The Ccr4-Not Protein Complex Regulates the Phase of the Neurospora Circadian Clock by Controlling WHITE COLLAR Protein Stability and Activity*

Received for publication, June 17, 2013, and in revised form, September 11, 2013 Published, JBC Papers in Press, September 12, 2013 DOI 10.1074/jbc.M113.494120

Guocun Huang†§, Qiang He†§, Jinhu Guo‡*, Joonseok Cha†, and Yi Liu‡

From the †Center for Circadian Clocks, Medical College, Soochow University, Suzhou 215123, China, the Departments of §Physiology and *Neuroscience, University of Texas Southwestern Medical Center at Dallas, Dallas, Texas 75390, the ‡Institute of Medicinal Biotechnology, Peking Union Medical College and Chinese Academy of Medical Sciences, Beijing 100050, China, and the *State Key Laboratory of Biocontrol, School of Life Sciences, Sun Yat-sen University, Guangzhou 510006, China

In the Neurospora circadian negative feedback loop, WHITE COLLAR 1 (WC-1) and WC-2 form the WC complex that activates frequency (frq) transcription. Here we show that Not1 is a WC-interacting protein and is important for maintaining WC levels. The not1 transcript displays a circadian oscillation with a similar phase as frq. Down-regulation of not1 leads to low levels of WC-1 and WC-2 and a delayed circadian phase as a result of increased protein degradation and increased WC activity. Protein purification of Not1 shows that it is part of the Neurospora Ccr4-Not complex. ccr4 is a clock-controlled gene and is regulated directly by the WC complex. Down-regulation of ccr4 results in a phase delay and period lengthening of the clock. Together, our findings suggest that the Ccr4-Not complex participates in the Neurospora clock function by interacting with and regulating the WC complex.

Background: The stability and activity of WCC is important for Neurospora circadian clock function.

Results: Not1 is a WC-interacting protein. Down-regulation of not1 and ccr4 result in low WC levels and delayed circadian phases.

Conclusion: The Ccr4-Not complex regulates the Neurospora clock by controlling WCC stability and activity.

Significance: This study identifies the Ccr4-Not complex as a new factor in the Neurospora circadian clock.

Eukaryotic circadian oscillators consist of autoregulatory negative feedback loops in which there are positive and negative elements (1–3). In the filamentous fungus Neurospora, two PER-ARNT-SIM-domain-containing transcriptional factors, WHITE COLLAR 1 (WC-1) and WHITE COLLAR 2 (WC-2), function as the positive elements in the core circadian negative feedback loop, whereas FREQUENCY (FRQ) and FRQ-interacting RNA helicase (FRH) act as the negative elements (4, 5). In constant darkness (DD) in Neurospora frq mRNA by binding to the Clock box in the frq promoter (6–8). FRQ protein binds FRQ-interacting RNA helicase to form the FRQ-FRQ-interacting RNA helicase complex (FFC), which represses WCC activity by promoting WC phosphorylation (8–12). Alternatively, FFC also promotes the degradation of frq mRNA posttranscriptionally (13). FRQ is progressively phosphorylated by casein kinase (CK)-1a and CKII and degraded by the ubiquitin–proteasome pathway mediated through the ubiquitin E3 ligase SCF FWD (8, 14–18).

Phosphorylation of WC proteins inhibits WCC activity (9, 10, 19). WC phosphorylation is regulated in both a FRQ-dependent (8, 10) and an FRQ-independent manner (20). FFC recruits CK-1a and CKII to the WCC to mediate the FRQ-dependent WC phosphorylation (8, 10). On the other hand, protein kinase A phosphorylates WC-1 independently of FRQ and serves as the priming kinase for the casein kinases (20). In addition to the negative feedback loop, there is a positive feedback loop in which FFC promotes the accumulation of WC proteins (21, 22). frq and frh mutations or down-regulation of frh result in low levels of both WC-1 and WC-2 (11, 12, 21, 22). These results are consistent with the hypothesis that transcriptionally active WCs are unstable and that the positive feedback loop is, at least in part, due to the consequence of the FRQ-mediated negative feedback process (23).

