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Methods to study the mechanism of the *Neurospora* Circadian Clock

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Abstract

Eukaryotic circadian clocks are comprised of interlocked auto-regulatory feedback loops that control gene expression at the levels of transcription and translation. The filamentous fungus *Neurospora crassa* is an excellent model for the complex molecular network of regulatory mechanisms that are common to all eukaryotes. In the heart of the network, post-translational regulations and functions of the core clock elements are of major interest. This chapter will discuss the methods that were recently used to study the *Neurospora* circadian oscillator mechanisms at the molecular level.

Introduction

Circadian clocks are self-sustaining timekeepers found in almost all organisms on earth (Dunlap 1999, Young and Kay 2001). Eukaryotic circadian oscillators employ complex networks of molecules to form interlocked feedback loops. Despite the evolutionary distance, the mechanism of circadian oscillator of the filamentous fungus *Neurospora crassa* is very similar to those of higher eukaryotes and *Neurospora* has served as an outstanding model organism for the field (Liu and Bell-Pedersen 2006, Heintzen and Liu 2007). Working with fungi is relatively simple, and analysis of this organism allowed identification of clock components and mechanisms through genetic, biochemical and molecular biological studies. Furthermore, the availability of a whole genome knockout collection and a bioluminescence reporter provided technical versatility that enabled dissection of the *Neurospora* circadian clock (Colot, Park, Turner, Ringelberg, Crew, Litvinkova et al. 2006, Gooch, Mehra, Larrondo, Fox, Touroutoudis, Loros et al. 2008).

In the *Neurospora* circadian clock, FREQUENCY (FRQ) forms the FFC complex with its partner FRQ-interacting RNA helicase (FRH) to function as the negative limb in the core negative feedback loop (Aronson, Johnson, Loros and Dunlap 1994, Cheng, He, He, Wang and Liu 2005). The transcription of *frq* gene is activated by the positive element, WHITE COLLAR complex (WCC), which is formed by two PER-ARNT-SIM (PAS) domain-containing transcription factors WC-1 and WC-2 (Crosthwaite, Dunlap and Loros 1997, Cheng, Yang, Gardner and Liu 2002, Cheng, Yang, Wang, He and Liu 2003, He and Liu

2005). Circadian expression of *frq* gene is achieved by rhythmic binding of WCC to its promoter and requires timely modulation of chromatin structure by multiple factors (Froehlich, Loros and Dunlap 2003, Belden, Loros and Dunlap 2007, Belden, Lewis, Selker, Loros and Dunlap 2011, Cha, Zhou and Liu 2013). It was also recently shown that RCO-1-mediated suppression of WC-independent transcription of *frq* is essential for the clock function (Zhou, Liu, Hu, Zhang, Sun, Cha et al. 2013).

FFC interacts with WCC to promote phosphorylation of WCs; this phosphorylation is primed by PKA in an FRQ-independent manner (Schafmeier, Haase, Kaldi, Scholz, Fuchs and Brunner 2005, He, Cha, Lee, Yang and Liu 2006, Huang, Chen, Li, Cha, Long, Li et al. 2007). The inhibition of WCC by FFC is the critical step in the circadian negative feedback, and its mechanism involves not only physical interactions but also enzymatic reactions to modify WC proteins post-translationally. Phosphorylations stabilize WCs, decrease their DNA binding ability, and result in export of these proteins to the cytoplasm (He, Shu, Cheng, Chen, Wang and Liu 2005, Cha, Chang, Huang, Cheng and Liu 2008, Hong, Ruoff, Loros and Dunlap 2008, Diernfellner, Querfurth, Salazar, Hofer and Brunner 2009). In addition to the well-known circadian oscillations of *frq* mRNA and FRQ protein, WCC phosphorylation status, occupancies on the *frq* promoter, and nuclear localization all display circadian rhythms in constant darkness. Protein phosphatases PP2A and PP4 are known to counterbalance the kinases to regulate the timely reactivation and relocation of WCC for the new cycle (Yang, He, Cheng, Wrage, Yarden and Liu 2004, Cha et al. 2008, Diernfellner et al. 2009).

