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The Genetics of Circadian Rhythms in *Neurospora*

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Abstract

This chapter describes our current understanding of the genetics of the *Neurospora* clock and summarizes the important findings in this area in the past decade. *Neurospora* is the most intensively studied clock system, and the reasons for this are listed. A discussion of the genetic interactions between clock mutants is included, highlighting the utility of dissecting complex mechanisms by genetic means. The molecular details of the *Neurospora* circadian clock mechanism are described, as well as the mutations that affect the key clock proteins, FRQ, WC-1, and WC-2, with an emphasis on the roles of protein phosphorylation. Studies on additional genes affecting clock properties are described and place these genes into two categories: those that affect the FRQ/WCC oscillator and those that do not. A discussion of temperature compensation and the mutants affecting this property is included. A section is devoted to the observations pertinent to the existence of other oscillators in this organism with respect to their properties, their effects, and their preliminary characterization. The output of the clock and the control of clock-controlled genes are discussed, emphasizing the phasing of these genes and the layers of control. In conclusion, the authors provide an outlook summarizing their suggestions for areas that would be fruitful for further exploration.

I. INTRODUCTION

A. The *Neurospora* clock system

Neurospora has been one of the primary organisms for circadian research for many years, and work on the *Neurospora* circadian system has often been reviewed (Brunner and Kaldi, 2008; Dunlap and Loros, 2006; Heintzen and Liu, 2007; Lakin-Thomas and Brody, 2004; Liu and Bell-Pedersen, 2006; Vitalini *et al.*, 2010). One output of the circadian system, which is easily assayed under constant environmental conditions (usually constant darkness and constant temperature), is the daily rhythm of conidiation (asexual spore formation) on agar growth medium: the conidiation rhythm is seen as a pattern of areas of thick conidiating growth (bands) alternating with thinner areas of growth (interbands). These areas are formed as the endogenous clock directs the growth front to produce primarily apical extensions of the filamentous mycelium for approximately 11 h (forming an “interband”) followed by approximately 11 h of extension of the apical filaments plus the production of aerial hyphae at right angles to the surface of the medium on which the spores develop (forming a “band” region). This “fossil record” of the developmental state of the growth front can be seen on

agar medium in petri plates (Fig. 3.1) or in long glass cylindrical tubes (“growth tubes” or “race tubes”). This conidiation rhythm has been the standard assay for the state of the circadian system in labs working with *Neurospora*. All known clock genes were identified and/or characterized by observing changes in the conidiation rhythm. Molecular rhythms can also be observed in the *Neurospora* system and are most often assayed in specialized cultures in which mycelial disks are submerged in liquid medium (Loros *et al.*, 1989; Nakashima, 1981). Conidiation is suppressed to some extent by these culture conditions, but the underlying clock mechanisms continue functioning. Molecular rhythms can also be assayed on solid agar medium in conditions more physiologically similar to those the organism would encounter in nature (Gooch *et al.*, 2008; Ramsdale and Lakin-Thomas, 2000; Schneider *et al.*, 2009).

This review focuses on what genetics has told us about the mechanism of the underlying circadian oscillator that drives the observed rhythms, with an emphasis on findings within the past 10 years. We will also consider one aspect of the output of the oscillator: the downstream effects of the clock on the rhythmic control of the expression of “clock-controlled genes,” or ccgs. Circadian systems must also have input pathways to synchronize the clock with the external environment, but a consideration of input pathway genetics is beyond the scope of this review; the reader is directed to recent reviews (Chen *et al.*, 2010; Liu, 2003; Price-Lloyd *et al.*, 2005).

B. *Neurospora* as a model organism for circadian research

The circadian system of *Neurospora* is arguably the most intensively studied of all clocks and has many advantages as an experimental system. As a microorganism that is easily grown in culture, *Neurospora* has an advantage over other organisms in that large amounts of material can be synchronously harvested, making biochemical analysis easier. As a haploid organism, *Neurospora* has advantages for genetic analysis in the ease of isolation of new mutations and the expression of mutant phenotypes in the haploid without the complications of dominance relationships between alleles in a diploid. This has resulted in a huge collection of mutant strains, cataloged by (Perkins *et al.*, 2001), now available online (<http://www.fgsc.net/2000compendium/NewCompend.html>), and continuously updated (http://bmbpcu36.leeds.ac.uk/~gen6ar/newgenelist/genes/gene_list.htm). The genome has been sequenced (Galagan *et al.*, 2003), transformation methods are routine, and most molecular genetics methods are available for *Neurospora* including the use of an inducible promoter for dosage control (Campbell *et al.*, 1994; Geever *et al.*, 1989) and RNAi for gene silencing (Ziv and Yarden, 2010). Modern imaging techniques can be applied to *Neurospora*, including the use of luciferase (Gooch *et al.*, 2008), GFP (Freitag *et al.*, 2004), and mCherry (Castro-Longoria *et al.*, 2010). Knockout mutants can be easily constructed (Colot *et al.*, 2006), and knockouts are available for most identified genes from the Fungal Genetics Stock Center (McCluskey, 2003).

Neurospora has an 80-year history as a lab organism (Perkins and Davis, 2000), and the first report of circadian rhythmicity in *Neurospora* was published in 1959 (Pittendrigh *et al.*, 1959). Classic circadian experiments describing the response of the *Neurospora* clock to pulses and steps of light and temperature were published in the 1960s and 1970s (Francis

and Sargent, 1979; Sargent and Briggs, 1967). The primary output, the conidiation developmental pathway, has been extensively studied (Correa and Bell-Pedersen, 2002; Springer, 1993), and the characterization of ccgs at the molecular level is underway (see Section VI). The development of a transcription/translation feedback model to describe the mechanism of the *Neurospora* circadian oscillator (see Section III) has contributed to the development of similar models for other organisms and the finding of analogous genes in other clock systems (see Chapters 2, 4, 5, 6 and 7 in this volume). *Neurospora* was also one of the pioneer lab organisms for biochemical investigations (Davis, 2000), and there is a wealth of information about metabolic pathways, organelles such as mitochondria, signaling pathways, etc.; as the attention of circadian researchers shifts away from transcription/translation mechanisms and toward metabolic pathways (Bass and Takahashi, 2010; Harrisingh and Nitabach, 2008; Hastings *et al.*, 2008), *Neurospora* will again prove its worth as a model organism.

II. EARLY GENETIC ANALYSIS

A. Identification of clock mutants

Neurospora was the second organism, after *Drosophila* (Konopka and Benzer, 1971), in which mutations affecting the circadian clock were isolated (Feldman and Hoyle, 1973). These were identified in brute force screenings for period changes as observed in the conidiation rhythm on solid agar medium. The first locus identified in these screens, *frequency* (*frq*), also turned out to be of extraordinary interest. Alleles with both shorter periods (*frq*¹, *frq*²) and longer periods (*frq*³, *frq*⁷) were found at the same locus (see Table 3.1 for periods). These were found to be point mutations, introducing single amino acid changes (Aronson *et al.*, 1994a), and were shown to be codominant in heterokaryons. Null alleles, either a truncated protein (*frq*⁹) (Loros and Feldman, 1986) or an engineered gene deletion (*frq*¹⁰) (Aronson *et al.*, 1994a), were found to be “conditionally rhythmic” (see Section V) and recessive in heterokaryons. A great deal of subsequent work has been carried out on the biochemistry and genetics of the *frq* gene and is summarized below (Section III).

A number of other clock-affecting loci were identified in screens for period mutants, including five *prd* (period) loci (*prd-1*, *prd-2*, *prd-3*, *prd-4*, and *prd-6*) and *chr* (chrono). Additional period-affecting mutations were identified by screening existing libraries of mutants, including several amino acid auxotrophs, drug resistance mutants, and polymerase mutants. Comprehensive lists of mutations and their effects on period can be found in earlier reviews (Lakin-Thomas *et al.*, 1990; Loros and Dunlap, 2001; Morgan *et al.*, 2001). With the few exceptions noted in later sections, the molecular bases for the effects of most of these mutations on the period of the circadian rhythm have not been determined.

Two mutations affecting lipid synthesis are of particular interest: *cel* (*fas*) and *chol-1*. The *cel* (chain elongation) mutation impairs fatty acid elongation, and this strain requires exogenous saturated fatty acids for normal growth. When supplemented with unsaturated fatty acids, the period of the conidiation rhythm lengthens and temperature compensation is impaired. The *chol-1* (choline) mutation impairs synthesis of the lipid phosphatidylcholine, and this strain requires choline for normal growth. On low-choline medium, the period of the rhythm lengthens in inverse proportion to the choline concentration in the medium, and

temperature compensation is impaired. The *cel* and *chol-1* strains will be discussed further in Sections II.B and V.

One large class of period-affecting mutants has a common target in mitochondrial functions and similar period-shortening effects. This class includes *olr* (oligomycin resistance), several cytochrome mutants, and both nuclear- and mitochondrially inherited genes (Lakin-Thomas *et al.*, 1990). The short period of these mitochondrial mutants can be phenocopied by treatment of cultures with inhibitors of mitochondrial function such as chloramphenicol (Brody, 1992). A common effect of several of these mitochondrial mutations was found to be an increase in mitochondrial mass per cell volume (Brody, 1992), but the relationship between this effect and the circadian clock mechanism has not been uncovered.

In the absence of detailed molecular information about the roles of period-affecting mutations, there are several approaches to formulating hypotheses about their effects on the circadian clock. One type of hypothesis focuses on molecular mechanisms and changes in rates: short-period mutations may increase the rate of turnover of a negative-acting clock component, or increase the rate of synthesis of a positive-acting component; long-period mutations may have the opposite effects. Another possibility is a change in affinity of proteins in a regulatory complex: short-period mutations may increase the affinity of two proteins or of a protein for a promoter element, thus leading to precocious activation, and long-period mutations may decrease such affinities. A complementary approach is to consider the amplitude of the oscillator: an increase or decrease in amplitude may cause an increase or decrease in period. This change in amplitude may be brought about by any of the molecular mechanisms listed above. Evidence for changes in amplitude in clock mutants has been discussed in the framework of limit cycle theory (Lakin-Thomas *et al.*, 1991; Shaw and Brody, 2000).

B. Interactions between clock mutants

With quantitative phenotypes such as circadian periods, it is possible to use genetics to ask questions about interactions between gene products in the absence of biochemical evidence. Constructing double- and triple-mutant strains combining several clock mutations in *Neurospora* has provided some insights into potential gene product interactions. The simplest interpretation of additive or multiplicative interactions (each mutation either adds/subtracts a fixed number of hours to the period or multiplies the period by a fixed factor) is that each gene product acts independently on the final phenotype (the period). Epistatic, synergistic, or intermediate effects indicate the possibility of interactions between the gene products, and at least a common pathway for effects on the period (Lakin-Thomas and Brody, 1985; Morgan *et al.*, 2001). It should be noted that most studies of genetic interactions have focused on period measurements and very little work has been reported on other clock properties such as phase resetting or temperature compensation.

The *cel* mutation described in Section II.A requires saturated fatty acids for normal growth, and when supplemented with unsaturated fatty acids, the period of the conidiation rhythm lengthens (Mattern *et al.*, 1982). A series of double mutant strains was constructed between *cel* and the series of *frq* alleles (Lakin-Thomas and Brody, 1985), and the data are presented in Table 3.1. A systematic interaction between *cel* and the *frq* alleles was found, with greater

divergence from the predicted additive or multiplicative periods found with the longer-period *frq* alleles (Lakin-Thomas and Brody, 1985). The prediction of complete epistasis of *cel* over *frq* (in which all *cel frq* double mutants would have the same period as *cel frq*⁺) also fails, but with the short-period *frq* alleles diverging the most from this prediction (Lakin-Thomas and Brody, 1985). In contrast, both the *prd-1* and the *olr*^r mutations were found to be epistatic to *cel*, blocking the period-lengthening effects of unsaturated fatty acids (Lakin-Thomas and Brody, 1985) (Table 3.1). These data suggest that the gene products of *frq*, *prd-1*, and *olr*^r all interact with the unknown mechanism that produces long periods in *cel*.