In addition to FFC, the LOV (light-oxygen-voltage) domain-containing protein VVD (VIVID) also interacts and stabilizes WCs and plays an important role in photoadaptation (24–26). vvd mutants exhibit increased light sensitivity and a phase delay of the circadian condensation rhythm following a light-to-dark transition (27), which is likely due to prolonged turnover of frq mRNA (28). Therefore, FFC and VVD independently regulate the activity and stability of WCC by interacting with WC proteins. In this study, we purified the WC complex from Neurospora to identify Not1 (negative on TATA) as a WC-interacting protein that regulates WC stability and activity. Furthermore, we demonstrated that the Neurospora Ccr4 (carbon catabolite repression)-Not complex is important for circadian phase
determination of the clock. Our results uncovered an unexpected link between the circadian clock and the Ccr4-Not complex.

EXPERIMENTAL PROCEDURES

Strains and Culture Conditions—All strains carry the bd mutation in this study. Liquid cultures were grown in medium containing 0.01 M QA, 1× Vogel's medium, 0.1% glucose, and 0.17% arginine. The dsnot1 and dscr4 strains were created by a method described previously (5). Two inverted fragments were ligated together to generate a hairpin with a loop. For the dsnot1 construct, one fragment was amplified by the primers 5′-ATTGGATCCATCTAGCCCTGGCTCCCTCAAGG-3′ (forward) and 5′-AGGCCCGGGGACAGCTTGAAGCGGCTT-GACAAAG-3′ (reverse). Another fragment was amplified by the primers 5′-ATTGGATCCATCTAGCCCTGGCTCCCTCAAGG-3′ (forward) and 5′-ATTGGATCCATCTAGCCCTGGCTCCCTCAAGG-3′ (reverse). For the dscr4 construct, one fragment was amplified by the primers 5′-ATTGGATCCATCTAGCCCTGGCTCCCTCAAGG-3′ (forward) and 5′-ATTGGATCCATCTAGCCCTGGCTCCCTCAAGG-3′ (reverse).

Protein Purification and MS Analysis—The wc-2KO, Myc-His-WC-2 strain was cultured in constant light (LL) or DD conditions in liquid medium. The purification was performed as described previously (29). The final c-Myc precipitates were separated in SDS-PAGE gel, and the gel was subsequently subjected to colloidal blue staining or silver staining according to the instructions of the manufacturer (SiverQuest, Invitrogen). The specific bands were excised and subjected to tryptic digestion and nano-HPLC/electrospray MS analysis.

Protein and RNA Analysis—Western blot analysis and an immunoprecipitation assay were carried out as described previously (21). RNA was prepared and analyzed by Northern blot analysis (30) or by quantitative RT-PCR as described previously (21). RNA was prepared and analyzed by Northern blot analysis (30) or by quantitative RT-PCR as described previously (21). RNA was prepared and analyzed by Northern blot analysis (30) or by quantitative RT-PCR as described previously (21).

ChIP Assay—A ChIP assay was performed as described (19). Immunoprecipitation was performed using WC-2 antibody, and immunoprecipitation without the WC-2 antibody was used as the negative control. The primers for the ccr4 promoter were 5′-GTCTAGGGCTGACAGCTTTG-3′ (forward) and 5′-GACAAACTGCGCTTGCTCAC-3′ (reverse).

RESULTS

Not1 Is a WC-interacting Protein—To identify novel WC-interacting proteins, we purified the WC complex from the wc-2KO, Myc-His-WC-2 strain (phenotypically similar to a wild-type strain) by nickel column purification, followed by immunoprecipitation using c-Myc monoclonal antibody (9, 20, 29). One protein band of high molecular weight was found to be specifically coimmunoprecipitated with WC complex in cultures grown in DD and, at a reduced amount, in LL (Fig. 1A). Mass spectrometry analysis identified this protein as Not1 (NCU04766), one subunit of the Ccr4-Not complex in the Neurospora crassa Database. Not1 shows significant homology across the entire open reading, with the yeast Not1 protein (27% identity, E value of 2e-178) and Not1 proteins in other fungal species. Yeast Not1 is a subunit of the Ccr4-Not complex, which is a multisubunit protein complex that is conserved in eukaryotic organisms (32, 33). The core complex is composed of nine proteins: Not1, Not2, Not3, Not4, Not5, Caf130, Caf40, Caf1, and Ccr4 in Saccharomyces cerevisiae (32). In Neurospora, the Not1 protein has no homology to other Not proteins. Not proteins were originally identified in a genetic screen as factors that inhibit the basic RNA polymerase II transcription machinery by repressing the TATA-less promoter in yeast (34, 35). Not1 is thought to be a scaffold in this complex, and Not4 is a RING domain E3 ubiquitin ligase (36) responsible for the polyubiquitination and degradation of the specific H3K4 me3 demethylase Jhd2 (37). Ccr4 has been demonstrated to be a cytoplasmic deadenylase required for RNA turnover (38, 39). Thus, the Ccr4-Not complex can regulate gene expression at both transcriptional and posttranscriptional levels. Whether the Ccr4-Not complex is involved in circadian clock function is not known.