After its synthesis, FRQ forms a homodimer and interacts with FRH, which is important for the stability and proper structure of FRQ (Cheng et al. 2005, Guo, Cheng, Yuan and Liu 2009, Guo, Cheng and Liu 2010, Shi, Collett, Loros and Dunlap 2010, Cha, Yuan and Liu 2011). Like its animal homolog PERIOD (Per), FRQ is progressively phosphorylated by kinases through the subjective day and evening, leading to its extensive phosphorylation and degradation by the ubiquitin/proteasome pathway (Liu, Loros and Dunlap 2000, Gorl, Merrow, Huttner, Johnson, Roenneberg and Brunner 2001, Yang, Cheng, Zhi and Liu 2001, He, Cheng, Yang, He, Yu and Liu 2003, Yang, Cheng, He, Wang and Liu 2003, He, Cheng, He and Liu 2005, He and Liu 2005, He et al. 2006, Pregueiro, Liu, Baker, Dunlap and Loros 2006). FWD-1, the E3 ubiquitin ligase for FRQ, recognizes phosphorylated FRQ and facilitates its degradation, whereas the COP9 signalosome regulates the activity and stability of SCF^{FWD-1} complex to indirectly affect FRQ stability. Protein Kinase A (PKA) also phosphorylates FRQ, but in contrast to the other kinases, it stabilizes FRQ; dephosphorylations of FRQ by PP1, PP2A, and PP4 are also important for its stability (Yang et al. 2004, Huang et al. 2007, Cha et al. 2008). We and others have identified more than 100 phosphorylation sites on FRQ by analyzing *in vitro* phosphorylations by casein kinases and by purification of phosphorylated FRQ from *Neurospora* (Baker, Kettenbach, Loros, Gerber and Dunlap 2009, Tang, Li, Long, Cha, Huang, Li et al. 2009). Extensive phosphorylation of FRQ may change the protein conformation allowing better access by SCF^{FWD-1}, and the presence of multiple motifs that interact with CKI suggest that conformational changes facilitate the degradation of FRQ (Querfurth, Diernfellner, Gin, Malzahn, Hofer and Brunner 2011). The phosphorylation of FRQ also modulates its

interactions with other proteins to affect its function in the negative feedback loop (Cha et al. 2011). Thus, the phosphorylation of FRQ is crucial for regulating the circadian negative feedback loop, and these modifications are fine-tuned by a series of regulators to determine FRQ stability and the period length of the clock.

Combinations of biochemical, genetic, and molecular approaches were used effectively to study the mechanisms of circadian clock (Liu 2005). Here we will describe some of newly developed methods used in the studies of the *Neurospora* circadian clock. These techniques may be useful for future studies and in other model organisms.

Description of Methods

Purification of epitope-tagged protein and interacting partners from *Neurospora* extracts

Purification of a protein of interest enables investigation of the molecular context of its cellular function by allowing identification of putative interaction partners. Epitope-tagged proteins can avoid the difficulty of purification of endogenous protein. We have successfully used a tandem affinity tag made of c-Myc and 6-His to demonstrate the interactions of clock proteins and to perform biochemical assays of the purified enzymes. The construction of tandem repeats was previously described (He et al. 2005), and the strain of interest can be generated by using the homologous recombination of the transgene expressing the epitope-tagged protein at the *his-3* locus.

To set up an affinity column for the 6x His-tag, wash an empty column with MilliQ water and add desired amount of Ni-NTA beads (Qiagen) or modified metal affinity resin such as TALON (Clontech). Let the column stand for 20–25 min, so that all beads sediment to the bottom. Wash the column with 10 column volumes (CVs) of MilliQ water and then with 10 CVs of extraction buffer (50 mM HEPES, pH 7.4, 137 mM NaCl, 10% glycerol (v/v)). Protease inhibitors (1 mg/mL pepstatin A, 1 mg/mL leupeptin, and 100 mM phenylmethylsulphonyl fluoride) should be added to this buffer just before protein extraction. After harvesting the *Neurospora* mycelia cultured in a desired way, it is grinded into fine powder in liquid nitrogen. Keeping the mortar and pestle cold is absolutely necessary to prevent denaturation of sensitive proteins during purification. Add 3 volumes of extraction buffer to the powder, mix well by vortexing with frequent chilling on ice, and centrifuge at 12,000 rpm at 4 °C for 15 min. Transfer the supernatant to fresh tube and centrifuge again (45,000 rpm, 4 °C, 30 min). Transfer the supernatant to a clean tube, measure the protein concentration, dilute to 2–4 mg/ml, and add imidazole to 0–20 mM depending on the target proteins. Load the extract onto the column and elute at a flow rate of around 1 ml/min. Collect the flow-through. Wash the column with 10 CVs of extraction buffer (salt concentration may need to be adjusted to improve the purity), and elute the attached proteins with 1 CV of elution buffer (50 mM HEPES, pH 7.4, 137 mM NaCl, 200 mM imidazole, and 20% glycerol (v/v)); this may require five to seven washes with elution buffer. Each fraction should be analyzed by western blot using anti-c-Myc antibody, and the most enriched fractions should be combined for the immunoprecipitation (IP) step. For IP, mix 1 ml of eluted fraction with 40 µl anti-c-Myc agarose beads (10 µl beads; Santa Cruz Biotechnology), and incubate on a rotator at 4 °C for 4 h. Collect beads by centrifugation (4,000 rpm, 4 °C, 1 min), and wash the beads with 1 ml of high salt buffer (20 mM Tris-