The *chol-1* mutant described above requires choline supplementation for normal growth and rhythmicity (Lakin-Thomas, 1996, 1998). Double mutants between *chol-1* and the series of *frq* alleles were constructed, and the data are presented in Table 3.1. As with *cel*, the prediction of complete epistasis of *chol-1* over *frq* (in which all *chol-1 frq* double mutants would have the same period as *chol-1 frq*⁺) also fails, again with the short-period *frq* alleles diverging the most from this prediction (Lakin-Thomas, 1998). The *chol-1 frq*¹ strain is significantly different from *chol-1 frq*⁺ (Lakin-Thomas, 1998); an additive prediction fits this double mutant best. The *chol-1 frq*² and *chol-1 frq*³ strains are not significantly different from additive, multiplicative, or epistatic predictions (Table 3.1). The *chol-1 frq*⁷ strain fits an epistatic prediction but not additive or multiplicative models (Table 3.1). These results indicate a complex series of interactions between the *frq* alleles and the period-lengthening effect of *chol-1*.

A large number of multiple mutant strains have been constructed using the series of *prd* mutants, *frq* alleles, and the *chr* mutant (Morgan and Feldman, 2001; Morgan *et al.*, 2001), and the periods of some of these strains are presented in Table 3.2. These authors found significant synergistic interactions among a group of mutations including *prd-2*, *prd-3*, *prd-6*, and *frq*⁷, suggesting these gene products mutually interact (Table 3.2A and B). Synergistic effects on temperature compensation were also found in several double mutant combinations (Morgan and Feldman, 2001). Epistasis between *prd-1* and *prd-2* places them in the same pathway (Table 3.2B). It is interesting to note that not all alleles at the *frq* locus produced evidence of interactions; in the case of *prd* double mutants, only the double mutants carrying the *frq*⁷ allele, and in one case, *frq*³, differed significantly from the additive or multiplicative models (Table 3.2A). The *chr* mutant did not produce significant interactions in double mutants (Table 3.2A), suggesting that the *chr* gene product affects the period through a pathway independent of *frq*. Triple mutants with various combinations of mutations have also been constructed (Table 3.2C), and although the results are more difficult to interpret, the periods are roughly as would be predicted from the interactions revealed by the data in Table 3.2A and B.

III. THE GENETICS AND BIOCHEMISTRY OF THE FRQ/WCC FEEDBACK LOOP

A. Basic mechanism of the feedback loop

The phenotypes of *frq* mutations described above identified the *frq* gene as potentially a central component of the mechanism of the circadian clock in *Neurospora*, and cloning of

the gene (McClung *et al.*, 1989) allowed biochemical and molecular analysis of its function to begin. When negative feedback of *frq* transcription by its protein product FRQ was discovered (Aronson *et al.*, 1994b), following the example of the *per* gene of *Drosophila* (Hardin *et al.*, 1990), a transcription/translation negative feedback model was developed for the circadian clock mechanism of *Neurospora* with the FRQ protein and *frq* RNA as the core components (Aronson *et al.*, 1994b). Additional clock components were later identified, the most important being the two white-collar proteins WC-1 and WC-2 (Crosthwaite *et al.*, 1997). These were incorporated into a model that can be called the FRQ–white-collar complex (FRQ/WCC) model. In a simplified version of the current model for the *Neurospora* clock mechanism (for a detailed description of the model, see Vitalini *et al.*, 2010), transcription of the *frq* gene is activated by a complex of the two white-collar proteins, WC-1 and WC-2, together called the white-collar complex (WCC; Froehlich *et al.*, 2002, 2003). The level of *frq* mRNA increases when WCC is active, FRQ protein is translated, and FRQ protein inhibits the activity of WCC, thereby negatively regulating its own transcription and leading to a fall in *frq* mRNA levels (He *et al.*, 2006; Schafmeier *et al.*, 2006). FRQ protein is degraded (Liu *et al.*, 2000), inhibition of transcription is relieved, and the cycle repeats as *frq* mRNA accumulates again. In a positive feedback loop, FRQ protein also acts to increase the levels of WCC, at posttranscriptional and/or posttranslational steps (Cheng *et al.*, 2001b, 2003; Lee *et al.*, 2000; Merrow *et al.*, 2001; Schafmeier *et al.*, 2006). It is the rhythmic activity of the WCC that drives the observed rhythms by regulating transcription of downstream genes (see Section VI). Interestingly, the levels of the *wc* transcripts do not cycle over the course of the day; however, WC-1 protein levels but not WC-2 protein do cycle (He *et al.*, 2002; Lee *et al.*, 2000; Merrow *et al.*, 2001). Experiments in which *wc-1* or *wc-2* were overexpressed from an inducible promoter demonstrate that, while cycling levels of WC-1 protein are not required for overt rhythmicity, the positive feedback loops are important to stabilize and maintain proper amplitude of the rhythm (Cheng *et al.*, 2001b).

B. Genetic analysis of FRQ and WCC

The earliest work on clock genes in *Neurospora* identified multiple alleles at the *frq* locus that altered the period, and subsequently, null alleles were found to be arrhythmic under standard growth conditions, or “conditionally rhythmic” (described in Section II.A). The two white-collar genes, *wc-1* and *wc-2*, had been previously identified as essential for blue light photoresponses and were subsequently identified as clock components through the arrhythmic phenotypes of null mutants (Crosthwaite *et al.*, 1997). Recent genetic studies have added to our understanding of the functions of these genes.

1. FRQ protein domains—No enzymatic activity has been attributed to the FRQ protein. Its function appears to be to help recruit enzymes involved in the negative feedback loop, including kinases, phosphatases, and a helicase. The full length FRQ protein is 989 amino acids long, with a coiled-coil domain near the N-terminus (Cheng *et al.*, 2001a), and a nuclear localization signal located downstream of the coiled-coil domain. FRQ dimerizes through its coiled-coil domain, and dimerization is required for binding to the WCC. Two alternatively translated forms of FRQ, large FRQ (l-FRQ) and small FRQ (s-FRQ), that differ by 99 amino acids at the N-terminus are translated from two in frame AUGs (AUG1

and AUG3) (Garceau *et al.*, 1997; Liu *et al.*, 1997) within different transcripts that result from alternative splicing events (Colot *et al.*, 2005; Diernfellner *et al.*, 2005, 2007). Low levels of FRQ are present in the nucleus, and nuclear localization is required for FRQ's role in the circadian oscillator (Luo *et al.*, 1998). Despite the presence of the nuclear localization signal, most FRQ protein is cytoplasmic, suggesting that there is an active process to export FRQ from the nucleus. Using FRQ deletion mutants, the C-terminus of FRQ that includes the FRQ/FRQ-interacting RNA helicase (FRH) interaction domain was found to play an important role for cytoplasmic localization of FRQ (Cha *et al.*, 2011). Other domains in FRQ include a casein kinase I (CK1) interaction domain located in the middle of the protein (Guo *et al.*, 2010) and two PEST domains that function in FRQ turnover (Görl *et al.*, 2001). The first PEST domain is just downstream of the CK1 interaction domain, and the second PEST domain is located at the carboxy-terminus of the protein. Between the two PEST domains is a binding site for FRH (Cheng *et al.*, 2005; Guo *et al.*, 2010). All FRQ protein interacts with FRH, and the FRQ/FRH complex is required for interaction of FRQ with the WCC and the inactivation of WCC in the negative feedback loop. The FRQ/FRH complex is also required for FRQ stability and for the positive activity of FRQ in promoting the accumulation of the WC proteins (Guo *et al.*, 2010; Shi *et al.*, 2010).

2. The WCC proteins—WC-1 and WC-2 are PAS domain-containing GATA-type zinc finger transcription factors, found primarily in the nucleus of cells (Ballario *et al.*, 1996; Lee *et al.*, 2000; Linden and Macino, 1997; Schwerdtfeger and Linden, 2000). The WC-1 protein has three PAS domains (A, B, and C), with the N-terminal PAS domain being a specialized LOV (light, oxygen-, voltage-sensing) domain that functions as a blue light sensory module (Briggs, 2007; Christie *et al.*, 1999; Liu *et al.*, 2003). The WC-2 protein has a single PAS domain. WC-1 and WC-2 bind to each other to form the WCC through the PASC domain of WC-1 and the PAS domain of WC-2 (Cheng *et al.*, 2002, 2003; Talora *et al.*, 1999).

3. *wc-1* simple sequence repeats—Wild-type *Neurospora* isolates collected from a variety of locations and environments can provide information about natural genetic and phenotypic variation. A survey of circadian phenotypes among 143 accessions found a correlation between period of the conidiation rhythm and polymorphisms in simple sequence repeat (SSR) regions in the *wc-1* gene (Michael *et al.*, 2007). A correlation between period and latitude of collection was also found. These results suggest that the variation in *wc-1* SSRs may be a target of selection for fitness by the local environment (Michael *et al.*, 2007).

C. Role of protein phosphorylation in the FRQ/WCC feedback loop

Current work on the FRQ/WCC feedback loop focuses on filling in details of this model. It has become increasingly clear that both the mechanism of negative feedback, whereby FRQ protein inhibits the transcriptional activity of WCC, and the mechanism of positive feedback, whereby FRQ promotes accumulation of WCC, depend on posttranslational mechanisms, specifically the phosphorylation and dephosphorylation of the three proteins FRQ, WC-1, and WC-2. The activity and subcellular localization of these three major components of the FRQ/WCC are all modulated by phosphorylation. Kinase and phosphatase genes that have been identified as playing roles in regulating rhythmicity in *Neurospora* are listed in Table 3.3.

1. FRQ phosphorylation—The FRQ protein is phosphorylated at multiple sites, and a robust rhythm of phosphorylation can be seen on Western blots (Garceau *et al.*, 1997). Newly synthesized FRQ protein is hypophosphorylated and undergoes progressive phosphorylation as it matures, until the hyperphosphorylated protein is degraded by the ubiquitin/proteasome pathway (He and Liu, 2005a). Recent work has identified numerous phosphorylation sites on the FRQ protein using MS techniques (Baker *et al.*, 2009; Tang *et al.*, 2009). At least 43 (Tang *et al.*, 2009) or 75 (Baker *et al.*, 2009) *in vivo* sites were identified, and the majority are progressively phosphorylated during maturation of the FRQ protein. Systematic mutagenesis of these sites did not identify individual residues essential for rhythmicity but did identify regions of the FRQ protein in which phosphorylation affects the period: mutations in the central region of FRQ lengthen the period, and mutations in the C-terminal region shorten the period; degradation rates of FRQ protein are correlated with period (Baker *et al.*, 2009; Tang *et al.*, 2009).