The WC purification result also indicated that the interaction between WCC and Not1 is substoichiometric. To confirm the association between WCC and Not1 protein, we created a strain that expresses the c-Myc-tagged Not1 and performed immunoprecipitation using WC-2 antibody. As shown in Fig. 1B, WC-2 immunoprecipitation specifically pulled down the WC-2 strain with the Myc-Not1 strain showing that not1 mRNA oscillates in a circadian manner. Densitometry is shown below. rRNA was used as the loading control. Data are mean ± S.D. n = 3.
control of the circadian clock. Northern blot analysis results showed that not1 mRNA displays a circadian oscillation in DD (Fig. 1C) with a similar phase as frq (Fig. 2D), suggesting that not1 is a clock-controlled gene in Neurospora.

**Down-regulation of not1 Results in a Phase Delay of FRQ Protein and frq mRNA Rhythms**—As in yeast (40), not1 is an essential gene in Neurospora because not1 knockout strains are not viable. To assess the function of Not1 in the Neurospora circadian clock, we created a dsnot1 strain that carries a construct that allows the expression of dsRNA specific for not1 (5). The dsnot1 construct is under the control of a QA-inducible promoter so that the endogenous not1 mRNA is silenced in the presence of QA (5, 20). As shown in Fig. 2A, no significant difference in conidia banding rhythms between the WT and mutant was observed in the absence of QA treatment on race tubes. The presence of QA in the race tube, however, resulted in dramatic inhibition of cell growth and the loss of circadian conidia rhythm for the dsnot1 strain. These results indicate that Not1 is critical for the cell growth of Neurospora and suggest that it may play a role in the Neurospora circadian clock.

To understand the role of Not1 in the clock, we characterized the circadian rhythms of the dsnot1 strain at the molecular level. As shown by the Western blot analysis results in Fig. 2, B and C, in the presence of QA, both FRQ amounts and its phosphorylation status displayed robust circadian rhythms in both WT and the dsnot1 strains in DD. However, the peak of FRQ protein phase was ~4 h delayed in the dsnot1 strain. After the light-dark transition, FRQ levels decreased in both strains. The newly synthesized FRQ appeared at DD12 in the WT strain, but it was observed at DD16 in the mutant.

We then compared the rhythms of frq mRNA expression in DD between WT and the dsnot1 strain. Similar to the FRQ protein oscillation, frq mRNA remained rhythmic in both strains, but the phase was also delayed in the dsnot1 strain (Fig. 2D). In addition, frq mRNA levels were elevated in the dsnot1
strain, suggesting that WC activity is up-regulated when Not1 is down-regulated.

We noticed that FRQ level at DD0 was higher and that it reached its trough ~4 h later, after the light-to-dark transition in the dsnot1 strain, suggesting that the delayed circadian phase of the dsnot1 strain may be due to its high FRQ levels in LL, which took longer to degrade to restart a new cycle. To confirm this result, we performed a side-by-side comparison of FRQ protein levels in the WT and dsnot1 strains in LL. As shown in Fig. 2E, the FRQ protein level was significantly higher in the mutant than in the WT strain in LL. Thus, the phase delay of the dsnot1 strain should be at least partly due to increased FRQ levels in LL. On the other hand, we found that the levels of FRQ were comparable in DD in the mutant and in the WT strain (Fig. 2B), suggesting that the effect of Not1 on FRQ transcription and the FRQ level is different in LL and DD.

To rule out the possibility that the dsnot1 strain carries another unknown effect that contributes to the phase-delay phenotype in DD, we compared the FRQ oscillation in the dsnot1 strain with or without QA treatments (Fig. 2F). As expected, the phase of FRQ rhythm was delayed in the presence of QA. In the absence of QA, the phase of FRQ rhythm was similar to that in the WT strain. Similarly, the phase of frq mRNA oscillation in the presence of QA was also delayed compared with that without QA (Fig. 2G). These results indicate that the phase delay observed in the dsnot1 strain is indeed caused by the down-regulation of not1 expression. Together, these results indicate that Not1 is important for the phase determination of the Neurospora clock by regulating FRQ levels.