HCl, pH 7.5, 500 mM NaCl) and then with low salt buffer (20 mM Tris-HCl, pH 7.5, 50 mM NaCl).

The beads should be resuspended in desired buffer: appropriate assay buffer for enzyme assays or 1× SDS-PAGE loading buffer to run a gel. To identify interacting proteins, the eluted proteins are separated by a 4–15% gradient gel (Bio-Rad) and stain the gel with silver (GE Healthcare). Cut out the bands of interest and elute/digest proteins from the gel for mass spectrometry (MS) analysis using protocols suggested by the MS operator.

Identification of phosphorylated residues of clock proteins

As mentioned earlier, FRQ is progressively and extensively phosphorylated over circadian time. To identify phosphorylated residues, we analyzed both *in vitro* and *in vivo* phosphorylated FRQ samples by MS and concluded that CKI and CKII are the major kinases that mediate most of the phosphorylations on FRQ (Tang et al. 2009). Furthermore, quantitative methods were used to reveal the preferentially phosphorylated residues in the hyperphosphorylated FRQ species, which are the important signals for FRQ degradation.

Mapping in vitro phosphorylation sites—His-tagged full-length FRQ was expressed and purified from Sf9 cells. CK-1a and CKA were also His-tagged at their N-termini, expressed in *E. coli*, and purified by Ni-NTA column. To perform *in vitro* phosphorylation, 6–8 µg of FRQ protein were incubated with 1–2 µg of kinase(s) in phosphorylation buffer (25 mM HEPES, pH 7.9, 10 mM MgCl₂, 2 mM MnCl₂, 25 µM ATP, and 10 µCi/ml [γ -³²P]ATP) at 37 °C for 2–4 h and then subjected to SDS-PAGE. After electrophoresis, the bands were visualized by colloidal blue staining. FRQ bands were excised and analyzed by MS.

Mapping in vivo phosphorylation sites—To purify FRQ from *Neurospora* for mapping of the *in vivo* phosphorylation sites, we inserted 5x c-Myc and 9x His tags at the C-terminus of FRQ. The construct was transformed into a *frq*-null strain and an *fwd-1^{RIP}* strain. The latter displays elevated levels of FRQ protein in hyperphosphorylated forms. Epitope-tagged FRQ was purified to near homogeneity and the bands corresponding to FRQ were excised from the gel for trypsin digestion and MS analyses.

MS analyses—Protein was digested in gel with 10 ng/L sequencing-grade trypsin in 50 mM NH₄HCO₃ (pH 7.8) at 37 °C overnight. The resulting peptides were extracted sequentially with 5% formic acid/50% acetonitrile and 0.1% formic acid/75% acetonitrile and concentrated to about 10 µl for the following steps. The trypsin-digested peptides were loaded onto a pre-column packed with 5–15 µm spherical C18, reversed-phase particles (YMC). The pre-column was connected by a piece of Teflon tubing to a homemade analytical column packed with YMC, 5-µm spherical, C18 reversed-phase particles. The eluted peptides were sprayed directly into a QSTAR XL mass spectrometer (MDS SCIEX) equipped with a nano-ESI ion source. The spectra were collected in Information Dependent Acquisition mode. Spray voltage was 2.1 kV, MS scan range was 400–2,000 Da, resolution was low for precursor ion isolation, and the top 3 most abundant precursor ions were selected for MS/MS scans with enhance-all mode. Database searches were performed using

an in-house Mascot server. After the database searches, all of the recognized phosphopeptides were manually checked to exclude false-positives.

