldi et al., 2006). Likewise, *wc-2* gene contains several transcription initiation sites, which result in the expression of full-length WC-2 and sWC-2 in antagonistic manner (Neiss et al., 2008). In constant darkness, WCC rhythmically binds to the C-box which contains two GATN repeats on *frq* and *ccgs* promoters and activates their transcription (Froehlich et al., 2003; He et al., 2005a; He and Liu, 2005b; Belden et al., 2007b). Disruption of *wc* genes results in very low *frq* mRNA level and complete abolishment of circadian rhythmicity (Crosthwaite et al., 1997; Cheng et al., 2002, 2003a).

In addition to the role in repressing the WCC function in the negative feedback loop, FRQ functions to promote the level of WC proteins (Lee et al., 2000; Cheng et al., 2001b; Brunner and Schafmeier, 2006; Diernfellner et al., 2007). FRQ supports the accumulation of WC-1 at post-translational level and activates transcription of *wc-2*. WC proteins also regulate their expression and form another interconnected feedback loop. WC-1 indirectly inhibits *wc-2* by controlling expression of a putative repressor, while in the presence of WC-1 and FRQ, it activates *wc-2* transcription (Cheng et al., 2001b, 2003b). Like FRQ, FRH is also required for these positive feedback loops (Cheng et al., 2005; Shi et al., 2009; Guo et al., 2010). These interconnected feedback loops are critical for maintaining the robust and stable clock function (Cheng et al., 2001a). Similar interlocked positive loops operate in mammalian and insect circadian systems (Glossop et al., 1999; Shearman et al., 2000).

In addition to its essential role in the circadian negative feedback loop, WC-1 also acts as blue-light receptor required for resetting and entrainment of the clock and almost all known light response in *Neurospora* (Froehlich et al., 2002; He et al., 2002, 2005b; Heintzen and Liu, 2007). Light induces rapid binding of the WCC to the promoters of *frq* and other light-induced genes (He et al., 2005b; Belden et al., 2007b).

Like other eukaryotic circadian oscillators, the *Neurospora* circadian oscillator is limit cycle-based. In *Neurospora*, temperature and light treatments can result in abolishment of the rhythmicity, a phenomenon called singularity behavior (Gooch et al., 1994; Huang et al., 2006). Both temperature step-up and a brief light exposure can trigger singularity (Huang et al., 2006). In addition, the change of FRQ level alone is sufficient to trigger the singularity behavior, indicating that FRQ is a state variable of the *Neurospora* limit cycle-based circadian clock. Furthermore, our results suggest that the singularity behavior is due to loss of the rhythms in all cells, after which cell population become desynchronized (Gooch et al., 1994; Huang et al., 2006).

POST-TRANSLATIONAL REGULATION OF THE *NEUROSPORA* CLOCK

Phosphorylation of FRQ

Phosphorylation of FRQ is essential for its clock function (Cha et al., 2007). Studies in the last decade have led to the understanding of function of FRQ phosphorylation and identification of many FRQ kinases and phosphotases (Table 1).

Like the animal PERIOD (PER) proteins, FRQ is progressively phosphorylated after its synthesis and becomes extensively phosphorylated before its degradation (Garceau et al., 1997). FRQ exists as many isoforms, with molecular sizes ranging from ~120 kDa to ~200 kDa result from the phosphorylation on different sites (Baker et al., 2009; Tang et al., 2009). In total, over 75 FRQ phosphorylated residues have been identified (Baker et al., 2009; Tang et al., 2009). Except for a few regions, most of the FRQ regions are phosphorylated, suggesting that FRQ adapts a non-globular 3D structure to allow most of protein to be accessible by kinases. Systematic mutagenesis of these phosphorylation sites demonstrated that multiple independent phosphorylation events along the FRQ ORF modulate the period of the clock by regulating FRQ stability. While most of the phosphorylation events promote FRQ degradation, the phosphorylation events near the C-terminal end stabilize FRQ, indicating that FRQ stability is a major determinant in circadian period length and phosphorylation can play opposing roles to fine-tune FRQ stability (Tang et al., 2009).