WCC Is Unstable, and Its Activity Is Up-regulated in the dsnot1 Mutant—High levels of frq mRNA and FRQ protein in the dsnot1 strain suggest that the WCC activity is up-regulated. To understand how Not1 regulates WC activity, we first compared WC levels between the WT and dsnot1 strains. In the presence of QA, both WC-1 and WC-2 protein levels were significantly lower in the dsnot1 strain than those in the WT strain (Fig. 3A). In addition, the reduction of WC levels was found to be QA-dependent in the dsnot1 strain (Fig. 3B). Furthermore, quantitative RT-PCR results revealed that levels of wc-1 and wc-2 mRNA were not reduced in the dsnot1 mutant (Fig. 3C), indicating that low levels of WCs in the mutant were due to a posttranscriptional regulation.

To test this possibility, we compared the WC stability in both the WT and mutant strains after the addition of the protein synthesis inhibitor cycloheximide. As shown in Fig. 3D, both WC-1 and WC-2 proteins degraded significantly faster in the dsnot1 strain than in the WT strain. These results indicate that Not1 stabilizes the WC proteins.

Previous studies suggested that low levels of unstable WC proteins are associated with increased WC transcriptional activity (8, 20, 23). To confirm this possibility, we performed a ChIP assay using WC-2-specific antibody. As shown in Fig. 3E, a higher level and delayed WCC binding to the frq promoter was found in the dsnot1 strain, indicating that WCC has a higher frq Clock box binding activity after not1 down-regulation.

Moreover, a Western blot analysis comparing the WC phosphorylation profiles identified that both WC-1 and WC-2 exhibited reduced phosphorylation after the down-regulation of not1 (Fig. 3F). Together, these results indicate that Not1 stabilizes WC proteins and inhibits WCC activity. The down-regulation of not1 results in increased WCC activity and higher FRQ levels, which underlies the delayed phase of circadian rhythms after the light-to-dark transition.

Not1 Associates with Ccr4, and Down-regulation of ccr4 Results in Low Levels of WC Proteins, Delayed Phases, and a Long Period of a Conidiation Banding Rhythm—In yeast, Not1 is part of the Ccr4-Not complex, which contains at least nine proteins (32). To determine whether the role of Not1 in the clock is due to its function in the Ccr4-Not complex, we decided to examine the Ccr4-Not complex in Neurospora. We made two Neurospora strains to identify protein components of the complex, Myc-His-Not1 and Myc-His-Not3, which allowed cross-validation of the results. Not3 (NCU03855) is the Neurospora sequence homolog of the yeast Not3, which is a subunit of the Ccr4-Not complex (32). Myc-Not1 and Myc-Not3 from these strains were purified by tandem affinity purification using a nickel column and c-Myc immunoprecipitation (41). Mass spectrometry analyses from both purifications revealed that Not1, Not3, and the Neurospora sequence homologs of Ccr4, (NCU07779), Caf1 (NCU09001), and Caf4 (NCU07071), were parts of the protein complex under our experimental conditions (Fig. 4A). No significant WC proteins were detected in the purifications, consistent with the fact that the WC-Not1 interaction is substoichiometric.

Because Ccr4 is an important component of the major cytoplasmic mRNA deadenylase in yeast (38, 39), we asked whether Ccr4 is regulated by the clock. Quantitative RT-PCR analysis showed that the level of the ccr4 transcript was rhythmic in DD, with a similar phase as not1 (Fig. 4B). To determine whether ccr4 is a clock-controlled gene (ccg) that is under the direct control of the WC complex, we performed a ChIP assay using WC-2 antibody. As shown in Fig. 4C, WCC bound to the ccr4 promoter rhythmically, peaking at DD14, corresponding to the time of peak ccr4 mRNA. These results suggest that levels of the Neurospora Ccr4-Not complex are regulated by the clock.