Several kinases have been identified as phosphorylating FRQ, including calcium/calmodulin-dependent kinase (CAMK-1) (Yang *et al.*, 2001), casein kinase 1a (CK-1a) (Görl *et al.*, 2001; He *et al.*, 2006), casein kinase II (CKII) (Yang *et al.*, 2002), and protein kinase A (PKA) (Huang *et al.*, 2007). CK-1a and CKII were shown to phosphorylate the majority of sites identified by MS analysis (Tang *et al.*, 2009). Knockout of the *camk-1* gene produces slight effects on period, phase, and light-induced phase shifting of the conidiation rhythm (Yang *et al.*, 2001). CK-1a physically associates with FRQ protein (Görl *et al.*, 2001; He *et al.*, 2006), and disruption of that association by mutation of the binding domain on FRQ results in hypophosphorylation of FRQ and arrhythmic conidiation (He *et al.*, 2006). A knock-in mutation of *ck-1a* that disrupts the kinase domain also results in hypophosphorylation of FRQ and long-period rhythms of conidiation, FRQ protein levels and phosphorylation, and *frq* RNA levels (He *et al.*, 2006). Disruption of *cka*, the gene coding for the catalytic subunit of CKII, abolishes rhythms of FRQ protein levels and phosphorylation and RNA levels of *frq* and two ccgs: *cgc-1* and *cgc-2* (Yang *et al.*, 2002). Disruption of *ckb1*, the gene coding for the regulatory subunit CKB-1 of CKII, produces mostly arrhythmic conidiation patterns with a few long periods, and long-period rhythms of FRQ protein and *frq* and *cgc-1* RNA (Yang *et al.*, 2003). With either a knockout of the gene encoding the major PKA catalytic subunit *pkac-1* or a mutation in the gene encoding the PKA regulatory subunit *mcb*, rhythms of conidiation, rhythms of FRQ levels and phosphorylation, and rhythms of *cgc-1* RNA are abolished (Huang *et al.*, 2007).

Three protein phosphatases, PP1, PP2A, and PP4, have been implicated in regulating the phosphorylation status of FRQ (Cha *et al.*, 2008; Yang *et al.*, 2004). The PP1 and PP2A proteins can dephosphorylate FRQ *in vitro* (Yang *et al.*, 2004). A partially functional mutation of *ppp-1*, the gene that codes for the catalytic subunit of PP1, shortens the period and advances the phase of the conidiation rhythm (Yang *et al.*, 2004). Disruption of *rgb-1*, the gene for a regulatory subunit of PP2A, produces low-amplitude, long-period rhythms of FRQ protein and *cgc-1* RNA (Yang *et al.*, 2004). Deletion of the *pp4* gene shortens the period of the conidiation rhythm and the FRQ protein rhythm (Cha *et al.*, 2008). FRQ protein is hyperphosphorylated and is at low levels in this mutant, and its degradation rate is faster than wild type (Cha *et al.*, 2008). A double mutant strain carrying both the *pp4*

deletion and the partially functional *ppp-1* mutation is arrhythmic for conidiation and for FRQ protein levels, and also displays hyperphosphorylated FRQ and a rate of FRQ degradation even faster than the *pp4* deletion strain (Cha *et al.*, 2008), indicating that both PP1 and PP4 normally dephosphorylate FRQ and stabilize it.

There may be several related functions for this phosphorylation of FRQ. Degradation of hyperphosphorylated FRQ protein is essential for the operation of the feedback loop to allow reactivation of WCC. A major role for FRQ phosphorylation is in determining the kinetics of FRQ degradation, and this, in turn, influences the period of the circadian oscillator (Baker *et al.*, 2009; Liu *et al.*, 2000; Ruoff *et al.*, 2005; Tang *et al.*, 2009).

A second role for FRQ phosphorylation may be modulating the interaction between FRQ and WCC. In the *cka* mutant that is defective in CKII, FRQ protein is hypophosphorylated and more FRQ was found to associate with WCC than in the wild type (Yang *et al.*, 2002). In a time series analysis of proteins interacting with FRQ, WCC was found to associate with FRQ preferentially during the times in the circadian cycle when FRQ is hypophosphorylated (Baker *et al.*, 2009). Lastly, mutation analysis of FRQ phosphorylation sites demonstrated that FRQ phosphorylation inhibits FRQ binding to the WCC and FRQ/CK-1a interactions (Cha *et al.*, 2011).

Phosphorylation of FRQ may also modulate the rate of nucleocytoplasmic shuttling of FRQ (Diernfellner *et al.*, 2009). Negative feedback requires hypophosphorylated nuclear FRQ (Schafmeier *et al.*, 2006), and the slow accumulation of cytoplasmic FRQ as it becomes phosphorylated may contribute to the slow kinetics of the circadian cycle; this is supported by the observation that the mutant FRQ⁷ protein remains in the nucleus longer than the wild-type protein, and this correlates with the slower phosphorylation kinetics of FRQ⁷ (Diernfellner *et al.*, 2009). However, recent data using FRQ kinase mutants and FRQ phosphorylation site mutations indicate that FRQ phosphorylation does not play a major role in FRQ localization in the cell (Cha *et al.*, 2011).

2. WCC phosphorylation—Both the WC-1 and the WC-2 proteins are phosphorylated *in vivo* in the dark, and their phosphorylation increases in response to light exposure. This light-dependent phosphorylation may play a role in the function of WCC as the blue light receptor in *Neurospora*, but this function of WCC can be separated from its function in the FRQ/WCC feedback loop of the circadian system. We will be concerned in this review only with the clock-related function of WCC.

FRQ/FRH regulates the activity of WCC by regulating its phosphorylation status, inhibiting WCC activity as its phosphorylation increases (He *et al.*, 2006; Schafmeier *et al.*, 2005). Five phosphorylation sites on the WC-1 protein downstream of the DNA-binding zinc finger domain have been identified as light independent, and mutation of these sites produces short-period or arrhythmic conidiation (He *et al.*, 2005b). Additional WC-1 phosphorylation sites have been identified, as well as one site on WC-2 (Sancar *et al.*, 2009). The dark phosphorylation of both WC-1 and WC-2 depends on FRQ/FRH, and hypophosphorylated WCC is more transcriptionally active than the hyperphosphorylated forms (He *et al.*, 2006;

Schafmeier *et al.*, 2005), supporting the conclusion that FRQ inactivates WCC in the negative feedback loop by promoting WCC phosphorylation.

Both CK-1 α and CKII phosphorylate WC-1 and WC-2 *in vivo* in a FRQ-dependent manner (He *et al.*, 2006). Mutations of FRQ that abolish the interaction between FRQ and CK-1 α result in hypophosphorylation of WC proteins, as do both a kinase-defective *ck-1a* knock-in mutant and a *cka* mutant defective in CKII (He *et al.*, 2006). The transcriptional activity of WCC is increased by mutations in either CK-1 α or CKII (He *et al.*, 2006). PKA also phosphorylates WC-1, but independently of FRQ (Huang *et al.*, 2007). WC-1 protein is at low levels and is hypophosphorylated in the *pkac-1* knockout, suggesting that PKA stabilizes WC-1 and primes it for phosphorylation by other kinases (Huang *et al.*, 2007).

The phosphatases PP2A and PP4 also play roles in regulating WCC phosphorylation status. WCC is a substrate for PP2A, and the *rgb-1* mutant, defective in a regulatory subunit of PP2A, increases phosphorylation of WC-1 and WC-2 (Schafmeier *et al.*, 2005). Both WC-1 and WC-2 are also hyperphosphorylated when the *pp4* gene is deleted, and the nuclear enrichment of WCC is lost (Cha *et al.*, 2008).

3. Positive feedback and phosphorylation—The mechanism of positive feedback whereby FRQ promotes accumulation of WCC is not completely known. In one simple model (Schafmeier *et al.*, 2008), positive feedback depends only on posttranslational regulation of WCC. Degradation of WCC may be triggered by DNA binding, so that FRQ can cause accumulation of WCC by inhibiting its binding to DNA through phosphorylation. In this way, the same mechanism (phosphorylation of WCC) that causes negative feedback (inhibition of DNA binding) may also cause positive feedback (accumulation of WCC). However, the system is more complex as FRQ-dependent transcriptional regulation of *wc-2* has been demonstrated (Cheng *et al.*, 2001b), and FRQ regulates WC-1 levels independent of WC-2 (Cheng *et al.*, 2001b, 2002; Lee *et al.*, 2000).

4. Nuclear localization and phosphorylation—As a transcription factor, WCC acts on its DNA targets in the nucleus, and therefore, the subcellular localizations and movements of WCC and FRQ are essential to the kinetics of the FRQ/WCC feedback loop. As indicated above, the phosphorylation and dephosphorylation cycle of FRQ is probably not a major player in this process. Instead, an unknown mechanism to actively export FRQ from the nucleus is thought to exist (Cha *et al.*, 2011). Using the fluorescent protein mCherryNC fused to FRQ, the accumulation of FRQ in the nucleus can be visualized across a circadian cycle; a major peak of nuclear accumulation is found at CT 5 and a much smaller peak at CT 19, suggesting two phases of FRQ nuclear transport (Castro-Longoria *et al.*, 2010). WCC is normally enriched in the nucleus, but this enrichment is lost when the *pp4* gene, which codes for protein phosphatase 4, is deleted (Cha *et al.*, 2008). There is conflicting evidence as to whether the *rgb-1* mutation, which inactivates a subunit of PP2A, affects the nuclear localization of WCC (Cha *et al.*, 2008; Schafmeier *et al.*, 2008). The movement of WCC to the cytosol depends on the presence of a functional FRQ protein (Cha *et al.*, 2008; Hong *et al.*, 2008). FRAP analysis of GFP-tagged WC proteins showed rapid shuttling in and out of the nucleus on a time scale of minutes, modulated by FRQ-induced phosphorylation and PP2A phosphatase-mediated dephosphorylation (Schafmeier *et al.*, 2008). These results

suggest that FRQ promotes phosphorylation of WCC, which is counteracted by PP2A and PP4, and phosphorylated WCC preferentially accumulates in the cytosol. FRQ also physically interacts with WCC to promote clearance of the complex from the nucleus, and this may also contribute to negative feedback (Cha *et al.*, 2008; Hong *et al.*, 2008).

D. Identification of additional genes regulating the FRQ/WCC feedback loop

Included in this category are genes with clock-affecting phenotypes, either period effects or disruption of rhythmicity, whose functions in the FRQ/WCC feedback loop are known. See Table 3.3 for a summary of the genes described below.

1. *frq* antisense RNA—Two antisense transcripts from the *frq* locus have been identified, and their roles in regulating *frq* expression have been investigated (Kramer *et al.*, 2003). Antisense *frq* RNA is rhythmically produced, but in antiphase to sense RNA, and its transcription is light induced. This light induction requires the WCC, which directly binds to the promoter of the antisense RNA following a short light pulse (Smith *et al.*, 2010). When antisense *frq* expression is abolished, there is a delay in the expression of sense *frq* and a similar delay in the onset of the conidiation rhythm. The most dramatic effect is an increased response to phase resetting by light of the conidiation rhythm in the strains without antisense *frq* (Kramer *et al.*, 2003). Antisense *frq* therefore seems to oppose the function of sense *frq* in the light response and may play a role in moderating the level of *frq* expression (Crosthwaite, 2004).

2. *frh*—A search for additional clock components that associate with FRQ using immunoprecipitation of FRQ protein revealed a protein that was given the name FRH. FRH forms a complex with the entire pool of FRQ protein and mediates the interaction of FRQ with WCC (Cheng *et al.*, 2005; Guo *et al.*, 2010). The *frh* gene is essential in *Neurospora*, but downregulation abolishes circadian conidiation rhythms as well as molecular rhythms in FRQ protein, *frq* RNA, and *cgc-2* RNA, identifying FRH as an essential component of the FRQ/WCC feedback loop (Cheng *et al.*, 2005). A genetic screen for mutations affecting negative feedback identified a mutant allele, *frh*^{R806H}, that disrupts the interaction between FRQ/FRH and WCC and abolishes conidiation rhythmicity and molecular rhythms without affecting growth and viability (Shi *et al.*, 2010). WC-1 phosphorylation and consequent stabilization appear to require the interaction of both FRQ and FRH with WCC: the *frh*^{R806H} mutant disrupts those interactions, and both WC-1 and WC-2 are found at low levels in hypophosphorylated forms in this mutant (Shi *et al.*, 2010).