Five kinases, CK-1a (casein kinase-1a), CKII, CAMK-1 (calmodulin kinase 1), PKA and PRD-4 (chk2), have been shown to be FRQ kinases (Garceau et al., 1997; Görl et al.,

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Table 1 Protein kinases and phosphotases in the *Neurospora* circadian clock

enzymes	substrates	molecular functions in clock	clock phenotypes of mutants
protein kinases			
casein kinase I (CK-1)	FRQ; WC-1; WC-2	a major FRQ kinasealso mediates FRQ-dependent WC phosphorylation; regulates the abundance of WCs	*ck-1a*^{L} exhibit > 30 h periodicity
casein kinase II (CK-2)	FRQ; WC proteins[a]	a major FRQ and WC kinase; stabilizes FRQ; represses the association between FRQ and WCC	arrhythmic and low amplitude long period rhythms
calmodulin kinase I (CAMK-1)	FRQ	phosphorylates FRQ	regulates the phase, period, and light-induced phase shifting of the conidiation rhythm
cyclic AMP-dependent protein kinase A (PKA)	FRQ; WC proteins[a]	phosphorylates and stabilizes FRQ; phosphorylates WCs and inhibits WCC activity; inhibits WC nuclear localization	arrhythmicity (*mcb* mutant) or low amplitude rhythms (*pkac-1* mutant)
protein kinase C (PKC)	WC-1	WC-1; decreases WC-1 level	regulates light response
checkpoint kinase 2 (chk2/PRD-4)	FRQ	phosphorylates FRQ	resets clock upon DNA damage
protein phosphotases			
protein phosphotase 1 (PP1)	FRQ	dephosphorylates and stabilizes FRQ	regulates phase; exhibits short period rhythms
protein phosphotase 2A (PP2A)	FRQ; WC proteins[a]	dephosphorylates FRQ and WCs	regulates period and amplitude of the rhythms
protein phosphotase 4 (PP4)	FRQ[a]	dephosphorylates FRQ and WCs; promotes nuclear entry of WCC	regulates period and amplitude of the rhythms

[a] The relevant enzymes regulate both WC-1 and WC-2.

2001; Yang et al., 2001, 2002, 2003; He et al., 2006; Pregueiro et al., 2006; Huang et al., 2007) (Table 1). Among these kinases, CK-1a and CKII contribute to most of the FRQ phosphorylation events (Yang et al., 2004; He et al., 2006; Tang et al., 2009). On the other hand, it has been shown that PRD-4 (chk2) mediates FRQ phosphorylation after DNA damage, while CAMK-1 affects the light entrainment of the clock (Yang et al., 2001; Pregueiro et al., 2006).

The *Drosophila* DOUBLETIME (DBT) and the mammalian CKI homologs, including CKIδ as well as CKIε, associates with and phosphorylates animal PER proteins (Kloss et al., 1998; Price et al., 1998; Lowrey and Takahashi, 2000; Xu et al., 2005). Similarly, CK-1a was found to be tightly associated with FRQ (Yang et al., 2003; Baker et al., 2009; Mehra et al., 2009b). A *ck-1a* mutant, which contains the same mutation as the *Drosophila dbt*^{L}, exhibits hypophosphorylated FRQ and more than 30 h period due to increased FRQ stability (He et al., 2006). In addition, CK-1a mediates the FRQ-depedent phosphorylation of WC-1 and WC-2 which inhibits the WCC activity to close the negative feedback loop (He et al., 2006; Querfurth et al., 2007). Although another *Neurospora* CK-1 homolog CK1-b can phosphorylate FRQ *in vitro*, the disruption of *ck-1b* gene resulted in no significant change in both FRQ phosphorylation and oscillation of FRQ, suggesting it's not essential for the clock functions (Yang et al., 2003).