In the yeast Ccr4-Not complex, Not1 is the only subunit that is essential for viability (40). However, both Not1 and Ccr4 are indispensable for cell viability in Neurospora. To examine the function of Ccr4, we created a dscrr4 strain in which the expression of crr4 can be inducibly silenced by dsRNA specific for ccr4. Similar to the dsnot1 mutant, both WC-1 and WC-2 levels were lower in the dscrr4 mutant than in the WT (Fig. 4D), indicating that Not1 and Ccr4 function to regulate WC as the Ccr4-Not complex. In addition, silencing of ccr4 resulted in a modest growth inhibition of the dscrr4 strain, more than 3 h of phase delay of the circadian conidiation rhythm following the light-to-dark transition, and a modest increase in period length (WT period, 22.45 h; mutant period, 24.34 h) (Fig. 4E). We noticed that the dscrr4 mutant also had a modestly lengthened period in the absence of QA treatments compared with the WT (WT period, 22.7 h; mutant period, 23.7 h), which is likely due to the leaky expression of the quinic acid promoter that drives the dscrr4 construct (Fig. 4E). The phase delay observed in both dsnot1 and dscrr4 after gene down-regulation demonstrate that...
FIGURE 3. Not1 stabilizes WCs and inhibits its binding to the frq promoter by promoting phosphorylation. A, both WC-1 and WC-2 protein levels are low in the dsnot1 strain. B, QA treatment resulted in low levels of WC-1 and WC-2 in DD in the dsnot1 mutant. C, quantitative RT-PCR results show that the down-regulation of not1 did not reduce the levels of wc-1 and wc-2 mRNA. The error bar represents the S.E. of three independent experiments. D, protein stability was determined by measuring WC levels after the addition of cycloheximide (CHX) (10 μg/ml). The densitometric results are shown in the respective bottom panels. Data are mean ± S.D. n = 3. E, ChIP assay results showing that Not1 inhibits WCC binding to the frq Clock box. Data are mean ± S.D. n = 3. F, down-regulation of not1 resulted in a hypophosphorylation status of WCs.
the Ccr4-Not complex is important for phase determination in the Neurospora circadian clock.

**DISCUSSION**

WCC is the positive element of the Neurospora core circadian oscillator. Previous studies have suggested that transcriptionally active WCCs are unstable and that active WCC leads to low WC levels (8, 10, 19, 20, 23, 42). Both FRQ-dependent and FRQ-independent phosphorylation of WCs inhibit WC activity and are important for maintaining the steady-state WC levels. On the other hand, VVD can physically interact with the light-activated WCC, inhibiting its activity and degradation (24–26).
Role of the Ccr4-Not Complex in the Neurospora Clock

In this study, we identified Neurospora Not1 as a new WC-interacting protein. Down-regulation of not1 results in low levels of WC proteins, high frq mRNA levels, and elevated levels of WC that bind to the frq promoter. These results indicate that Not1 inhibits WCC activity and, thus, stabilizes WC proteins. As a result of high WC activity after down-regulation of not1, the FRQ protein level is higher in LL. Upon transferring into DD, the high level of FRQ takes longer to degrade to its trough level and restart a new circadian cycle, resulting in a phase delay of the circadian clock. These results demonstrate that Not1 contributes to the phase determination of the clock by regulating WC activity. Similar to that seen in Neurospora, in a genomewide RNAi screen in human cells, CNOT1 (the mammalian homolog of the yeast not1) knockdown resulted in a phase delay and low amplitude of clock-controlled luciferase rhythm (43).

Purification of Neurospora Not1 showed that, as in other organisms, Not1 is part of the Ccr4-Not complex. Similar to the knockdown of not1, silencing of ccr4 also caused low levels of WCs and a delayed phase of the circadian conidiation rhythm. These results suggest that Not1 regulates WC activity as part of the Ccr4-Not complex. Because the Ccr4-Not complex is conserved among all eukaryotes, a similar mechanism in clock regulation may also be applied to other organisms.

Not1 was originally identified as a transcriptional repressor in yeast and other organisms (34, 35, 44). However, the underlying mechanism is not known. Although we currently do not know how Not1 regulates WC activity in Neurospora, it is possible that it affects the transcriptional ability of WCC phosphorylated by WC kinases. Consistent with this notion, we found that WCs are hypophosphorylated after down-regulation of not1 (Fig. 3F). The unstable WC and high WC activity in the dson1 strain is reminiscent of the pkac-1KO mutant in which the catalytic subunit of PKA is deleted (20). It was reported that the Ccr4-Not complex components could regulate PKA activity in S. cerevisiae (45), raising the possibility that the Ccr4-Not complex may affect phosphorylation of WC by PKA. Because PKA also stabilizes FRQ in DD (20), a change in PKA activity in the dson1 strain might affect FRQ stability, resulting in levels similar to the WT FRQ levels despite having higher levels of frq mRNA in DD (Fig. 2B).

Protein purification of Not1 showed that it associates with Ccr4, Not3, Caf1, and Caf40 in Neurospora. In yeast and other organisms, Ccr4 functions as a deadenylase that regulates mRNA stability (38, 39, 46), and Caf1 bridges Ccr4 to the rest of the Ccr4-Not complex for integrity of Ccr4 function (47). It is not clear whether Ccr4 is involved in the deadenylation of mRNAs in Neurospora. Because of the association of Not1 with the WCs, it is possible that the Ccr4-Not complex regulates WC activity independently of its mRNA deadenylation.