Quantitative MS—To understand how the extensive phosphorylations on FRQ are regulated in a circadian cycle or to distinguish FRQ-dependent and from FRQ-independent phosphorylations on WCs, we developed a quantitative method of MS employing whole-cell ^{15}N metabolic labeling in *Neurospora* (Huang et al. 2007, Tang et al. 2009). For the purification of Myc-His-FRQ for the quantitative MS experiments, NH_4Cl or $^{15}\text{NH}_4\text{Cl}$ (Cambridge Isotope Labs) was used to replace NH_4NO_3 in Vogel's medium. Unlabeled or labeled cultures of 2 to 3 L were used to prepare extracts, and both extracts were mixed in equal protein amounts before the purification step. Epitope-tagged FRQ was analyzed by MS, and the resulting files from the Mascot search were imported into the open-source software MSQuant (<http://msquant.sourceforge.net>). The ^{15}N -labeled and unlabeled peptide pairs were recognized automatically by MSQuant based on the information from Mascot search results and their differences in mass-to-charge ratios. Peptide ratios were obtained by calculating the extracted ion chromatograms of the peptide pairs for quantification, and the results were also manually verified. The average ^{15}N to ^{14}N ratios of unphosphorylated peptides were used as the correction factor to determine the ratios of the phosphorylated peptides.

Isolation of *Neurospora* nuclei to analyze localization of clock proteins

Post-translational regulations of WCs and FRQ are involved in the mechanism controlling the nucleo-cytoplasmic trafficking of these proteins (Luo, Loros and Dunlap 1998, Cha et al. 2008, Hong et al. 2008, Schafmeier, Diernfellner, Schafer, Dintsis, Neiss and Brunner 2008, Diernfellner et al. 2009, Cha et al. 2011). Given that the transcriptional activation of *frq* by WCC and the FRQ-mediated inhibition of DNA-binding are essential for the circadian negative feedback loops, analyses of nuclear localization of clock proteins are critical to our understanding of the molecular mechanisms of the clock.

To prepare nuclear extract, grind frozen *Neurospora* cells with acid-washed glass beads (Sigma-Aldrich) in liquid nitrogen. We used equal weights of glass beads and dehydrated tissues. Slowly pour the cell powder into 10 ml of buffer A (1 M sorbitol, 7% Ficoll, 20% glycerol, 5 mM magnesium acetate, 3 mM CaCl_2 , 50 mM Tris-HCl, pH 7.5) on ice while stirring. Filter the resuspended sample through cheesecloth (pre-wet with buffer A) into a fresh flask on ice. Add 2 volumes of cold buffer B (10% glycerol, 5 mM magnesium acetate, 25 mM Tris-HCl, pH 7.5) slowly with gentle shaking on ice. Layer the mixed solution onto the bed of 10 ml of cold buffer A/B (4:6.6) in the centrifuge tube, and centrifuge (3,000 g, 4 °C, 7 min). Layer the supernatant (total extract) onto a bed of 5 ml of buffer D (1 M sucrose, 10% glycerol, 5 mM magnesium acetate, 25 mM Tris-HCl, pH 7.5) and centrifuge (9,400 g, 4 °C, 15 min). Discard the supernatant (cytosolic fraction), and resuspend the pellet (nuclear fraction) in half volume of buffer D. Add same volume of the extraction buffer and sonicate briefly to disrupt the nuclear membrane. Centrifuge (12,000 rpm, 4 °C, 15 min); resolve the supernatant by SDS-PAGE and analyze by western blot.

Chromatin immunoprecipitation (ChIP)

The association of transcription factors, histones, and RNA polymerase II with target sites can provide important information on how they regulate gene expression. WCC rhythmically binds to the *frq* promoter to drive its circadian transcription (Froehlich et al. 2003, He et al. 2006). CSW-1 and CATP are required to generate a circadian rhythm of chromatin state at the *frq* locus to ensure proper WCC-driven transcription (Belden et al. 2007, Cha et al. 2013). The ChIP assay has been widely used to determine whether the protein of interest is associated with a specific genomic region in the cell. We analyzed formaldehyde-fixed chromatin for the occupancies of WCC and modified histones at the *frq* gene and other clock-controlled gene loci.