Protein kinase CKII, also termed casein kinase-2, is a ubiquitous protein kinase consisting of two catalytic (α and/or α') subunits and two regulatory subunits. Like CKI, CKII exhibits conserved function in circadian clock functions in plant, fungi, as well as animal (Yang et al., 2002; Allada and Meissner, 2005). In *Neurospora* CKII was biochemically purified as a major FRQ kinase (Yang et al., 2002). In a mutant, in which the catalytic subunit of CKII (*cka*) was disrupted, FRQ protein is hypophosphorylated and stabilized, and the circadian rhythms are completely abolished (Yang et al. 2002). Similarly, the disruption of *ckb1,* the gene encoding for the regulatory subunit, led to hypophosphorylation and stabilization of FRQ as well as and long periods but low amplitude oscillations (Yang et al., 2002, 2003).

The *Neurospora* CKII also regulates the temperature compensation of circadian clock (Mehra et al., 2009b). Temperature compensation is one of the several hallmarks of circadian clocks, which allows circadian clocks to function precisely at different temperatures. Reduction of CKII resulted in changes of temperature compensation in a dosage dependent manner. In the *chrono* and *prd3* mutants which bore a mutation in ckb1 and *cka* subunits, respectively, the temperature compensation was partially impaired (Mehra et al., 2009b).

The eukaryotic PKA (cyclic AMP-dependent protein kinase A) holoenzyme consists of catalytic subunits and inhibitory regulatory subunits. The binding of cyclic AMP (cAMP) to the regulatory subunit of PKA results in its activation (Taylor et al.,

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2005). Mutants of both of the PKA catalytic (*pkac-1*) and regulatory (*mcb*) subunit genes exhibit loss of circadian rhythms and altered expression levels of FRQ and WC proteins (Huang et al., 2007). PKA mediates the phosphorylation of both FRQ and WC proteins. The phosphorylation of FRQ by PKA results in stabilization of FRQ, which is in contrast to the function of the casein kinases (Huang et al., 2007). A similar role of phosphorylation in stabilization of clock proteins was also seen with the phosphorylation of the FASP site in mammals (Vanselow et al., 2006; Huang et al., 2007; Xu et al., 2007). Thus, phosphorylation of clock proteins at different sites can play opposite roles in regulating FRQ stability. It is possible that the phosphorylation by PKA at certain sites of FRQ can inhibit the efficient ubiquitilation or phosphorylation of FRQ.

In addition to these kinases, FRH can also indirectly regulate the phosphorylation profile of FRQ. In the *frh* knockdown strain and those strains in which the FRH-FRQ interaction was abolished, FRQ becomes hypophosphorylated and the amount of FRQ is dramatically reduced due to rapid degradation (Guo et al., 2010). It is likely that FRH can regulate the protein-protein associations between FRQ and its kinases.

FRQ inhibits WCC activity by promoting WC phosphorylation

Phosphorylation of WC proteins is essential for the negative feedback loop process in *Neurospora* (Lauter and Russo, 1990; Schafmeier et al., 2005; He et al., 2005b, 2006). Several kinases have been demonstrated to mediate the phosphorylation of WC proteins. These kinases include CKI, CKII, PKA and PKC (Table 1) (Yang et al., 2001, 2002, 2003; Franchi et al., 2005; He et al., 2006; Huang et al., 2007; Querfurth et al., 2007). WC proteins are phosphorylated in constant darkness and hyperphosphorylated in the light (He et al., 2005b). Hypophosphorylated WCC becomes active for the transcription of *frq* and *ccg* genes while hyperphosphorylated WCC is inactive (Bell-Pedersen et al., 1996; Crosthwaite et al., 1997; Cha et al., 2008; Schafmeier et al., 2008). Phosphorylation of WC proteins inhibits its DNA binding activity *in vitro* and *in vivo* and both WC-1 and WC-2 are hypophosphorylated in the *frq* null strain, suggesting that FRQ inhibits WCC activity by promoting the phosphorylation of WC proteins (He and Liu, 2005b; He et al., 2005b; Schafmeier et al., 2005; Hong et al., 2008). The inhibition of WCC transcriptional activity by FRQ-dependent phosphorylation closes the circadian negative feedback loop.