We showed that both not1 and ccr4 are clock-controlled genes, suggesting that the activity of the complex is rhythmic. Interestingly, the vertebrate ccr4-like gene, Nocturnin (Noc), is also a clock-controlled gene (48), and CLOCK (circadian locomotor output cycles kaput), CYR1, and CBP (CREB-binding protein) could bind to the CNOT1 promoter region in murine liver (49). Similar to Neurospora ccr4, the transcription of the human Noc gene is regulated by CLOCK/BMAL1 (50). The rhythmic expression of not1 and ccr4 in Neurospora raised the possibility that the Ccr4-Not complex may be involved in posttranscriptional regulation of clock-controlled processes.

Acknowledgments—We thank Dr. Jennifer Mohawk and Jeremy Stubblefield for critical comments regarding the manuscript.

REFERENCES

1. Baker, C. L., Loros, J. J., and Dunlap, J. C. (2012) The circadian clock of Neurospora crassa. FEMS Microbiol. Rev. 36, 95–110
2. Bell-Pedersen, D., Cassone, V. M., Earnest, D. J., Golden, S. S., Hardin, P. E., Thomas, T. L., and Zoran, M. J. (2005) Circadian rhythms from multiple oscillators. Lessons from diverse organisms. Nat. Rev. Genet. 6, 544–556
3. Heintzen, C., and Liu, Y. (2007) The Neurospora crassa circadian clock. Adv. Genet. 58, 25–66
4. Dunlap, J. C. (2006) Proteins in the Neurospora circadian clockworks. J. Biol. Chem. 281, 28489–28493
5. Cheng, P., He, Q., He, Q., Wang, L., and Liu, Y. (2005) Regulation of the Neurospora circadian clock by an RNA helicase. Genes Dev. 19, 234–241
6. Crosthwaite, S. K., Dunlap, J. C., and Loros, J. J. (1997) Neurospora WC-1 and WC-2. Transcription, photoresponses, and the origins of circadian rhythm. Science 276, 763–769
7. Froehlich, A. C., Loros, J. J., and Dunlap, J. C. (2003) Rhythmic binding of a WHITE COLLAR-containing complex to the frequency promoter is inhibited by FREQUENCY. Proc. Natl. Acad. Sci. U.S.A. 100, 5914–5919
8. He, Q., Cha, J., He, Q., Lee, H. C., Yang, Y., and Liu, Y. (2006) CK1 and CKII mediate the FREQUENCY-dependent phosphorylation of the WHITE COLLAR complex to close the Neurospora circadian negative feedback loop. Genes Dev. 20, 2552–2565
9. He, Q., Shu, H., Cheng, P., Chen, S., Wang, L., and Liu, Y. (2005) Light-independent phosphorylation of WHITE COLLAR-1 regulates its function in the Neurospora circadian negative feedback loop. J. Biol. Chem. 280, 17526–17532
10. Schafmeier, T., Haase, A., Káldi, K., Scholz, J., Fuchs, M., and Brunner, M. (2005) Transcriptional feedback of Neurospora circadian clock gene by phosphorylation-dependent inactivation of its transcription factor. Cell 123, 235–246
11. Guo, J., Cheng, P., and Liu, Y. (2010) Functional significance of FRH in regulating the phosphorylation and stability of Neurospora circadian clock protein FRQ. J. Biol. Chem. 285, 11508–11515
12. Shi, M., Collett, M., Loros, J. J., and Dunlap, J. C. (2010) FRQ-interacting RNA helicase mediates negative and positive feedback in the Neurospora circadian clock. Genetics 184, 351–361
13. Guo, J., Cheng, P., Yuan, H., and Liu, Y. (2009) The exosome regulates circadian gene expression in a posttranscriptional negative feedback loop. Cell 138, 1236–1246
14. He, Q., Cheng, P., Yang, Y., He, Q., Yu, H., and Liu, Y. (2003) FWD1-mediated degradation of FREQUENCY in Neurospora establishes a conserved mechanism for circadian clock regulation. EMBO J. 22, 4421–4430
15. Tang, C. T., Li, S., Long, C., Cha, J., Huang, G., Li, L., Chen, S., and Liu, Y. (2009) Setting the pace of the Neurospora circadian clock by multiple independent FREQUENCY phosphorylation events. Proc. Natl. Acad. Sci. U.S.A. 106, 10722–10727
16. Yang, Y., Cheng, P., and Liu, Y. (2002) Regulation of the Neurospora circadian clock by casein kinase II. Genes Dev. 16, 994–1006
17. Baker, C. L., Kettenbach, A. N., Loros, J. J., Gerber, S. A., and Dunlap, J. C. (2009) Quantitative proteomics reveals a dynamic interactome and phase-specific phosphorylation in the Neurospora circadian clock. Mol. Cell 34, 354–363
18. Görü, M., Merrow, M., Huttner, B., Johnson, J., Roenneberg, T., and Brunner, M. (2001) A PEST-like element in FREQUENCY determines the length of the circadian period in Neurospora crassa. EMBO J. 20, 7074–7084
19. He, Q., and Liu, Y. (2005) Molecular mechanism of light responses in Neurospora. From light-induced transcription to photoadaptation. Genes Dev. 19, 2888–2899
Role of the Ccr4-Not Complex in the Neurospora Clock

