Degradation of the *Neurospora* circadian clock protein FREQUENCY through the ubiquitin–proteasome pathway

Q. He and Y. Liu
Department of Physiology, University of Texas Southwestern Medical Center, 5323 Harry Hines Blvd, Dallas, TX 75390, U.S.A.

Abstract
Phosphorylation of the *Neurospora* circadian clock protein FREQUENCY (FRQ) promotes its degradation through the ubiquitin–proteasome pathway. Ubiquitination of FRQ requires FWD-1 (F-box/WD-40 repeat-containing protein-1), which is the substrate-recruiting subunit of an SCF (SKP/Cullin/F-box)-type ubiquitin ligase. In the *fwd-1* mutant strains, FRQ degradation is defective, resulting in the accumulation of hyper-phosphorylated FRQ and the loss of the circadian rhythmicities. The CSN (COP9 signalosome) promotes the function of SCF complexes in *vivo*. But *in vitro*, de neddylation of cullins by CSN inhibits SCF activity. In *Neurospora*, the disruption of the *csn-2* subunit impairs FRQ degradation and compromises the normal circadian functions. These defects are due to the dramatically reduced levels of FWD-1 in the *csn-2* mutant, a result of its rapid degradation. Other components of the SCF FWD-1 complex, SKP-1 and CUL-1 are also unstable in the mutant. These results establish important roles for SCF FWD-1 and CSN in the circadian clock of *Neurospora* and suggest that they are conserved components of the eukaryotic circadian clocks. In addition, these findings resolve the CSN paradox and suggest that the major function of CSN is to maintain the stability of SCF ubiquitin ligases in *vivo*.

Introduction
Circadian clocks regulate a wide variety of physiological and molecular activities in almost all eukaryotic and certain prokaryotic organisms. The filamentous fungus *Neurospora crassa* has one of the best understood circadian systems. Like other eukaryotic organisms, the circadian oscillator of *Neurospora* consists of autoregulatory negative feedback loops in which there are positive and negative elements [1–3]. In the dark, a heterodimeric complex made of two PAS (Per/Arnt/Sim) domain-containing transcription factors, WC-1/WC-2, activates *frequency* (*frq*) transcription by binding to its promoter [4–8]. On the other hand, together with FRH [FREQUENCY (FRQ)-interacting RNA helicase], an RNA helicase, FRQ represses its own transcription through its physical interactions with the WC-1/WC-2 complex [4,9–12]. Thus the WC complex is the positive element, while FRQ acts as a negative element of this circadian negative feedback loop. Such a negative feedback loop generates endogenous circadian oscillations of *frq* mRNA and FRQ protein that regulate rhythmicities in *Neurospora*.

Regulation of clock protein degradation by protein phosphorylation is critical for the functions of eukaryotic circadian clocks from fungi to animals [1,3,13]. FRQ protein is phosphorylated immediately after its synthesis and becomes progressively phosphorylated over time. After FRQ proteins are extensively phosphorylated, their levels decrease, suggesting that phosphorylation of FRQ may lead to its degradation. Phosphorylation of FRQ is mediated by several kinases, including the protein kinase CK1 (casein kinase 1), CK2 and a calcium/calmodulin-dependent kinase [14–17]. On the other hand, PP1 (protein phosphatase 1) and PP2A have been shown to dephosphorylate FRQ and counteract the effects of these kinases [18].

In addition to regulating the transcriptional repressor activity of FRQ by its phosphorylation, several lines of evidence indicate that FRQ phosphorylation promotes its degradation. First, when FRQ phosphorylation is inhibited by a kinase inhibitor *in vivo*, FRQ degradation is slowed down, resulting in the long period of the clock [19]. Secondly, mutations of the FRQ phosphorylation sites also lead to stabilization of the FRQ protein and lengthening of the periods of the clock [16,17,19]. Furthermore, disruption of the *Neurospora* CK2 catalytic subunit or one of its regulatory subunits resulted in higher FRQ protein levels and slower FRQ degradation rate [14,17].

FRQ is ubiquitinated *in vivo*
To understand the degradation mechanism of FRQ, we examined whether FRQ is degraded through the ubiquitin–proteasome pathway [20]. The commonly used proteasome inhibitors, including LLN (N-acetyl-leucyl-leucyl-norleucinal), MG115, MG132 and lactacystin that failed to enter the *Neurospora* cells, cannot be used to examine the
ubiquitination of FRQ in vivo. However, when ophiobolin A, a known calmodulin inhibitor, was added to the Neurospora culture, we noticed a high-molecular-mass FRQ-specific smear in these samples, reminiscent of the poly-ubiquitinated proteins typically seen after treatments of the proteasome inhibitors. Since the same ophiobolin A treatment also led to a significant increase in the ubiquitinated proteins, these results suggest that FRQ can be ubiquitinated in Neurospora cells.