FRQ/FRH associates with the exosome that regulates RNA processing and promotes decay of *frq* RNA (Guo *et al.*, 2009). FRQ/FRH may therefore play two roles in the FRQ/WCC feedback loop: inhibiting WCC activity and promoting decay of *frq* RNA. Downregulating a component of the exosome, RRP44, increases the stability of *frq* RNA and lengthens the period of the molecular rhythms of FRQ protein and *frq* RNA, demonstrating the role of the exosome in regulating *frq* RNA decay (Guo *et al.*, 2009). The exosome may also regulate rhythmicity of some *cgc*s by regulating their RNA stability: *rrp44* RNA is rhythmic, and downregulating *rrp44* affects the rhythmic expression of two *cgc*s (Guo *et al.*, 2009). FRH has recently been shown to play a role in stability of FRQ protein as well (Guo *et al.*, 2010):

FRQ is degraded rapidly in the absence of FRQ–FRH interaction, by a pathway that is independent of FWD-1 (see Section III.D.3).

3. *fwd1* and *csn*—The degradation of FRQ protein is an essential process in the functioning of the FRQ/WCC feedback loop. In *frq* mutants with different periods, the rate of degradation correlates with the period (Ruoff *et al.*, 2005). Protein degradation in eukaryotes is often carried out by the ubiquitin–proteasome pathway, and this pathway has been shown to mediate FRQ degradation in the circadian feedback loop. FRQ protein is ubiquitinated, and the FWD1 protein was identified as the substrate-recruiting subunit of the SCF ubiquitin ligase complex responsible for FRQ degradation (He *et al.*, 2003). Disruption of the *fwd1* gene disrupts rhythms of FRQ protein, *frq* RNA, *cgc-1* RNA, and conidiation rhythms (He *et al.*, 2003). The COP9 signalosome (CSN) is a multisubunit complex that, among other functions, regulates the stability and activity of ubiquitin ligases. Disruption of the *csn-2* gene that encodes a CSN subunit impairs degradation of FRQ, abolishes FRQ protein oscillations, and produces conidiation rhythms with very long periods (He *et al.*, 2005a). A series of knockouts of other CSN subunits (*csn-1*, -2, -4, -5, -6, and -7) produces similar phenotypes of long-period and irregular conidiation rhythms (Wang *et al.*, 2010). It should be noted that there is also evidence for another pathway for FRQ degradation independent of FWD-1, although the details of this pathway are not yet known (Guo *et al.*, 2010).

4. *csw-1*—The WCC is the transcriptional activator that is responsible for activating *frq* expression, but little is known about events at the promoter. Chromatin remodeling and histone modifications are likely to be involved as they are in the activation of many genes. A survey of knockouts of putative chromatin-remodeling genes in *Neurospora* identified one gene, *csw-1* (clockswitch), with a clock-associated phenotype (Belden *et al.*, 2007b). The knockout produces abnormal, sporadic conidiation patterns and a loss of rhythmicity of *frq* RNA and FRQ protein expression. The CSW protein localizes to the *frq* promoter and affects chromatin structure and WCC association with the *frq* promoter (Belden *et al.*, 2007b).

E. Temperature compensation

One of the basic properties of circadian oscillators is the property of temperature compensation: the period of the rhythm changes very little at different constant environmental temperatures. The wild-type *Neurospora* clock is well compensated in the physiological temperature range of 16–30 °C and poorly compensated between 30 and 36 °C (Gardner and Feldman, 1981; Gooch *et al.*, 2008; Sargent *et al.*, 1966). Not all period-affecting mutations in *Neurospora* have been assayed for their effects on temperature compensation; but of those that have, many have been found to have some effect on this property, either impairing compensation such that the period becomes longer at low temperature or in a few cases causing “overcompensation” in which the period becomes somewhat shorter at lower temperatures (Gardner and Feldman, 1981). Effects on temperature compensation can be slight, such as for *prd-4* (Gardner and Feldman, 1981), or may be greater, as for *cel* (Mattern *et al.*, 1982). Impaired temperature compensation may be uniform across a broad temperature range, as for *frq*⁷ (Gardner and Feldman, 1981), or may

affect only a limited range of temperatures, as for *chol-1* (Lakin-Thomas, 1998). The observation that many different gene products influence temperature compensation with a range of different effects may indicate that temperature compensation is a property of the system as a whole, rather than a special function of a particular temperature-independent regulatory mechanism. It also seems likely that some components of the circadian system may have evolved to play larger roles in maintaining temperature compensation.

The *frq* gene plays an important role in temperature compensation of the period, as shown by the impaired compensation in the null *frq* mutants *frq⁹* (Loros and Feldman, 1986) and *frq¹⁰* (Aronson *et al.*, 1994a) and altered temperature compensation in period-affecting *frq* mutants (Gardner and Feldman, 1981). In *frq* mutants, FRQ protein stability is related to temperature compensation (Ruoff *et al.*, 2005). Casein kinase 2 (CK2 or CKII, see Section III.C.1) has recently been identified as a regulator of temperature compensation through its phosphorylation of FRQ protein (Mehra *et al.*, 2009). Two clock-affecting genes identified in early genetic screens as period-affecting mutants, *chr* (chrono) and *prd-3* (period-3), also display unusual temperature compensation: *chr* extends temperature compensation beyond the wild-type range, and *prd-3* is overcompensated (Gardner and Feldman, 1981). These two genes have now been identified as coding for subunits of CK2 (Mehra *et al.*, 2009): *chr* (*ckb-1*) encodes subunit CKB-1 and *prd-3* (*cka*) encodes subunit CK2 α . The mutations were demonstrated to be hypomorphs that result in reduced FRQ phosphorylation and could be phenocopied by mutations in putative CK2 phosphorylation sites on FRQ. Manipulating the dosage of the CKB-1 protein alters temperature compensation and affects FRQ stability as predicted (Mehra *et al.*, 2009). Importantly, not every kinase that phosphorylates FRQ affects temperature compensation. For example, manipulation of the levels of CK1 α alters the period of the developmental rhythm, but the clock is still temperature compensated (Mehra *et al.*, 2009). Together, these data suggest a specific role for CK2 in regulating the pace of the clock at different temperatures via temperature-dependent phosphorylation of FRQ altering FRQ stability.

IV. OTHER CLOCK-ASSOCIATED GENES

Included in this category are genes whose identity is known that affect rhythmicity but do not appear to directly affect the functioning of the FRQ/WCC feedback loop, and genes that affect the period of the conidiation rhythm but whose identity and function are as yet unknown. While the significance and underlying mechanisms for many of these observations are unknown, they provide fertile ground for future investigation. See Table 3.3 for a summary of the genes described below.

A. *csp-1*

This gene codes for a light-inducible transcription factor that affects the conidiation process by preventing conidial separation (Lambreghts *et al.*, 2009; Smith *et al.*, 2010). Strains carrying the *csp-1* mutation show periods about 1 h shorter than wild type (Schneider *et al.*, 2009).

B. *csp-2*

Mutations in *csp-2* also prevent conidial separation, but unlike *csp-1*, this mutation lengthens the period by about 1.5 h (Brody *et al.*, 2010). The *csp-2* gene has been found by mapping and complementation to be allelic with *ghh*, the *Neurospora* homolog of the animal epidermal integrity gene Grainyhead; the period of the *ghh* mutant is about 3 h longer than wild type (A. Paré and B. McGinnis, personal communication).

C. *prd-4*

The *prd-4* (period) gene was identified in the early screens for mutations affecting the period of the conidiation rhythm. The original *prd-4* mutation shortens the period by about 3 h (Gardner and Feldman, 1981), but a null mutation (Pregueiro *et al.*, 2006) displays a wild-type period and normal rhythmicity, indicating that *prd-4* is not essential to the clock mechanism. Cloning of the gene (Pregueiro *et al.*, 2006) provided an identity and function. *Prd-4* is an ortholog of checkpoint kinase 2 (Chk2) that activates a cell cycle checkpoint in response to DNA damage in eukaryotes. DNA-damaging agents reset the clock in *Neurospora*, and this resetting requires PRD-4 (Pregueiro *et al.*, 2006). PRD-4 promotes phosphorylation of FRQ protein in response to DNA damage, thereby resetting the clock. The *prd-4* mutant is semidominant, suggesting a gain of function. Consistent with that, FRQ protein is phosphorylated earlier than normal during the circadian cycle in the *prd-4* mutant, accounting for the decreased period (Pregueiro *et al.*, 2006).

D. *ras^{bd}* (formerly *bd*)

Almost all strains used in laboratories that study the circadian rhythm of conidiation in *Neurospora* carry the *bd* (band) mutation to make the rhythm easier to assay. The effect of the *bd* mutation was originally described as making the conidiation rhythm resistant to inhibition by high levels of CO₂ that accumulate in closed culture vessels such as race tubes (Sargent and Kaltenborn, 1972). Cloning of the *bd* gene (Belden *et al.*, 2007a) revealed that it is an allele of *ras-1*, the small G-protein involved in many signaling pathways in eukaryotes, and the mutation has been renamed *ras^{bd}*. The addition of a source of reactive oxygen species (ROS) such as menadione to the growth medium can phenocopy the effects of *ras^{bd}*, suggesting a signaling role for ROS in the conidiation output pathway (Belden *et al.*, 2007a).

E. *rco-1*

This gene is a regulator of conidiation and photoadaptation (Olmedo *et al.*, 2010a; Yamashiro *et al.*, 1996). In the *ras^{bd}* background, it lengthens the conidiation rhythm to 34–55 h, depending on the culture conditions (Brody *et al.*, 2010).

F. *Sod-1*

This gene codes for superoxide dismutase, an enzyme that removes ROS. Mutation or deletion of this gene phenocopies the *ras^{bd}* mutation in that a robust conidiation rhythm with a slightly shorter period can be seen under conditions where the wild type appears arrhythmic (Belden *et al.*, 2007a; Yoshida *et al.*, 2008). The addition of ROS to the growth medium also phenocopies *ras^{bd}* (Belden *et al.*, 2007a), while the removal of ROS reverses

the banding phenotype of *sod-1* (Yoshida *et al.*, 2008), implicating ROS in the conidiation output pathway.

G. *ult*

This newly isolated mutation (*ult*, ultradian) shortens the conidiation period to about 12 h (see also Section V) and is the shortest clock mutant yet reported (Lombardi *et al.*, 2007). It is dominant in heterokaryons and is not yet mapped and cloned. It produces a pattern of alternating thin and thick bands of conidiation (Lombardi *et al.*, 2007). The addition of caffeine to the growth medium doubles the period of the *ult* mutant, suggesting a role for the cAMP pathway in the *ult* phenotype (Brody *et al.*, 2010).

H. *vvd*

The *vvd* (vivid) gene has been previously identified as a component of the light input pathway in *Neurospora*. The gene is rapidly transcribed in response to light, and the protein functions to downregulate responses to light (Heintzen *et al.*, 2001; Schwerdtfeger and Linden, 2003; Shrode *et al.*, 2001). Wild-type cultures of *Neurospora* do not express the conidiation rhythm in constant light (LL), but *vvd* mutants express short-period (~11 h) conidiation rhythms in LL (Schneider *et al.*, 2009). With increasing light intensity, temperature compensation is gradually lost and the period decreases to as little as 6–7 h. Upon transfer to the dark, the period gradually lengthens until a final steady-state period of 22 h is reached. The expression of rhythmicity depends on the culture: rhythms are seen in petri plates but not in race tubes (Schneider *et al.*, 2009). A mutation in the *csp-1* gene lengthens the period and reduces the sensitivity of the period to light effects. Cultures rhythmic in LL can be phase shifted by dark pulses. The level of FRQ protein is high and constant in *vvd* cultures when they are rhythmic in LL, indicating that a rhythm in FRQ is not essential for the conidiation rhythm; this result is consistent with the finding that the *frq* gene is not required for the *vvd* rhythm in LL (Schneider *et al.*, 2009) (see Section V).