FRQ recruits both CK-1a and CKII to mediate FRQ-dependent WC phosphorylation and inhibition (He et al., 2006). In FRQ mutants which the FRQ-CK-1a interaction is disrupted, both WCs become hypophosphorylated. In addition, both WC-1 and WC-2 are also hypophosphorylated in *ck1-a* and *cka* (catalytic subunit of CKII) mutants, and WCC constitutively binds to the *frq* promoter at high levels (He et al., 2006). These results indicate the importance of FRQ, CK-1a and CKII in closing the circadian negative feedback loop.

There are five major *in vivo* WC-1 phosphorylation sites which are located immediately downstream of the DNA binding domain (Ser-988, Ser-990, Ser-992, Ser-994, and Ser-995), which are critical for the clock function of WC-1 (He et al., 2005b). Quantitative mass spectrometry analyses revealed that phosphorylation of these sites is a sequential process: first by FRQ-independent phosphroylation at Ser-990 by a priming kinase and then by FRQ-dependent phosphorylation events at the other sites (Huang et al., 2007). PKA acts as the priming kinase that phosphorylates on S990 of WC-1, which converts the rest of the serine sites into good CK1 or CKII sites (Huang et al., 2007). In *pkac-1^{ko}* (the catalytic subunit of PKA) strain, WC-1 and WC-2 are hypophosphorylated and the levels are extremely low. In addition, the *frq* mRNA levels are high despite low WC levels in this mutant. In contrast, although WC levels are high, *frq* mRNA levels are very low in the *mcb* mutants (the regulatory subunit of PKA). Together, these results demonstrate that PKA phosphorylates and inhibits WCC activity (Huang et al., 2007). Similarly, hPER2 was shown to be sequentially phosphorylated near the FASPS site by CK1 and an unidentified priming kinase (Vanselow et al., 2006; Xu et al., 2007).

Dephosphorylation of clock proteins

Phosphorylation of FRQ and WC proteins is reversibly regulated by several phosphotases. So far, phosphotase 1 (PP1), phosphotase 2A (PP2A) and phosphotase 4 (PP4) have been identified as clock-related phosphatases in *Neurospora* (Yang et al., 2004; Cha et al., 2008) (Table 1).

PP1 and PP4 antagonize the functions of the casein kinaseson FRQ by protein stabilization. In both *ppp-1* and *pp4* (catalytic subunits of PP1 and PP4, respectively) mutants FRQ protein is less stable, resulting in advanced phase and short period phenotypes (Yang et al., 2004; Cha et al., 2008).

in vitro experiments suggest that FRQ is a substrate for both PP1 and PP2A (Yang et al., 2004). The mutation in *rgb-1* (the regulatory subunit of PP2A) resulted in low abundance of both *frq* mRNA and FRQ protein (Yang et al., 2004), indicating its essential role in the circadian negative feedback loop. One circadian role of PP2A is achieved by its dephosphorylation and reactivation of WC proteins (Yang et al., 2004; Schafmeier et al., 2005). In the *rgb-1* mutant, WC-2 is hyperphosphorylated, consistent with low WCC activity. In addition to its role in dephosphorylating FRQ, PP4 also dephosphorylates WC proteins and activates their activity by promoting the nuclear entry of WCC (Cha et al., 2008). Together, these results suggest that these phosphotases play important but distinct roles in the *Neurospora* circadian clock by acting on different clock proteins or on different phosphorylation sites. Similarly, both PP1 and PP2A have been shown

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to play similar roles in *Drosophila* and mammalian circadian clocks (Cohen, 2002; Sathyanarayanan et al., 2004; Fang et al., 2007).