20. Huang, G., Chen, S., Li, S., Cha, J., Long, C., Li, L., He, Q., and Liu, Y. (2007) Protein kinase A and casein kinases mediate sequential phosphorylation events in the circadian negative feedback loop. Genes Dev. 21, 3283–3295
21. Cheng, P., Yang, Y., and Liu, Y. (2001) Interlocked feedback loops contribute to the robustness of the Neurospora circadian clock. Proc. Natl. Acad. Sci. U.S.A. 98, 7408–7413
22. Lee, K., Loros, J. J., and Dunlap, J. C. (2000) Interconnected feedback loops in the Neurospora circadian system. Science 289, 107–110
23. Schafmeier, T., Diernfellner, A., Schäfer, A., Dintsis, O., Neiss, A., and Brunner, M. (2008) Circadian activity and abundance rhythms of the Neurospora clock transcription factor WCC associated with rapid nuclear-cytoplasmic shuttling. Genes Dev. 22, 3397–3402
24. Chen, C. H., DeMay, B. S., Gladfelter, A. S., Dunlap, J. C., and Loros, J. J. (2010) Physical interaction between VIVID and white collar complex regulates photoadaptation in Neurospora. Proc. Natl. Acad. Sci. U.S.A. 107, 16715–16720
25. Hunt, S. M., Thompson, S., Elvin, M., and Heintzen, C. (2010) VIVID interacts with the WHITE COLLAR complex and FREQUENCY-interacting RNA helicase to alter light and clock responses in Neurospora. Proc. Natl. Acad. Sci. U.S.A. 107, 16709–16714
26. Malzahn, E., Ciprianidis, S., Káldi, K., Schafmeier, T., and Brunner, M. (2010) Photoadaptation in Neurospora by competitive interaction of activating and inhibitory LOV domains. Cell 142, 762–772
27. Heintzen, C., Loros, J. J., and Dunlap, J. C. (2001) The PAS protein VIVID defines a clock-associated feedback loop that represses light input, modulates gating, and regulates clock resetting. Cell 104, 453–464
28. Elvin, M., Loros, J. J., Dunlap, J. C., and Heintzen, C. (2005) The PAS/LOV protein VIVID supports a rapidly dampened daytime oscillator that facilitates entrainment of the Neurospora circadian clock. Genes Dev. 19, 2593–2605
29. He, Q., Cheng, P., Yang, Y., Wang, L., Gardiner, K. H., and Liu, Y. (2002) White collar-1, A DNA binding transcription factor and a light sensor. Science 297, 840–843
30. Aronson, B. D., Johnson, K. A., Loros, J. J., and Dunlap, J. C. (1994) Negative feedback defining a circadian clock. Auto-regulation of the clock gene frequency. Science 263, 1578–1584
31. Choudhary, S., Lee, H. C., Maiti, M., He, Q., Cheng, P., Liu, Q., and Liu, Y. (2007) A double-stranded-RNA response program important for RNA interference efficiency. Mol. Cell Biol. 27, 3995–4005
32. Collart, M. A., and Panasenko, O. O. (2012) The Ccr4-not complex. Gene 492, 42–53
33. Miller, J. E., and Reese, J. C. (2012) Ccr4-Not complex. The control freak of eukaryotic cells. Crit. Rev. Biochem. Mol. Biol. 47, 315–333
34. Collart, M. A., and Struhl, K. (1993) CDC39, an essential nuclear protein that negatively regulates transcription and differentially affects the constitutive and inducible HIS3 promoters. EMBO J. 12, 177–186
35. Collart, M. A., and Struhl, K. (1994) NOT1(CDC39), NOT2(CDC36), NOT3, and NOT4 encode a global-negative regulator of transcription that differentially affects TATA-element utilization. Genes Dev. 8, 525–537