**FWD-1 (F-box/WD-40-repeat-containing protein 1) as the substrate-recruiting subunit of the ubiquitin ligase for FRQ**

To understand the importance of FRQ ubiquitination in the circadian clock, we searched for the FRQ-specific ubiquitin ligase through a candidate approach [20]. Because of the known roles of FWDs in regulating the degradation of phosphor-proteins and in mediating PERIOD protein degradation in Drosophila [21,22], we disrupted the *fwd-1* gene, which encodes the Neurospora homologue of the Drosophila Slimb and the mammalian β-TrCP proteins. In the *fwd-1* mutant, FRQ is hyperphosphorylated and accumulates to high levels due to impaired FRQ degradation. The circadian rhythms of the FRQ protein levels and its phosphorylation patterns are abolished, leading to loss of normal circadian rhythms of conidiation and the expression of clock-controlled genes. However, *frq* expression can still respond to light and the conidiation process can still be driven by light/dark cycles. These results indicate that FWD-1 is a critical component in the Neurospora circadian clock.

To confirm that FWD-1 directly acts on FRQ, we showed that FWD-1 is associated with FRQ in vivo by immunoprecipitation assay. However, tight interaction between FRQ and FWD-1 is only observed in an *fwd-1 Δ* strain containing the FWD-1 protein lacking its F-box domain, which stabilizes the interaction between the WD-40 domain and its substrates. Thus the interaction between FRQ and FWD-1 is only transient in a wild-type strain and FRQ is rapidly ubiquitinated and degraded by the 26 S proteasome after its binding with FWD-1. Therefore the affinity between FRQ and FWD-1 and the stability of FRQ are probably regulated by the extent of FRQ phosphorylation. The progressive FRQ phosphorylation events at multiple sites, thus, are a critical process that provides a critical delay needed in the circadian negative feedback loop that is important for period length determination. Taken together, these results suggest that FWD-1 is the substrate-recruiting subunit of an SCF (SKP Cullin F-box)-type ubiquitin ligase mediating FRQ ubiquitination and degradation.

**Conservation of the clock protein degradation pathway in eukaryotic clocks**

Slimb and β-TrCP, the Drosophila and mammalian homologues of FWD-1 respectively, have also been shown to be components of the SCF ubiquitin ligases (E3) that mediate the ubiquitination of phosphorylated PER (a clock protein in *Drosophila*) proteins [21–23]. In addition, phosphorylation of FRQ and PER regulated by homologous kinases and phosphatases is important for their degradation and their roles as transcriptional repressors [14,16–18,24]. The conservation of these post-translational regulators and their functions in divergent circadian systems suggests that the molecules mediating the modification and degradation of clock proteins may be the common foundation that allows the evolution of circadian clocks in eukaryotic systems.

**The role of CSN in the Neurospora circadian clock**

The CSN (COP9 signalosome) is a conserved multisubunit complex found in all eukaryotes [25–27]. CSN has eight different subunits (CSN1–CSN8) and is structurally related to the 19 S lid of the 26 S proteasome. Biochemically, CSN can remove the ubiquitin-like molecule, NEDD8 (neural-precursor-cell-expressed, developmentally down-regulated 8), from the cullin subunit of SCF-type ubiquitin ligases [28,29], a process that inhibits the SCF ubiquitin ligase activity in vitro. Paradoxically, overwhelming genetic evidence indicates that CSN is important for the SCF-mediated degradation of substrates in vivo [26,27,30]. To reconcile this paradox, CSN has been proposed to mediate the assembly/disassembly and maintenance of cullin-containing ubiquitin ligases [25–27].

Because of the known roles of CSN in mediating light-regulated developmental processes and in regulating SCF activities, we examined its function in the Neurospora circadian clock. We disrupted the Neurospora *csn-2* gene, which is one of the most conserved CSN subunits that is essential for CSN function. Unlike in higher eukaryotic organisms, the disruption of *csn-2* is not lethal in Neurospora, but the *csn-2* mutant strains (*csn-2Δ*?) exhibit growth and developmental phenotypes: they grow more slowly and produce fewer aerial hyphae and conidia than the wild-type strain. In addition, *CULLIN-1*, the cullin subunit of the SCF complexes, becomes hyperneddylated in the *csn-2* mutant, indicating that *csn-2* is essential for the function of CSN in Neurospora.