Regulation of cellular localization of FRQ and WC proteins

Translocation of FRQ

FRQ contains a functional nuclear localization signal (195–200 aa) and mutation of which causes loss of the circadian rhythmicity (Luo et al., 1998). However, the majority of FRQ protein (> 90%) is present in the cytoplasm (Cheng et al., 2005; Schafmeier et al., 2005). The loss of circadian rhythm by elimination of FRQ NLS suggests that nuclear FRQ is essential for its clock function (Luo et al., 1998; Diernfellner et al., 2009). Shortly after its synthesis, FRQ enters the nucleus to fulfill its function both in the negative feedback loop to repress the WCC activity and in the positive limb to promote the WC proteins level (Luo et al., 1998; Cheng et al., 2001b; Diernfellner et al., 2009). FRQ shuttles rapidly between the nucleus and cytoplasm and its phosphorylation appears to regulate its nucleocytoplasmic shuttling (Diernfellner et al., 2009). Hypophosphorylated FRQ enters the nuclei more efficiently while the nuclear import efficiency of hyperphosphorylated FRQ is low. In contrast to the role of nucleic FRQ in closing the negative feedback loop of clock, the function of the cytoplasmic FRQ remains elusive. The association between FRQ, FRH and the exosome components suggests a potent function of the cytoplasmic FFC complex in the circadian regulation of RNA metabolism (Guo et al., 2009).

Translocation of WC proteins

Like FRQ, nucleocytoplasmic shuttling of WC proteins is regulated by phosphoryation, which is critical for their clock function. The nucleocytoplasmic shuttling of WC proteins is regulated by their phosphorylation states. Hypophosphorylated WCC is enriched in nuclei which functions to bind DNA and activate the transcription of the target genes including *frq* and *ccgs* (Cha et al., 2008; Hong et al., 2008; Schafmeier et al., 2008; Diernfellner et al., 2009). In contrast, hyperphosphorylation of WCC results in low abundance of nuclear WCs.

PKA and PP4 have been demonstrated to be regulators in modulating the nucleocytoplasmic shuttling of WC proteins (Cha et al., 2008). PP4 functions to promote the nuclear entry of WCC while PKA inhibits WC nuclear localization. In addition, FRQ plays a role in mediating WCs nucleocytoplasmic shuttling by regulating the phosphorylation and abundance of WCs. It's been proposed that the accumulation of cytoplasmic FRQ recruits kinases to phosphorylate WCC and inhibit its nuclear entry (Cha et al., 2008; Hong et al., 2008; Schafmeier et al., 2008; Diernfellner et al., 2009).

REGULATION OF FRQ DEGRADATION

FRQ protein undergoes progressive phosphorylation that leads to its eventual degradation, and this turnover process is essential in maintaining the circadian cycles and is a major determinant in circadian period length (Liu et al., 2000; He et al., 2003, 2005a; Baker et al., 2009; Mehra et al., 2009a; Tang et al., 2009). This phosphorylation-dependent turnover process is controlled by the ubiquitin/proteasome pathway, which is conserved in eukaryotic clocks (He et al. 2003; He and Liu, 2005a; Mehra et al., 2009a).

In *ck-1a* and *cka* mutants, FRQ was hypophosphorylated and more stable which indicating that a major role of FRQ phosphorylation by CK-1a and CKII is to promote FRQ degradation (Yang et al., 2002, 2003, He et al., 2006). On the other hand, PP1 and PP4 dephosphorylate and stabilize FRQ to oppose the effects of the casein kinases (Yang et al., 2004; Cha et al., 2008). Overall, the combined effects of these kinases and phosphatases set the phosphorylation state and stability of FRQ.

The phosphorylation-dependent FRQ degradation by the ubiquitin/proteasome pathway is mediated an SCF (Skp1/Cullin/F-box protein) ubiquitin ligase (E3), in which the F-box/WD40-repeat-containing protein FWD-1 is the substrate-recruiting subunit (He et al. 2003). Disruption of *fwd-1* results in hyperphosphorylation of FRQ, high FRQ levels and circadian arrhythimicity. The high level of FRQ in the mutant is due to blocked degradation. In addition, FWD-1 bound tightly with FRQ when the F-box of FWD-1 is deleted. On the other hand, the FRQ-FWD-1 interaction was not detected in a wild-type strain, indicating that the tight FRQ-FWD-1 association results in rapid FRQ ubiquitination and degradation. The FWD-1 homologs in *Drosophila* (Slimb) and mammals (beta-TRCPs) are also components of SCF ubiquitin ligases that mediate the phosphorylation-dependent degradation of PER proteins (Grima et al., 2002; Ko et al., 2002; He et al., 2003; Eide et al., 2005; Shirogane et al., 2005).