To crosslink the proteins with the chromatin, add 1% formaldehyde directly into the liquid culture and incubate 15 min. To stop the crosslinking, add 125 mM glycine (pH 7.5) and incubate 5 min. Then wash the mycelia by transferring to wash buffer (50 mM HEPES, pH 7.5, 137 mM NaCl). Harvest the culture, and grind cells in liquid nitrogen. Add 1 ml of lysis buffer (50 mM HEPES, pH 7.5, 137 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% deoxycholate, 0.1% SDS) with protease inhibitors to around 200 µl of cell powder, and mix thoroughly. Sonicate the chromatin using Bioruptor (Diagenode) for 15 min with a 30 s:30 s cycle. The sonication conditions should be adjusted based on the size of the sheared chromatin. We obtained <200-bp fragments with the conditions above. Centrifuge (10,000 g, 4 °C, 15 min), and transfer the supernatant to the fresh tubes. Measure the protein concentration, and dilute 1 mg protein into 0.5 ml lysis buffer, add pre-blocked Gammabind G Sepharose (GE Healthcare; incubated with 100 mg/ml BSA and 100 mg/ml salmon sperm DNA at 4°C with rotation for 4 h), and incubate at 4 °C for 1 h to remove the non-specific binding proteins. Spin down the beads (4,000 rpm, 2 min), and transfer the supernatant to the fresh tubes. 50 µl can be removed for the ‘input’, and add appropriate antibody to the remaining chromatin. Incubate at 4°C for 4 h (or overnight), add 40 µl (10 µl beads only) Gammabind G Sepharose, and incubate for 1 h. Collect the beads (4,000 rpm, 2 min), and wash the beads sequentially with low salt washing buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 150 mM NaCl, 20 mM Tris-HCl, pH 8), high salt washing buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 500 mM NaCl, 20 mM Tris-HCl, pH 8), LND buffer (0.25 M LiCl, 1% NP40, 0.1% deoxycholate, 1 mM EDTA, 10 mM Tris-HCl, pH 8) and TE buffer (1 mM EDTA, 10 mM Tris-HCl, pH 8).

To elute DNA from the ChIP samples, we use Chelex 100 resin (Bio-Rad) and incubated at 94 °C for 15 min. Chill the samples on ice and then spin down (1,000 g, 1 min); the supernatant is the ChIP DNA. ‘Input’ samples were incubated at 65 °C to reverse the crosslink, and DNAs were extracted using phenol/chloroform. Quantitative real-time PCR (qPCR) was used to quantify the enrichment of the target site, and the efficiency of primers should be verified by analysis of a dilution series of input DNAs.

Monitoring bioluminescence reporter expression during the circadian cycle

Codon-optimized luciferase has been successfully used as a bioluminescence reporter in *Neurospora* (Gooch et al. 2008). Luciferase reporter activity can be greatly improved by codon optimization of the bacterial luciferase gene based on the codon usage of *Neurospora*.

With this optimization, the light produced from expression driven by a highly expressed promoter can be visible to naked eyes. *frq*-promoter-driven luciferase activity rhythm can be monitored in the conventional race tube assay setting. A liquid nitrogen-cooled or electronically cooled camera can be used to capture the image of the race tube every 10 to 30 min over the time course, and the time-lapse video shows an oscillating intensity of luminescence. For the quantitative analysis, the region of interest – which can be the whole tube or a small part of the growth front – can be analyzed from the time lapse images, and the period of different strains can be compared with or without the presence of conidial banding of the strains. Thus, the circadian rhythms of the strains defective in conidiation can be monitored on this relatively simple platform, which is a huge advantage given that the sexual crosses necessary to obtain the *bd* strain can take more than 4 weeks and sexual reproduction of some strains may be very difficult or impossible.

We also constructed a *pfrq-luc-I* (the precursor was a generous gift from Dr. Jay Dunlap) reporter in a plasmid that contains the *basta/ignite resistance (bar)* gene that can be used to transform *his*⁺ strains. Instead of using the race tube setting, the transformants from either *his-3* or *bar* transformation are inoculated on autoclaved FGS-Vogel's (AFV) media (0.05% fructose, 0.05% glucose, 2% sorbose, 1×Vogel's, 50 µg/L biotin, and 1.8% agar) containing 50 µM firefly luciferin (BioSynt). The culture is incubated in constant light overnight and then transferred to LumiCycle (Actimetrics), which has four independent photomultiplier tubes (PMTs) to record data on 32 samples simultaneously. The data are analyzed using LumiCycle Analysis software supplied by the manufacturer. The activity curve is normalized by subtracting the baseline luciferase signal which changes as cells grow.