36. Albert, T. K., Hanzawa, H., Legtenberg, Y. I., de Ruwe, M. J., van den Heuvel, F. A., Collart, M. A., Boelens, R., and Timmers, H. T. (2002) Identification of a ubiquitin-protein ligase subunit within the CCR4-NOT transcription repressor complex. EMBO J. 21, 355–364
37. Mersman, D. P., Du, H. N., Fingerman, J. M., South, P. F., and Briggs, S. D. (2009) Polyubiquitination of the demethylase Fdh2 controls histone methylation and gene expression. Genes Dev. 23, 951–962
38. Tucker, M., Valencia-Sanchez, M. A., Staples, R. R., Chen, J., Denis, C. L., and Parker, R. (2001) The transcription factor associated Ccr4 and Cof1 proteins are components of the major cytoplasmic mRNA deadenylase in Saccharomyces cerevisiae. Cell 107, 377–386
39. Tucker, M., Staples, R. R., Valencia-Sanchez, M. A., Mulhrad, D., and Parker, R. (2002) Ccr4p is the catalytic subunit of a Ccr4p/Pop2p/Notp mRNA deadenylase complex in Saccharomyces cerevisiae. EMBO J. 21, 1427–1436
40. Maillet, L., Tu, C., Hong, Y. K., Shuster, E. O., and Collart, M. A. (2000) The essential function of Not1 lies within the Ccr4-Not complex. J. Mol. Biol. 303, 131–143
41. He, Q., Cheng, P., He, Q., and Liu, Y. (2005) The COP9 signalosome regulates the Neurospora circadian clock by controlling the stability of the SCFFWD-1 complex. Genes Dev. 19, 1518–1531
42. Hong, C. I., Ruoff, P., Loros, J. J., and Dunlap, J. C. (2008) Closing the circadian negative feedback loop. FRQ-dependent clearance of WC-1 from the nucleus. Genes Dev. 22, 3196–3204
43. Zhang, E. E., Liu, A. C., Hirota, T., Miraglia, L. J., Welch, G., Pongsawatkul, P. Y., Liu, X., Atwood, A., Huss, J. W., 3rd, Jones, J., Su, A. I., Hognesch, J. B., and Kay, S. A. (2009) A genome-wide RNAi screen for modifiers of the circadian clock in human cells. Cell 139, 199–210
44. Winkler, G. S., Mulder, K. W., Bardwell, V. J., Kalkhoven, E., and Timmers, H. T. (2006) Human Ccr4-Not complex is a ligand-dependent repressor of nuclear receptor-mediated transcription. EMBO J. 25, 3089–3099
45. Lenssen, E., Oberholzer, U., Labarre, J., De Virgilio, C., and Collart, M. A. (2002) Saccharomyces cerevisiae Ccr4-Not complex contributes to the control of Msn2p-dependent transcription by the Ras/cAMP pathway. Mol. Microbiol. 43, 1023–1037
46. Chen, J., Chiang, Y. C., and Denis, C. L. (2002) CCR4, a 3'-5' poly(A) RNA and ssDNA exonuclease, is the catalytic component of the cytoplasmic deadenylase. EMBO J. 21, 1414–1426
47. Goldstrohm, A. C., Seay, D. J., Hook, B. A., and Wicksens, M. (2007) PUF protein-mediated deadenylation is catalyzed by Ccr4p. J. Biol. Chem. 282, 109–114
48. Green, C. B., and Besharse, J. C. (1996) Identification of a novel vertebrate circadian clock-regulated gene encoding the protein nocturnin. Proc. Natl. Acad. Sci. U.S.A. 93, 14884–14888
49. Koike, N., Yoo, S. H., Huang, H. C., Kumar, V., Lee, C., Kim, T. K., and Takahashi, J. S. (2012) Transcriptional architecture and chromatin landscape of the core circadian clock in mammals. Science 338, 349–354
50. Li, R., Yue, J., Zhang, Y., Zhou, L., Hao, W., Yuan, J., Qiang, B., Ding, J. M., Peng, X., and Cao, J. M. (2008) CLOCK/BMAL1 regulates human noc-tin gene transcription through binding to the E-box of nocturnin promoter. Mol. Cell Biochem. 317, 169–177