The study of the circadian functions of the COP9 signalosome (CSN) in *Neurospora* further confirmed that the importance of the ubiquitin-mediated FRQ degradation (He et al., 2005a). The CSN is a conserved deneddylation complex in eukaryotes, which contains eight distinct subunits (from CSN1 to CSN8) (Cope and Deshaies, 2003; He et al., 2005a). In *Neurospora*, the CSN plays a role in modulating the clock by regulating the stability of the SCF^{FWD-1} complex (He et al., 2005a; Cha et al., 2007). Disruption of the essential CSN component *csn-2* hindered the FRQ degradation and severely impaired circadian rhythmicity due to extremely low FWD-1 levels. The low FWD-1 levels in the *csn-2* mutant was caused by increased auto-ubiquitination of the SCF complex (He et al., 2005a).

The mammalian FWD-1 homolog beta-TRCPs recognize a high affinity DpSGΦXpS motif on their substrates (Wu et al., 2003). Like mammalian PERs, FRQ lacks such a motif. The

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involvement of multiple independent phosphorylation events in FRQ degradation suggests that FWD-1 does not recognize FRQ by a single high affinity site (Baker et al., 2009; Tang et al. 2009). Instead, we propose that there are multiple suboptimal FWD-1 binding sites on FRQ and the phosphorylation of FRQ at multiple sites can progressively promote the interaction between FRQ and FWD-1. Thus, extensive FRQ phosphorylation leads to tight FRQ-FWD-1association and rapid FRQ ubiquitination and degradation (Tang et al., 2009).

Our recent study also suggests an alternative pathway for FRQ degradation that is independent of FWD-1. When *frh* is silenced or when the interaction between FRH and FRQ is abolished, FRQ protein becomes hypophosphorylated and undergoes rapid degradation. Such an effect of FRH on FRQ phosphorylation and stability is independent of FWD-1 since the knock-down of *frh* in the *fwd-1* mutant also results in rapid degradation of FRQ, indicating the existence of an alternative FRQ degradation pathway (Guo et al., 2010). Since FRH is necessary for the maintenance of FRQ phosphorylation status and stability, it is likely that the proper protein conformation of FRQ is critical for its stability and function.

POST-TRANSCRIPTIONAL REGULATION OF THE *NEUROSPORA* CLOCK

In addition to the transcriptional and post-translational mechanisms that control the *Neurospora* clock, regulation at the post-transcriptional levels also plays important role. The *frq* gene locus transcribes multiple endogenous non-coding antisense transcripts *qrfs*. The expression of *qrf* cycles is in anti-phase manner to that of the sense *frq* transcripts (Kramer et al., 2003; Tralau et al., 2007). The expression of the *qrfs* was shown to contribute to the light entrainment of the clock by regulating *frq* expression.

FRQ and FRH form the FFC complex that represses *frq* mRNA levels. FRH is a homolog of Mtr4p in *Saccharomyces cerevisiae,* which is an essential nuclear co-factor for the exosome complex, a large complex consisting of several 3′→ 5′ exonucleases important for RNA metabolism by mediating 3′→ 5′ processing and degradation (LaCava et al., 2005; Vanácováet al., 2005; Houseley et al., 2006). Our recent results suggest that FFC and exosome are involved in a post-transcriptional negative feedback loop that is interlocked with the transcriptional loop (Guo et al., 2009).

In this post-transcriptional loop, FRQ promotes *frq* RNA decay and the level of FRQ determines *frq* RNA stability, resulting in a daily rhythm of *frq* RNA stability. *frq* RNA is more stable in the frq^9 strain and at a time point when FRQ level is low. In addition, the silencing of FRH resulted in abnormally high *frq* RNA level due to impaired RNA degradation. Furthermore, FRQ was shown to associate with *frq* RNA specifically and with the core exosome component RRP44 (rRNA processing 44). Finally, the downregulation of RRP44, the catalytic subunit of the exosome, resulted in an increase of *frq* RNA and severe impairment of the circadian rhythmicities. These results suggest that FFC and exosome are part of a post-transcriptional negative feedback loop that regulates *frq* RNA level. Therefore, both the transcriptional and the post-transcriptional processes mediated by FFC contribute to the circadian negative feedback loop process (Guo et al., 2009). In this scenario, when FRQ levels are high, *frq* mRNA levels are decreased by both the inhibition of the WCC activity and rapid degradation of *frq* RNA. When FRQ levels are low, *frq* mRNA levels increase rapidly due to WCC activation and increased *frq* RNA stability.