The *frq*-driven reporter construct has been modified to perform additional assays. The translational fusion of luciferase to the FRQ ORF displays a rhythm as robust as that of the transcriptional reporter and expands the application of the reporter to the analysis of post-translational regulations on FRQ protein (Larrondo, Loros and Dunlap 2012). More recently, it was shown that introduction of PEST sequence of FRQ, which contains the major phosphorylation sites to induce FRQ degradation, significantly destabilized the luciferase protein (Cesbron, Brunner and Diernfellner 2013). The destabilized luciferase reporter reveals rapid luciferase activity changes that otherwise would be masked by the long-lasting reporter activity. Furthermore, the development of 96-well plate-based high-throughput platform can accelerate the high-throughput analysis of circadian promoters or even replace RNA analyses, which demands more time and suffers from lack of reproducibility.

Analysis of protein conformation changes by limited digestion and freeze-thaw cycles

It has been previously suggested that the conformation adopted by FRQ affects its stability (Guo, Cheng and Liu 2010, Querfurth, Diernfellner, Gin, Malzahn, Höfer and Brunner 2011). A growing number of proteases are available with varied amino acid recognition sequences that can be used for digestion analyses to probe protein structure changes. For example, trypsin cuts after the amino acids arginine and lysine, and the environment in which these sites reside (loose or tight, exposed or buried structures) impacts the trypsin sensitivity of the protein. Other commercially available proteases have different preferential amino acid sequences. Repeated freeze-thaw cycles are also known to destroy proteins,

probably through irreversible denaturation and loss of secondary and tertiary structures (Gabellieri 1996, Zhang, Qi, Singh and Fernandez 2011). We and others have used these methods to demonstrate that the post-translational regulation or changes to codons in the *frq* ORF affect conformation and thereby change the protein stability (Querfurth et al. 2011, Zhou, Guo, Cha, Chae, Chen, Barral et al. 2013).

Limited protease digestion—Prepare *Neurospora* protein extracts in extraction buffer (50 mM HEPES, pH 7.4, 137 mM NaCl, and 10% glycerol, no protease inhibitors). Dilute the protein extract with extraction buffer to a total protein concentration of 2.5 µg/µl. Treat 100 µl extract with trypsin (final concentration depends on protein, ~0.3 µg/ml for wild-type FRQ is recommended) at 25 °C in a water bath with gentle shaking. A 20 µl sample is taken from the reaction at each time point (0, 5, 15 and 30 min) after addition of trypsin. Each 20-µl sample is then mixed with protein loading buffer, and protein samples are resolved on a 12.5% SDS-PAGE gel. Western blot is performed to examine the target protein levels at each time point. ImageJ is used to quantify the results and plot the degradation curve.

A similar method can be also applied with C-terminal TAP-tagged FRQ protein purified from *Neurospora* culture in different phosphorylation states (Querfurth et al. 2011). After purification using a tandem affinity column, treat the same amount of hypo- and hyperphosphorylated FRQ with 30 ng of Factor Xa protease (New England Biolabs) at 20 °C for 10 min. Stop the reaction by adding SDS-PAGE sample buffer and boiling at 95 °C for 3 min, and subject the samples to SDS-PAGE. The pattern of digested fragments can be visualized by western blot using a polyclonal anti-FRQ antibody.

Freeze-thaw assay—Prepare *Neurospora* protein extracts and dilute to a total protein concentration of 2.5 µg/µl (the same extraction buffer described in the *Limited trypsin digestion assay* section is used). For each culture, 200 µl protein extract is used in this assay. In each freeze-thaw cycle, the extract is snap frozen by immersion in liquid nitrogen, and thawed at 37 °C for 15 min. A 20-µl sample is taken from the extract before the first freeze-thaw, and after the second and fourth freeze-thaw cycles, respectively. If the target protein is quite stable, additional freeze-thaw cycles should be used. Each 20-µl sample is mixed with protein loading buffer, and subjected to SDS-PAGE and western blot analysis (Zhou et al. 2013).

Concluding Remarks

Neurospora has been an outstanding model organism for circadian clock research and enables use of a wide range of methodologies to study the molecular mechanisms of eukaryotic circadian clocks. Various techniques have been used to identify the mechanisms that are critical for the core negative feedback loop. These mechanisms regulate *frq* expression and FRQ activity and stability at transcriptional, post-transcriptional, co-translational, and post-translational levels. The knowledge provided from the *Neurospora* studies has helped us understand how the circadian period length and clock entrainment are determined at the molecular level. The remarkable similarities between the *Neurospora* clock and clocks of higher eukaryotes can help us tackle more complex problems in animal and plant clocks.

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