I. Quantitative trait loci

A method for identifying new clock-affecting genes is to exploit the genetic variation in natural populations. Free-running period and entrained phase were used to map quantitative trait loci (QTL) in three populations of progeny derived from crosses of isolates collected in different geographic locations (Kim *et al.*, 2007). Thirty QTL were found that did not map near previously identified clock genes, and these regions are candidates for the locations of new clock-affecting genes (Kim *et al.*, 2007).

J. Screening *Neurospora* knockout library for defects in circadian rhythms

Currently, almost all *Neurospora*'s predicted 10,000 open reading frames (ORFs) have been deleted, providing new tools for assaying the effects of specific gene knockouts on circadian rhythmicity. Screens are underway in several labs to assay alterations in the developmental rhythms in the knockout strain collection, as well as assaying rhythmic reporter genes for alterations in rhythmicity in the mutant strains. These approaches promise to provide a global view of the influence of the genome on circadian clock function.

V. FRQ-LESS RHYTHMS

A. “Conditional rhythmicity” of *frq* and *wc* null mutants

Although the vast majority of research carried out on the *Neurospora* circadian system is focused on the FRQ/WCC feedback loop, it has been known for many years that rhythmic conidiation can be seen in the absence of a functional *frq* gene (Loros and Feldman, 1986). More examples of these FRQ-less rhythms have been reported over the years, and there is now a large collection of conditions and genetic backgrounds in which FRQ-less rhythmicity can be demonstrated. Although null mutants of *frq*, *wc-1*, or *wc-2* are often described as “arrhythmic,” it might be more accurate to call them “conditionally rhythmic.” Rhythmicity can be induced in these null mutants by conditions such as introducing additional mutations, by adding chemicals to the growth medium, or by simply changing the geometry of the culture vessels.

Rhythmicity in the absence of FRQ/WCC function must be driven by an oscillator, and there has been much discussion over the years as to the relationship between the FRQ/WCC and the FRQ-less oscillator(s) (FLOs). The possibility that FRQ functions to transduce environmental information as part of an input pathway to another oscillator was discussed as early as 1997 (Lakin-Thomas *et al.*, 1997). In 1998, Roenneberg and Mellow proposed the “zeitnehmer” model in which FRQ/WCC functions as a rhythmic input pathway to an oscillator (Roenneberg and Mellow, 1998). Evidence for multiple oscillators was discussed with reference to the restoration of rhythmicity to *frq* null strains by Lakin-Thomas and Brody in 2000 (Lakin-Thomas and Brody, 2000). Subsequently, the term FLOs was coined to describe the oscillator(s) driving FRQ-less rhythms (Iwasaki and Dunlap, 2000). While several labs have obtained a significant amount of data describing the FRQ-less oscillations, we still do not know the identity of the components of the FLOs. Until we do, we can only speculate on the role and organization of the FLOs in the circadian system.

B. FRQ-less conidiation rhythms and their properties

Most reports of FRQ-less rhythms have assayed rhythms of conidiation, and these are described below in approximate chronological order. In some cases, a mutation in the genetic background is required to observe the rhythm, and those genes that have been reported in the past 10 years are included in Table 3.3.

1. Long race tubes—FRQ-less conidiation rhythms were first reported in a *frq⁹* null mutant (Loros and Feldman, 1986). Rhythms take several days to appear, so extra-long race tubes were used to gather more data, and not all cultures are rhythmic. The period is more variable than wild type and is affected by the nutritional composition of the medium. Temperature compensation of the period is defective. The rhythm can be damped out by constant light and entrained to cycles of light pulses. When the knockout *frq¹⁰* became available, it was found to produce similar rhythms (Aronson *et al.*, 1994a).

2. *cel* and *chol-1*—The two lipid-defective mutations *cel* and *chol-1* described in Section II can reveal FRQ-less rhythms under appropriate conditions. When double mutants of *cel* and either *frq¹⁰* or *wc-2* are grown at low temperature or supplemented with unsaturated

fatty acids, long-period conidiation rhythms are robustly produced (Lakin-Thomas and Brody, 2000). Double mutants of the choline-requiring mutant *chol-1* with *frq*, *wc-1*, or *wc-2* null mutants produce robust conidiation rhythms when choline supplementation is limited (Lakin-Thomas and Brody, 2000). At intermediate choline concentrations, the periods are in the circadian range and lengthen as choline is decreased. The period of the *chol-1 frq¹⁰* double mutant is temperature compensated between 22 and 28 °C and lengthens below 22 °C. None of the double mutant *cel* or *chol-1* strains are damped by constant light or entrain to light/dark cycles (Lakin-Thomas and Brody, 2000). Recent studies examining *frq*-driven luciferase rhythms in the *chol-1* mutant strain revealed that under conditions in which the strain shows a long-period developmental rhythm, the *frq:luc* rhythms cycled with a 22-h period (Shi *et al.*, 2007). Although it has been suggested that these data indicate independent functioning of two oscillators (Shi *et al.*, 2007), the possibility of coupling between two oscillators in a frequency demultiplication relationship has not been fully explored.

3. Temperature entrainment—The circadian oscillator in *Neurospora* can be entrained to cycles of high and low temperature, and it was reported that such cycles could also entrain the conidiation rhythm in a *frq* null strain (Merrow *et al.*, 1999; Roenneberg *et al.*, 2005), suggesting that the same oscillator functions in both wild-type and *frq* null strains. This interpretation was challenged when a similar data set was analyzed with different methods and the authors concluded that the conidiation peaks represented “driven” or “masking” responses, not the output of an oscillator (Pregueiro *et al.*, 2005). These experiments used symmetric temperature cycles, with equal periods of warm and cool temperature, which makes it difficult to separate masking effects from entrained peaks. An improved method using short pulses of high temperature demonstrated definitively that the conidiation peaks behave as if they are produced by an entrained oscillator (Lakin-Thomas, 2006a). This improved method uncovered both temperature-driven masking effects and temperature entrainment for both *frq¹⁰* and a *wc-1* null mutant (Lakin-Thomas, 2006a).

4. Farnesol and geraniol—The addition of the chemicals farnesol or geraniol to the growth medium produces reliable rhythms in *frq*, *wc-1*, and *wc-2* null mutants with periods in the circadian range or somewhat longer (Granshaw *et al.*, 2003). It takes several days before conidiation bands appear. Temperature compensation of the period is defective, and the carbon source in the growth medium affects the appearance of banding. Phase resetting by temperature pulses produces a strong type 0 phase response curve in both the *frq¹⁰* and the *frq⁺* strains grown on geraniol. However, *frq¹⁰* did not entrain to light/dark cycles, unlike *frq⁺* (Granshaw *et al.*, 2003).

5. *ult*—This short-period mutant produces robust 12 h rhythms in *frq¹⁰* double mutants. In a *wc-2* null mutant background, *ult* produces rhythms with short periods of about 16.5 h (Lombardi *et al.*, 2007).

6. *sod-1*—The *frq¹⁰*, *sod-1* double mutant strain, displays a conidiation rhythm in constant darkness, although the triple-mutant *frq¹⁰*; *bd*; *sod-1* is arrhythmic (Yoshida *et al.*, 2008).

Light/dark cycles induce a conidiation rhythm that behaves as if it is produced by masking effects of light and not by entrainment of an oscillator (Yoshida *et al.*, 2008).

7. *vvd*—The short-period conidiation rhythms expressed in constant light (LL) by the *vvd* mutant are still expressed in the *vvd; frq¹⁰* double mutant with no significant change in period, although the rhythms are not as robust as in *vvd* (Schneider *et al.*, 2009). Introduction of the *wc-1* knockout abolishes the LL rhythms in *vvd*, introducing *wc-2* reduced the clarity of the *vvd* rhythms (Schneider *et al.*, 2009). This suggests that *wc-1* is required as the light receptor for the expression of rhythmicity in *vvd; frq¹⁰* in LL.

8. Menadione—This chemical generates ROS and has been shown to phenocopy the *ras^{bd}* mutation (see Section IV). When added to the growth medium, menadione induces conidiation rhythms in null mutants *frq¹⁰*, *wc-1*, and *wc-2*, in both DD and LL (Brody *et al.*, 2010). The periods of *frq¹⁰* and *wc-1* are short (14–15 h), and *wc-2* is long (25 h). In 24-h LD cycles, *frq¹⁰* entrains and *wc-1* produces two bands per cycle, while *wc-2* fails to entrain. The addition of caffeine to the medium doubles the period of *frq¹⁰*. A mutation in the *csp-1* gene shortens the period of the null *frq⁹* mutant by about 1 h. The period of *wc-2* is temperature compensated in the range 16–28 °C, and *frq¹⁰* is compensated in the range 22–28 °C on menadione (Brody *et al.*, 2010).

C. FRQ-less molecular rhythms and their properties

Several molecular species have been shown to be rhythmic in FRQ-less strains. Because these rhythms have been assayed under different growth conditions in different laboratories, it is not yet clear whether they are related to each other, and how they are related to FRQ-less conidiation rhythms.

1. DAG—The levels of the neutral lipid diacylglycerol (DAG) are rhythmic in wild-type *Neurospora* with a period that reflects the conidiation rhythm (Ramsdale and Lakin-Thomas, 2000). In a *frq* null strain grown on solid agar, the DAG rhythm has a period of about 12 h, similar to short-period conidiation rhythms seen in these cultures (Ramsdale and Lakin-Thomas, 2000).

2. Nitrate reductase—In ammonia-free medium, a rhythm in nitrate reductase activity can be measured in *frq* null and *wc-1* null strains in both DD and LL (Christensen *et al.*, 2004). This rhythm may be an indicator of rhythmic metabolism, or may be an autonomous oscillator constructed from the negative feedback of glutamine on nitrate reductase expression (Christensen *et al.*, 2004).

3. *ccg-16*—The mRNA levels of a *ccg*, *ccg-16*, are rhythmic in DD in a *frq* knockout strain but are not seen in null mutants of either *wc-1* or *wc-2* (Correa *et al.*, 2003). This FRQ-less rhythm is also seen in LL when FRQ protein levels are constantly elevated and is more responsive to temperature than to light cues for synchronization (de Paula *et al.*, 2006). The period is temperature compensated between 22 and 27 °C (de Paula *et al.*, 2006). A knockout of *ccg-16* does not affect the conidiation rhythm in a *frq⁺* background and does not affect the rhythm of WC-1 protein in a *frq* null background (see Section V.C.4), thereby

defining *ccg-16* as an output of the FLO and not a component of either the FRQ/WCC oscillator or the FLO (de Paula *et al.*, 2006).

4. WC-1—A low-amplitude rhythm in WC-1 protein levels has been reported in *frq*⁺ strains (de Paula *et al.*, 2006; Lee *et al.*, 2000). This rhythm can also be seen in LL in wild-type strains, and in both DD and LL in a *frq* null strain, although the average levels are lower than in wild type (de Paula *et al.*, 2006).

D. Mutations affecting FRQ-less rhythms

If additional mutations are introduced into *frq* or *wc* null strains and these double mutant strains are assayed under conditions in which FRQ-less rhythms can be seen, the effects of the additional mutations on the FLO(s) can be assayed. Several mutations have been found to affect FRQ-less rhythms, and they are summarized in Table 3.3.