The mRNA of *rrp44* is also a clock-controlled gene with a phase that is very similar to *frq* (Guo et al., 2009). The levels of *rrp44* are reduced in *wc* mutants and WCC was shown to bind to the promoter of *rrp44* rhythmically. These results demonstrate that like *frq*, the expression of *rrp44* is directly controlled by WCC. In addition to its role regulating *frq* RNA degradation, RRP44 also control the expression levels of a sub-set of *ccgs*, suggesting that the rhythmical activity of the exosome also plays an important role in regulating the circadian output pathways by controlling mRNA decay (Guo et al., 2009).

In mammals and *Drosophila*, NONO is a RNA/DNA binding protein that binds to clock protein PER1. Silencing of NONO led to abolishment of rhythmicity (Brown et al., 2005). Whether NONO regulates the clock in a post-transcriptional manner remains to be demonstrated.

EPIGENETIC REGULATION OF THE *NEUROSPORA* CLOCK

Epigenetic mechanism plays an important role in regulating eukaryotic gene expression. Mounting evidence has suggested that various chromatin modifications are important for eukaryotic circadian clock functions. In eukaryotes, acetylation of histones H3 and H4 promotes the promoter accessibility for transcription. In *Neurospora* and mammals, it has been shown that the histone acetylation is controlled in a circadian manner (Etchegaray et al., 2003; Belden et al., 2007b). By screening of the ATP-dependent chromatin-remodeling enzymes in *Neurospora*, *csw-1* (*clockswitch 1*), one of homologs to yeast genes *Sth2* and *mi-2*, was identified to be important for clock function (Belden et al., 2007a). The loss of *csw-1* resulted in sporadic conidiation as well as impaired circadian rhythms (Belden et al., 2007b). Even though CSW-1 is not rhythmically expressed, it may binds to *frq* promoter to facilitate the rhythmic binding of WCC.

CONCLUSIONS AND PERSPECTIVES

The filamentous fungus *Neurospora* is one of the best understood circadian model systems for clock research due to its durability and simplicity. The understanding of the molecular of the *Neurospora* circadian clock has contributed significantly to how eukaryotic clocks work in general. The

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basic circadian mechanisms share remarkable similarities and conservations between *Neurospora* and animal circadian oscillators. Despite of our current understanding the *Neurospora* circadian oscillator, many important questions remain. These questions include the mechanism that controls of FRQ and WC cellular localization, mechanism of temperature compensation of the clock, the roles of post-transcriptional regulation in the control of circadian rhythms, and roles of non-*frq* based oscillators in the clock, etc. In the future, studies using *Neurospora* will lead to the revelation of new regulatory mechanisms at different levels and *Neurospora* will continue to serve as a premier eukaryotic model system for clock research.

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ABBREVIATIONS

CAMK-1, calmodulin kinase 1; cAMP, cyclic AMP; ccgs, clock-controlled genes; CK-1a, casein kinase-1a; CSN, COP9 signalosome; csw-1, clockswitch 1; DBT, DOUBLETIME; FFC, FRQ-FRH complex; FRH, FRQ-INTERACTING RNA HELICASE; FRQ, FREQUENCY; lFRQ, large FRQ; NATs, natural antisense transcripts; PAS, PER-ARNT-SIM; PER, PERIOD; PKA, cyclic AMP-dependent protein kinase A; PP1, phosphotase 1; PP2A, phosphotase 2A; PP4, phosphotase 4; RRP44, rRNA processing 44; SCF, Skp1/Cullin/F-box protein; sFRQ, smallFRQ; WCC, WC complex; WC-1, WHITE COLLAR 1; WC-2, WHITE COLLAR 2

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