Examination of FRQ expression in the *csn-2* mutants showed that FRQ levels are high and they accumulate in their hyperphosphorylated forms due to an impaired degradation process. Thus, similar to other eukaryotic systems, CSN is also important for the degradation of the substrates of SCF-type ubiquitin ligases. However, the degradation of FRQ is only partially abolished in the *csn-2* mutant, and the CSN-independent degradation of FRQ requires newly synthesized proteins. When protein synthesis is blocked by cycloheximide, FRQ degradation is completely blocked in the *csn-2* mutant.

Under the standard growth condition in liquid culture (growth up to 60 h), FRQ levels are high and the circadian rhythms of FRQ levels and its phosphorylation states are severely compromised in the *csn-2* mutant. However, residual FRQ oscillations in the mutant can still persist with low
amplitude under this condition. When a nutrient-depleting condition was used by growing the cells for one additional day (growth up to 84 h), which led to a slower growth rate, FRQ oscillations were completely abolished in the mutant. On race tubes, despite the slow growth of the mutants, they exhibited an abnormal 2 day conidiation pattern. Surprisingly, unlike the wild-type strain, such abnormal conidiation patterns in the mutants cannot be entrained by light/dark and temperature cycles and they even persist under the constant light condition. These results suggest that the normal circadian clock function and the light- and temperature-regulated conidiation processes were impaired in the csn-2 mutant. In addition, the abnormal conidiation banding pattern of the csn-2 mutant is probably driven by an FRQ-independent oscillator (or FRQ-less oscillator, FLO) [31].

Because of the conservation of the degradation pathway of PER and FRQ, we propose that CSN is likely to be another conserved component of the eukaryotic circadian clocks. If true, this would raise the possibility that a partial deficiency in CSN function might contribute to the circadian and sleep disturbance phenotypes observed in patients with SMS (Smith–Magenis syndrome). The SMS is a multiple congenital anomalies/mental retardation syndrome due to a hemizygous deletion of human chromosome 17, band p11.2, a region that includes the human CSN3 (COPS3) subunit [32–34].

The CSN paradox: role of CSN in maintaining the stability of SCF complexes

To understand the mechanism of CSN function in the clock and to resolve the CSN paradox, we compared the FWD-1 levels between the wild-type strain and the csn-2 mutant. We found that FWD-1 levels were dramatically lower in the csn-2 mutant strain due to its rapid degradation. The half-life of FWD-1 changed from 6–9 h in the wild-type to approx. 45 min in the mutant. In addition, the level of FWD-1 in the mutant depends on the growth conditions, as nutrient-depletion conditions led to its further reduction. Furthermore, we showed that the rapid turnover of FWD-1 in the mutant is due to its autoubiquitination since the removal of its F-box stabilized FWD-1 and led to its accumulation to high levels in the csn-2 mutant. These results explain impaired FRQ degradation in the csn-2 mutant and indicate that CSN is important for maintaining the stability of FWD-1 in Neurospora by preventing its autoubiquitination.

To examine whether the other components of the SCF complexes are regulated similarly by the CSN, we identified CUL-1 and SKP-1 as the components of the FWD-1 complex by biochemical purification. Comparison of the wild-type and the mutant strains showed that both CUL-1 and SKP-1 are unstable in the csn-2 mutant. In addition, examination of another FWD, SCON-2, revealed also that it is very unstable in the csn-2 mutant. Therefore CSN is important for maintaining the stability of the entire FWD complex and other SCF complexes in Neurospora.

Taken together, these results reconcile the CSN paradox in Neurospora and provide strong experimental support for the CSN cycling model [25–27]. In a wild-type strain, the de neddylation of CUL-1 by CSN allows it to bind to CAND-1, a process that inactivates the active ligase complex and removes the SKP-1–FWD-1 from the complex, preventing their autoubiquitination and destruction. But in csn mutants, the active SCF complexes cannot be disassembled, leading to the autoubiquitination and destruction of the SCF components, and the functional SCF complexes depend largely on the newly synthesized components. Therefore the low levels of cullin ubiquitin ligases in the csn mutants result in impaired degradation of their substrates.

Conclusion

Like other eukaryotic circadian systems, regulation of clock protein degradation plays critical roles in the Neurospora circadian clock function. The work described above established FWD-1 as a conserved clock component from fungi to mammals and identified CSN as another clock gear in eukaryotic clock systems. Furthermore, our study on CSN resolves the paradox between its in vivo and in vitro functions. The simplicity of the Neurospora system and the conservation of clock mechanisms between Neurospora and other higher eukaryotes will continue to make it an excellent eukaryotic model system to study circadian rhythmicity.

References


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