1. *frq*—It may seem paradoxical to claim that *frq* mutations affect the period of FRQ-less rhythms. However, under some conditions, it appears that it is primarily the FLO that is controlling rhythmicity when there is a functional *frq* gene, and mutations in *frq* can affect the FLO, supporting the idea that the FLO functions downstream of the FRQ/WCC feedback loop. In the *chol-1* strain, described above, long-period rhythms are seen when choline is limiting. Long-period rhythms are also seen in a *chol-1 frq* null double mutant, and these rhythms have characteristics similar to the rhythms in the *frq*⁺ background: the period is sensitive to the choline concentration and shows a similar pattern of temperature compensation (Lakin-Thomas and Brody, 2000). It is therefore reasonable to assume that the same oscillator is driving conidiation rhythms in both *frq*⁺ and *frq* null on limiting choline. As described in Section II.B and in Table 3.1, *frq* mutations can have significant effects on the long period in *chol-1*, indicating that the FLO can be affected by *frq*.

Additional evidence that mutations in the FRQ/WCC feedback loop can affect the FLO comes from the results with menadione in *frq* and *wc* null strains (Brody *et al.*, 2010), as described in Section V.B.8. In the presence of menadione, the period of conidiation rhythms in *frq*¹⁰ and *wc-1* is about 14–15 h, while the period in *wc-2* is about 25 h, indicating that the alleles at these loci influence the period of the FRQ-less rhythm under these conditions.

2. *ult*—The periods of the FRQ-less rhythms produced by geraniol in *frq*¹⁰ and *wc-2* mutants are shortened by the addition of the short-period *ult* mutation (Lombardi *et al.*, 2007).

3. *prds*—The lipid-deficient *cel* strain produces long-period conidiation rhythms at low temperature and when supplemented with unsaturated fatty acids. These long-period rhythms continue when *frq* and *wc* null mutations are introduced (Lakin-Thomas and Brody, 2000), and therefore the long-period rhythms in *cel frq*⁺ strains may be said to be driven by the FLO. When the *prd-1* mutation is introduced into *cel*, the period-lengthening effect of fatty acids is abolished, and therefore *prd-1* appears to block the normal functioning of the FLO in *cel* (Lakin-Thomas and Brody, 1985).

The *frq¹⁰* null mutant is rhythmic when supplemented with geraniol (described above), and this system was used to assay the effects of a series of *prd* mutations on the FLO (Lombardi *et al.*, 2007). The *prd-1* and *prd-2* mutations, both long period in a *frq⁺* background, also lengthen the period in the *frq¹⁰* background. The short-period *prd-6* mutation shortens the *frq¹⁰* period (Lombardi *et al.*, 2007). No change in period is seen with *prd-3* or *prd-4* in the *frq¹⁰* background.

In a similar experiment, triple mutants were constructed between *chol-1 frq¹⁰* and a series of *prd* mutations, and the effects of the *prds* on the long-period conidiation rhythm in *chol-1* were assayed (Li and Lakin-Thomas, 2010). Both *prd-1* and *prd-2* severely disrupt rhythmicity in choline-depleted cultures, but *prd-3* and *prd-4* have only subtle effects on period and robustness of the conidiation rhythm. These strains were also grown on high choline (which repairs the *chol-1* defect) to assay the effects of the *prd* mutations on the heat-entrainable oscillator in *frq¹⁰*. A similar pattern was seen: both *prd-1* and *prd-2* significantly affect the timing of the heat-entrainable peak, while *prd-3* and *prd-4* have little effect (Li and Lakin-Thomas, 2010).

Both *prd-3* and *prd-4* are known to encode subunits of protein kinases that phosphorylate FRQ protein (see Section III.C.1), and their lack of effect on FRQ-less rhythms is consistent with the assumption that they act primarily on the FRQ/WCC oscillator. The effects of *prd-1* and *prd-2* mutations on multiple FRQ-less rhythms may indicate that these genes act primarily on the FLO. The effects of both *prd-1* and *prd-2* on the period of the conidiation rhythm when FRQ/WCC is functional suggest that their effects on FLO can also affect the period of the intact system.

4. UV90—Using the *chol-1 frq¹⁰* double mutant, a mutagenesis screen was carried out to look for genes that affect the FRQ-less rhythm on low choline (Li *et al.*, 2011). A new mutation was found, named UV90, that abolishes the FRQ-less conidiation rhythm seen in low-choline cultures. UV90 also severely affects the heat-entrainable conidiation rhythm in choline-sufficient cultures. In a *frq⁺* background, UV90 damps the amplitude of the conidiation rhythm and also damps the FRQ protein rhythm. The response of *frq⁺* cultures to phase resetting by pulses of either light or high temperature is increased in the UV90 mutant background, consistent with a decrease in the amplitude of the circadian oscillator (Li *et al.*, 2011). These results suggest that the UV90 gene product is an important component of the FLO and is also required to maintain the amplitude of the FRQ/WCC oscillator, indicating an influence of the FLO on FRQ/WCC.

E. Multiple independent FLOs, or an integrated system?

The existence of FRQ-less rhythms implies the existence of one or more oscillators (FLOs) that drive these rhythms in the absence of a functional FRQ/WCC oscillator. If we wish to fully understand the circadian system of *Neurospora*, one of the most fundamental and vigorously debated questions at the moment is: how many oscillators make up the complete system? There are several possible interpretations of the data. One view (Shi *et al.*, 2007) describes FLOs as “metabolic oscillators” and proposes that there are different FLOs driving the observed rhythms for every reported condition; these multiple metabolic oscillators (of

which there would need to be more than a dozen at the latest count) are not considered to be connected to the circadian system (Shi *et al.*, 2007). Another view proposes multiple FLOs that, under normal circumstances, interact with one another and with the FRQ/WCC to produce a network of coupled oscillators, but that individual FLOs may act independently to drive a particular output (de Paula *et al.*, 2007). If there are multiple oscillators, there is a formal possibility that they may function either “upstream” or “downstream” of FRQ/WCC; in either case, they would need to bypass FRQ/WCC to provide output to the conidiation pathway and biochemical rhythms when FRQ/WCC is disabled. A third view proposes a single FLO, which may be the central rhythm generator for the circadian system, mutually coupled to the FRQ/WCC, which supplies stability, period control, and rhythmic input (Li and Lakin-Thomas, 2010; Roenneberg and Merrow, 1998).

The FRQ-less rhythms described above appear to have different properties, lending support to models in which every reported FRQ-less rhythm is driven by a different FLO. However, properties such as temperature compensation, response to alterations in growth media, and ability to entrain to light/ dark or temperature cycles have not been fully characterized for most of the reported FRQ-less rhythms. Claims of failure to entrain to environmental stimuli are particularly suspect unless a wide range of cycle lengths and stimulus strengths have been tested, as oscillators will fail to entrain to stimuli that are too weak or whose entraining periods are too far from the intrinsic period of the oscillator (Johnson *et al.*, 2003). Because of the many different conditions and experimental paradigms, it is difficult to compare the properties of FRQ-less rhythms reported by different laboratories and impossible to say what they have in common.

Obviously, the best way to address the questions of how many FLOs there are and whether they function independently of the FRQ/WCC oscillator is to identify components of the FLOs. The analysis of genetic interactions in multiple mutant strains can suggest participation in common pathways, as described in Section II.B. The complex interactions between the series of *frq* alleles and the long periods in both *cel* and *chol-1* (Lakin-Thomas, 1998; Lakin-Thomas and Brody, 1985) indicate that the FRQ gene product interacts with the FLO(s) that produce long periods in both *cel* and *chol-1*. The epistasis of *prd-1* over *cel* (Lakin-Thomas and Brody, 1985) places *prd-1* in the same pathway as the FLO in *cel*. The series of interactions among *prd* mutations and *frq* alleles (Morgan and Feldman, 2001; Morgan *et al.*, 2001) place *prd-1* and *prd-2* in the same pathway, interacting with *prd-6* and *frq*. All these interactions point to a system in which FRQ/WCC is not independent of the FLO(s) affected by *cel*, *chol-1*, *prd-1*, *prd-2*, and *prd-6*.

Additional genetic evidence comes from assaying the effects of mutations on more than one FRQ-less rhythm. Four different FRQ-less rhythms have been assayed in *prd-1* and all four were found to be affected: in *cel*, in *chol-1*, with geraniol, and with heat pulses. Three FRQ-less rhythms have been assayed in *prd-2* and all were affected: in *chol-1*, with geraniol, and with heat pulses. Two FRQ-less rhythms are affected by UV90: in *chol-1* and with heat pulses. So far, the evidence indicates that these mutations all affect multiple FRQ-less rhythms. It may be that these gene products participate in several different FLOs, but the simplest explanation is that a single FLO is behind multiple observed rhythms.

It has recently been proposed (Brody *et al.*, 2010) that the many conditions and mutations that allow expression of FRQ-less rhythms may all converge on a pathway that includes ROS and the activation of a RAS–cAMP–protein kinase signaling pathway leading to conidiation. Several of the mutations and treatments that affect the expression of FRQ-less rhythms can be linked to the production of or response to ROS, such as menadione, farnesol, and *sod-1* (Brody *et al.*, 2010). Menadione, by phenocopying *ras^{bd}*, links ROS to the RAS signaling pathway (Belden *et al.*, 2007a). The clock effects of genes involved in conidiation, such as *rco-1* and *csp-1*, and the effects of caffeine may implicate a cAMP pathway leading to conidiation as a candidate for a component of the FLO (Brody *et al.*, 2010). It is interesting to note that connections are now being reported between the circadian clockworks and the cAMP pathway, and metabolic regulation in general, in other organisms (Bass and Takahashi, 2010; Harrisingh and Nitabach, 2008; Hastings *et al.*, 2008).

A model for the *Neurospora* circadian system is proposed in Fig. 3.2. In this model, the FRQ/WCC feedback loop is coupled to a single FLO. The mechanism of the FLO is unknown and may or may not include a transcription/translation feedback loop. Both oscillators are required in the intact system to maintain the period, temperature compensation, amplitude, and stability of the output. When the FRQ/WCC oscillator is defective in *frq* or *wc* null mutants, the FLO can drive the output at a low amplitude and without the full range of properties such as temperature compensation and period stability. The conditions and genotypes listed above that allow expression of FRQ-less rhythms may act on various parameters of the FLO to increase its amplitude. The differences in properties between different FRQ-less rhythms can be explained by effects of the various conditions and background genotypes on different parameters of the FLO. Versions of this model have been discussed previously (Lakin-Thomas, 2006b; Lakin-Thomas and Brody, 2004; Li and Lakin-Thomas, 2010).

VI. CLOCK-CONTROLLED GENES

A. Output from the circadian oscillator: The hands of the clock

In *Neurospora*, circadian rhythms in several physiological properties have been documented (Bell-Pedersen *et al.*, 1996a; Lakin-Thomas *et al.*, 1990); however, it was the easily observable rhythm in the development of macroconidia that led to further investigation of the *Neurospora* clock mechanisms (Pittendrigh *et al.*, 1959). Mutations that altered the pattern of rhythmic development led to the identification of FRQ/WCC oscillator components (Feldman *et al.*, 1979). Thus, the study of circadian output pathways not only yields information on the cellular functions that are regulated by the clock and the mechanism of this regulation but also is instrumental in discovering oscillator components.

B. Identification and characterization of ccgs

Molecular investigation of circadian output pathways originated in *Neurospora* by using subtractive hybridization to isolate rhythmically expressed genes (Loros *et al.*, 1989). Two genes were identified that expressed mRNA that accumulated with a circadian rhythm with a period that matched the genotype of the strain. In other words, in a wild-type strain, the period of the mRNA rhythm in DD at 25 °C was 22 h, whereas in the long-period *frq⁷*

mutant, the period of the mRNA rhythm was about 29 h. This landmark study provided evidence that the genes were *bone fide* ccgs and provided the criteria to establish a gene as a ccg. Subsequent screens for ccgs (Bell-Pedersen *et al.*, 1996b; Zhu *et al.*, 2001), including microarray studies, led to the identification of over 400 ccgs (Correa *et al.*, 2003; Dong *et al.*, 2008; Nowrousian *et al.*, 2003). These data suggested that as much as 25% of the *Neurospora* transcriptome is under clock control, and that the dominant peak in rhythmic expression for most of the ccgs occurs just before dawn. However, ccgs were found to peak at all possible phases of the day (Correa *et al.*, 2003).

C. The functions of the ccgs provide insights into the role of the clock

The predicted functions of the proteins encoded by the identified *Neurospora* ccgs have yielded key insights into processes that are clock regulated, many of which are conserved in higher eukaryotes, including development, metabolism, cell cycle, transport, and stress responses (Fig. 3.3). For example, genes encoding enzymes involved in carbon and nitrogen metabolism show circadian rhythms in mRNA accumulation, with peaks of expression occurring in the late night to early morning (Correa *et al.*, 2003). In addition, the glycogen phosphorylase, mannitol-1-phosphate dehydrogenase, and a low-affinity glucose transporter genes peak in expression in the early night, suggesting that flux into the glycolytic pathway peaks at this time of day to prepare for increased energy requirements related to the consequent development of conidiospores. Several ccgs that are involved in stress responses, including genes encoding antioxidant enzymes that prevent damage due to ROS, are also under clock control, such as glutamate dehydrogenase, glutamine synthetase, oxidoreductase, and catalase. Genes encoding these enzymes peak in expression during the daytime, suggesting that the clock increases antioxidative defense mechanisms to cope with increases in free radicals that can result from light exposure.

In support for a role of the clock in providing a mechanism for anticipating stress responses, the highly conserved *Neurospora* osmosensing p-38 MAPK pathway (the OS pathway), essential for osmotic stress responses, functions as a circadian output pathway that regulates daily rhythms in expression of several downstream target genes of the pathway (de Paula *et al.*, 2007; Vitalini *et al.*, 2007). In this pathway, a circadian signal is relayed to the MAPK OS-2, resulting in rhythmic phosphorylation and activation of the MAPK. Phospho-OS-2, in turn, regulates downstream effector kinases, transcription factors, proteins involved in chromatin remodeling, and translation factors that then regulate rhythmic expression of downstream target genes of the pathway.

Two morning-specific ccgs known to be induced by osmotic stress under control of the OS-MAPK pathway include *ccg-1* and *ccg-9* (Vitalini *et al.*, 2007; Yamashita *et al.*, 2008) (D. Bell-Pedersen, unpublished data). Rhythms in *ccg-1* (a gene encoding a protein of unknown function) are dependent on rhythmic activation of OS-2, and the ATF transcription factor that is activated in response to osmotic stress by phospho-OS-2 binds to the *ccg-1* promoter (Yamashita *et al.*, 2008). The *ccg-9* gene encodes trehalose synthase, which catalyzes the synthesis of the disaccharide trehalose. Trehalose plays an important role in protecting cells from environmental stresses, including osmotic stress (Shinohara *et al.*, 2002). A role in development for *ccg-9* is also suggested by the finding that inactivation of *ccg-9* results in

altered conidiophore morphology and abolishes the normal circadian rhythm of conidial development. *ccg-9*-null strains have normal FRQ cycling, phosphorylation, and light induction, indicating that loss of the conidiation rhythm may be a defect in circadian output and is not due to changes in either the FRQ/WCC oscillator or light input into the clock (Shinohara *et al.*, 2002). The phenotype of the *ccg-9* deletion strain differs from *os-2* null mutants (Vitalini *et al.*, 2007), suggesting multiple levels of regulation of *ccg-9*.

Taken together, these data indicate that clock regulation of MAPK signaling pathways provides a mechanism to coordinately control major groups of genes such that they peak at the appropriate times of day to provide a growth and survival advantage to the organism likely by anticipating stresses (de Paula *et al.*, 2007). Consistent with this idea, global gene profiling in *Arabidopsis thaliana* revealed that approximately 68% of the ccgs overlap with genes that are differently regulated in response to osmotic- and cold stresses (Kreps *et al.*, 2002).

D. Signaling time-of-day information from the oscillator to the ccgs

A major goal has been to characterize, at the molecular and biochemical levels, the pathways by which the clock regulates phase-specific gene expression. One mechanism by which the output pathways are rhythmically controlled was believed to be through the rhythmic activity of the WCC transcription factor. Consistent with this idea, many ccgs contain a WCC binding site (LRE) in their promoters (Dong *et al.*, 2008), and WCC was found to bind directly to the promoters of several ccgs, including *vvd*, *al-3*, *fl*, and *sub-1* (Bell-Pedersen *et al.*, 2005; Carattoli *et al.*, 1994; Chen *et al.*, 2009; Correa *et al.*, 2003; He and Liu, 2005b; Olmedo *et al.*, 2010b).

To further explore the idea that rhythmic WCC activity is the primary output signal from the FRQ/WCC, global approaches were used to identify the direct targets of the WCC. Using ChIP-sequencing (ChIP-seq) of WC-2 following a light pulse to activate the WCC, more than 400 regions of the chromosome were found to bind the WCC, with about 200 of these binding sites present in predicted promoters of at least one gene (Smith *et al.*, 2010). A consensus WCC binding site (GATCGA) was derived from these studies, with two or more copies typically present in the binding site region. Consistent with previous studies on ccgs, the direct target genes of the WCC were enriched from genes with functions in the cell cycle, transcription, protein binding, and responses to the environment. As expected, most of the direct targets of the WCC were found to be light induced and/or clock regulated, peaking in the early morning. Interestingly, about 20% of the transcription factors known to be present in the *Neurospora* genome were found to be direct targets of the WCC. These 24 transcription factors include known activators and repressors of gene expression, that could, in theory, directly account for the differentially phased ccgs (Fig. 3.4). Further studies on one of the transcription factors, ADV-1, demonstrated that the *adv-1* gene is both light induced and expressed with a circadian rhythm with peak transcript and protein levels in the early morning (Smith *et al.*, 2010). Deletion of *adv-1* had only a minor affect on the development of spores, but similar to the *ccg-9* deletion strain, resulted in the complete loss of the circadian rhythm of spore development. FRQ protein rhythms were unaltered in the

adv-1 deletion strain, suggesting that ADV-1 functions directly downstream of the FRQ/WCC oscillator to regulate the developmental rhythm.

Taken together, the data suggest a “flat” hierarchical network in which the WCC activates the expression of a suite of second tier transcription factors, which, in turn, activate or repress their individual target genes. Some of these second tier targets may include additional transcription factors that activate or repress the next level. Conservatively, assuming that each of the 24 transcription factor regulates a set of about 20 downstream genes (and that WCC directly binds to regulate rhythms in about 180 additional morning-specific targets), this WCC-directed regulatory network can account for all of the known ccgs and their varied phases. Efforts are now underway to identify the direct targets of these 24 transcription factors using ChIP-seq to comprehensively map the WCC output network. In addition, it is clear that chromatin remodeling, posttranscriptional, translational, and posttranslational mechanisms provide additional combinatorial control of circadian rhythmicity, such as that observed for clock control of the MAPK signaling pathway, and these areas provide fertile ground for future work to decipher the mechanisms for clock control.

E. Output pathways can feed back to regulate oscillator activity

The modulating action of the clock-controlled component VVD on light input pathways provides a clear example of how output pathways can feed back to influence the circadian oscillator (Elvin *et al.*, 2005; Heintzen *et al.*, 2001). In addition to *vvd*, the ccg *prd-4* was shown to influence input to the FRQ/ WCC oscillator in *Neurospora* (Gardner and Feldman, 1981; Pogueiro *et al.*, 2006). PRD-4 is an ortholog of mammalian *chk-2*, which is involved in the response to DNA damage (see Section IV). *Prd-4* mRNA accumulates rhythmically, peaking during the day. This regulation would allow cells to respond maximally to DNA damage caused by ionizing radiation during the day, resulting in the arrest of the cell cycle following PRD-4 phosphorylation in response to this damage. PRD-4 feeds back to the clock through an input pathway that signals DNA damage information to the FRQ/WCC oscillator via FRQ phosphorylation. This output to input loop involving *prd-4* is predicted to provide a time-of-day-specific “gate” during which time DNA-damaging agents can affect the phase of the FRQ/WCC oscillator, which would, in turn, affect *prd-4* expression.

In summary, while it is clear that different organisms use their clocks to regulate different biological processes, many of the output signaling pathways, such as the MAPK pathway and certain transcription factors, are conserved. Thus, a detailed understanding of the circadian output network in *Neurospora* will have a major impact on our understanding of circadian regulation in higher eukaryotes, including humans. In addition, these studies may provide key insights into the design of novel therapies for circadian disorders that result in changes in overt circadian rhythms and desynchrony.

VII. OUTLOOK

Great strides have been made in our understanding of the *Neurospora* “clock works” over the past 40 years, moving from biological phenomenology to the molecular and biochemical mechanism of the circadian clock system. We now have considerable detail regarding the

nature of the core molecular feedback loops, their light input pathway, and their output. Further, the list of “clock-affecting” mutants continues to grow at a fast pace. More “effectors” are likely to continue to be discovered using the unusual resources that are available for *Neurospora*, such as the gene knockout collections. These genetic tools are likely to be supplemented by cytological and imaging techniques. Below is a partial list of some areas/techniques that are currently being studied, or are on the horizon.

- A.** Much remains to be discovered about the mechanism of the oscillator. More details will likely be uncovered regarding clock protein interactions. Perhaps these will be able to be described in biochemical terms, that is, k_m , k_i , flux rates, etc. 3-D crystal structures of the components would be useful in this regard. The nature of the FLO components needs to be established, and how the FLO(s) relate to the FRQ/WCC oscillator. Studying the connection of circadian oscillators to the regulation of the biochemistry of the cell should prove worthwhile, particularly if the FLO(s) are not transcription/translation feedback loops. The role of redox sensing and low molecular weight molecules has been relatively neglected in the clock field. However, a start has been made to establish the light and clock metabolome (<http://fungicyc.broadinstitute.org:1555/overviewsWeb/celOv.shtml>).
- B.** More studies are likely on subcellular localization of clock components. Currently, little is known about the role of subcellular organelles, such as mitochondria, peroxisomes, Golgi, etc., in the clock, although mutations that affect the function of the organelles can alter circadian period.
- C.** The mechanism of temperature compensation will continue to be a fruitful area for studies in *Neurospora*. This will be aided by the already available mutants that affect temperature compensation and the identification of the mutated genes. A full description of temperature compensation will likely be aided by mathematical modeling.
- D.** Significant progress has been made in understanding the circadian output pathways using new sequencing technologies. Clearly, the complexities of these processes and their links to cellular biochemistry and metabolism will occupy many labs for quite a while.
- E.** Chromatin remodeling is an area that will continue to be of interest in understanding rhythmic gene expression. *Neurospora* is a good model system for these analyses as well, as it provides the opportunity to combine genetic, genomic (through ChIP-seq of histone and DNA modifying proteins), and biochemical techniques with imaging of histones using GFP, RFP, etc.
- F.** #x02022; (F) A systems biology approach to monitor rhythmicity in RNA, proteins, and the metabolome will be useful. This has been initiated for the transcriptome (Dong *et al.*, 2008), but additional studies are needed to

investigate the proteome and metabolome under different growth/lighting conditions and in specific genetic backgrounds.

- G.** Finally, there is a special feature of the *Neurospora* clock system that is fascinating to study and one that may be applicable to understanding synchrony in tissue-specific multioscillator model systems discussed in other chapters. This property is that *Neurospora* grows as a syncytium, with cytoplasm and nuclei streaming throughout the colony. It is intriguing how the growing front of the colony stays synchronized, and how stable the clock signals (phase information) are within a given area of the colony. Experiments involving fluorescent labeling of clock proteins and following their movement in a colony would provide one approach to study this unique feature, and genetics could be used to identify key molecules required for this synchrony.

The progress in understanding the details of the mechanism of the circadian clock, particularly with regard to the oscillator feedback loop, the roles of accessory proteins, temperature compensation, light input, and output, would not have been possible without the use of genetics. *Neurospora* is the undisputed leader in this arena. Importantly, many of these conceptual advances made in *Neurospora* have provided the foundation for our understanding of the clock mechanism in higher organisms. Undoubtedly, genetic tools, coupled with new technologies, will continue to provide the tools needed to further advance the field into uncharted areas of circadian biology.

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Figure 3.1.

The developmental rhythm of conidiation. (A) A 15-cm petri plate was inoculated at the lower edge with the *bd; vvd^P pan-2* strain of *Neurospora* and allowed to grow in constant light. Yellow bands of conidiation formed at approximately 11-h intervals. This strain is unusual in that it continues to form conidiation bands in constant light with a short period. (B) Two 30-cm race tubes were inoculated at the left with the *csp-1, bd* strain, exposed to light for 24 h and transferred to constant darkness for the remainder of growth. Growth was from left to right. The upper two images are photographs of the tops of the tubes; the lower two images are scans of the bottoms of the tubes. The black marks indicate the positions of the growth fronts of the colonies at 24-h intervals. The period of these cultures was approximately 21.5 h.

Figure 3.2.

Model for the integrated circadian system of *Neurospora*. Circles represent oscillators; arrows represent causal influences. See text for details.

Figure 3.3.

Representation of the phase of expression of functional classes of ccgs. (Above) The developmental rhythm in *Neurospora* grown on race tubes in a 12-h light: 12-h dark cycle. Growth is from left to right. The vertical black lines on the tube depict the dark to light transition every 24 h. (Below) Representative functional groups of ccgs with respect to the approximate phases in which they peak in expression.

Figure 3.4.

Model of differential phase regulation of ccgs. The WCC directly activates the expression of 24 transcription factors in the morning, including activators and repressors of transcription. The repressors would be active during the day, and therefore the targets of the repressors would be expressed in the night. In addition, the activators would turn on the first tier targets during the morning, resulting in morning-specific gene expression. One or more of the first tier targets may include transcription factors that activate or repress the second tier targets to regulate mid-day-specific ccgs, etc.

Table 3.1Interactions Involving *cel* and *chol-1* Mutations in *Neurospora*

	wt ^a	cel ^a	wt ^b	chol-1 ^b
<i>frq</i> ⁺	20	42	21	63
<i>frq</i> ¹	14	34 ^{*,**}	16	55 ^{**}
<i>frq</i> ²	17	38 ^{*,**}	18	57
<i>frq</i> ³	23	41 ^{*,**}	23	70
<i>frq</i> ⁷	28	39 ^{*,**}	29	63 [*]
<i>prd-1</i>	24	22 ^{*,**}		
<i>oli</i> ^r	19	19 ^{*,**}		

Notes: Periods are rounded to the nearest whole hours. wt, wild type at other loci.

* Significantly different from both additive and multiplicative models.

** Significantly different from model of epistasis of *cel* or *chol-1* over *frq*.

^a Grown on unsaturated fatty acid supplement; data from Lakin-Thomas and Brody (1985).

^b Grown on medium without choline supplementation; data from Lakin-Thomas (1998).

Periods (in Hours) of *Neurospora* Double and Triple Clock Mutants from the Data of Morgan and Feldman (2001)

Table 3.2

A. <i>prd</i> <i>frq</i> and <i>chr</i> <i>frq</i> double mutants						
	<i>frq</i> ⁺	<i>frq</i> ¹	<i>frq</i> ²	<i>frq</i> ³	<i>frq</i> ⁷	
<i>prd</i> ⁺	22	17	19	24	29	
<i>prd-1</i>	26	19	23	28	35	
<i>prd-2</i>	26	19	22	28	38*	
<i>prd-3</i>	25	19	23	30*	41*	
<i>prd-4</i>	18	14	16	20	24	
<i>prd-6</i>	18	15	15	20	21*	
<i>chr</i>	24	17	21	26	33	

B. <i>prd</i> <i>prd</i> and <i>chr</i> <i>prd</i> double mutants						
	<i>chr</i> ⁺	<i>chr</i>	<i>prd-2</i>	<i>prd-3</i>	<i>prd-4</i>	<i>prd-6</i>
<i>prd</i> ⁺	22	24				
<i>prd-1</i>	26	28	26*	30	21	24
<i>prd-2</i>	26	29		33*	21	18
<i>prd-3</i>	25	27			20	18*
<i>prd-4</i>	18	19				16
<i>prd-6</i>	18	19				

C. Triple mutants		
<i>prd-1; chr; frq</i> ⁷		36
<i>prd-3; prd-1; frq</i> ⁷		49
<i>prd-3; prd-2; frq</i> ⁷		48
<i>prd-2 prd-6; frq</i> ⁷		21
<i>prd-3; prd-2 prd-6</i>		18

Note: Data from L. Morgan (personal communication).

* Significantly different from both additive and multiplicative models.

Table 3.3Recently Identified Clock-Associated Genes in *Neurospora*

Gene name	Rhythm phenotypes of mutants	Function/Identity	References
<i>camk-1</i>	Small effects on period, phase, and light-induced phase shifting of the conidiation rhythm	Calcium/calmodulin-dependent protein kinase	Yang <i>et al.</i> (2001)
<i>chr (ckb-1)</i>	Long period, low-amplitude rhythms of conidiation, altered temperature compensation, long-period rhythms of FRQ protein, <i>frq</i> , and <i>cgc-1</i> RNA	Regulatory subunit of casein kinase 2 (CKII)	Mehra <i>et al.</i> (2009), Yang <i>et al.</i> (2003)
<i>ck-1a</i>	Long-period rhythms of conidiation, FRQ protein, and <i>frq</i> RNA	Casein kinase 1a	He <i>et al.</i> (2006)
<i>csn-2</i>	Loss of FRQ protein rhythm, lengthened period of conidiation rhythm	Subunit of COP9 signalosome, involved in protein degradation	He <i>et al.</i> (2005a)
<i>csn-1</i> <i>csn-4</i> <i>csn-5</i> <i>csn-6</i> <i>csn-7</i>	Irregular and long- period conidiation rhythms	Subunits of COP9 signalosome, involved in protein degradation	Wang <i>et al.</i> (2010)
<i>csp-1</i>	Slightly shorter period of conidiation rhythm	Light-induced transcription factor, conidial separation	Schneider <i>et al.</i> (2009)
<i>csp-2</i>	Slightly longer period of conidiation rhythm	Conidial separation	Brody <i>et al.</i> (2010)
<i>csw-1</i>	Abnormal conidiation rhythm, loss of FRQ protein, and <i>frq</i> RNA rhythms	Chromatin remodeling enzyme	Belden <i>et al.</i> (2007b)
<i>frh</i>	Loss of conidiation rhythm and rhythms of FRQ protein, <i>frq</i> RNA, and <i>cgc-2</i> RNA	RNA helicase, component of exosome, regulates RNA processing	Cheng <i>et al.</i> (2005), Guo <i>et al.</i> (2009, 2010), Shi <i>et al.</i> (2010)
<i>fwd1</i>	Loss of conidiation rhythm and rhythms of FRQ protein, <i>frq</i> RNA, and <i>cgc-1</i> RNA	Component of SCF-type ubiquitin ligase complex, involved in protein degradation	He <i>et al.</i> (2003)
<i>mcb</i>	Loss of rhythms of conidiation, FRQ protein, and <i>cgc-1</i> RNA	Regulatory subunit of protein kinase A	Huang <i>et al.</i> (2007)
<i>pkac-1</i>	Loss of rhythms of conidiation, FRQ protein, and <i>cgc-1</i> RNA	Catalytic subunit of protein kinase A	Huang <i>et al.</i> (2007)
<i>ppp-1</i>	Short period and advanced phase of conidiation rhythm	Catalytic subunit of protein phosphatase 1	Yang <i>et al.</i> (2004)
<i>pp4</i>	Short period of conidiation and FRQ protein rhythms	Protein phosphatase 4	Cha <i>et al.</i> (2008)
<i>pp4 ppp-1</i>	Arrhythmic for conidiation and FRQ protein rhythms	Double mutant for protein phosphatases 1 and 4	Cha <i>et al.</i> (2008)
<i>prd-1</i>	Long period of conidiation rhythm in <i>frq⁻</i> , loss of fatty acid effect on FRQ-less rhythm in <i>cel</i> , long period of FRQ-less rhythm with geraniol, loss of FRQ-less rhythm in <i>chol-1</i> , severe effect on heat- entrainable FRQ-less rhythm	Unknown	Lakin-Thomas and Brody (1985), Li and Lakin-Thomas (2010), Lombardi <i>et al.</i> (2007)
<i>prd-2</i>	Long period of conidiation rhythm in <i>frq⁻</i> , long period of FRQ- less rhythm with geraniol, loss of FRQ- less rhythm in <i>chol-1</i> , severe effect on heat- entrainable FRQ-less rhythm	Unknown	Li and Lakin-Thomas (2010), Lombardi <i>et al.</i> (2007)
<i>prd-3 (cka)</i>	Long period of conidiation rhythm, altered temperature compensation, loss	Catalytic subunit of casein kinase 2 (CKII)	Mehra <i>et al.</i> (2009), Yang <i>et al.</i> (2002)

Gene name	Rhythm phenotypes of mutants	Function/Identity	References
	of FRQ protein rhythm, and RNA rhythms of <i>frq</i> , <i>cgg-1</i> , and <i>cgg-2</i>		
<i>prd-4</i>	Short period of conidiation rhythm	Checkpoint kinase 2 (Chk2), links DNA damage to cell cycle arrest	Pregueiro <i>et al.</i> (2006)
<i>prd-6</i>	Short period of FRQ- less rhythm with geraniol	Unknown	Lombardi <i>et al.</i> (2007)
<i>ras^{bd}</i> (formerly <i>bd</i>)	Increased conidiation output, rhythm resistant to CO ₂	<i>ras-1</i> , small G-protein involved in signaling pathways	Belden <i>et al.</i> (2007a)
<i>rco-1</i>	Very long period	Transcription factor, regulates conidiation and photoadaptation	Brody <i>et al.</i> (2010), Olmedo <i>et al.</i> (2010a), Yamashiro <i>et al.</i> (1996)
<i>rgb-1</i>	Low-amplitude, long- period rhythms of FRQ protein and <i>cgg-1</i> RNA	Regulatory subunit of protein phosphatase 2A	Yang <i>et al.</i> (2004)
<i>rrp44</i>	Long period of FRQ protein and <i>frq</i> RNA molecular rhythms	Component of exosome, regulates RNA processing	Guo <i>et al.</i> (2009)
<i>sod-1</i>	Phenocopies <i>ras^{bd}</i> , slightly shorter period, double mutant with <i>frq¹⁰</i> is rhythmic	Superoxide dismutase, removes reactive oxygen species	Belden <i>et al.</i> (2007a), Yoshida <i>et al.</i> (2008)
<i>ult</i>	Short-period conidiation rhythm, double mutant with <i>frq¹⁰</i> or <i>wc-2</i> is rhythmic, shortens the period of the FLO in the presence of geraniol	Unknown	Lombardi <i>et al.</i> (2007)
UV90	Loss of FRQ-less rhythm in <i>chol-1</i> , severe effect on heat- entrainable FRQ-less rhythm, damping of amplitude of FRQ/ WCC oscillator	Unknown	Li <i>et al.</i> (2011)
<i>vvd</i>	Short-period conidiation rhythms in constant light in <i>frq⁻</i> and <i>frq¹⁰</i>	Photoreceptor involved in downregulation of light responses	Schneider <i>et al.</i> (2009)