4 From Genetics to Molecular Oscillations: The Circadian Clock in *Neurospora crassa*

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I. Introduction: *Neurospora crassa* is an Important Model Organism for Research in Circadian Rhythms

Many organisms have evolved an anticipatory mechanism called the **circadian** clock to predict the consistent and dramatic daily changes in the levels of light, temperature, and UV radiation imposed by the Earth’s rotation. Importantly, there is a strong correlation between the maintenance of a **molecular clock** that generates circadian (around 24 h) oscillations in a variety of behavioral and molecular processes and the biological fitness of an organism. For example, when different cyanobacteria strains are grown together, the strain whose circadian period is most closely aligned to an artificially generated light-dark cycle will outcompete the other strains to become the predominant organism in the environment (Ouyang et al. 1998). In addition, squirrels and chipmunks with functioning clocks have a higher survival rate against predators as compared to those with a dysfunctional circadian clock (DeCoursey et al. 1997, 2000; DeCoursey 2014). Proper circadian timing also increases the virulence of some fungi during plant infection (Hevia et al. 2015). Indeed, we know that innumerable biological functions are timed by the clock to occur at their optimal phase of the day (e.g., Hurley et al. 2014, 2018; Panda et al. 2002), and the benefits of these **circadian rhythms** have led them to be conserved in almost all branches of life (Dunlap 1999).

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In identifying the behaviors that are regulated by the circadian clock, researchers have set four main criteria for an **oscillation** to be defined as truly circadian (Johnson et al. 2004). First, the **period** of the rhythm must be at or near 24 h, thereby coinciding with the external 24 h day/night cycle on Earth. Second, the rhythm must be entrainable, meaning that it is responsive to external cues, such as light, which can reset its phase relative to the current environment. For example, when traveling to a different time zone, a person will gradually begin to wake up at the local dawn time as a result of their internal clock adjusting to local lighting cues, rather than continue to wake up during the dawn phase of their previous time zone. Third, the rhythm must be able to persist without external cues. This characteristic demonstrates that the rhythm is an endogenous, anticipatory process and not simply a response to changes in the environment. Finally, the rhythm must be able to maintain a consistent period under different environmental conditions, a property called **compensation**. Since biochemical reactions speed up at higher temperatures, if the circadian clock were not buffered, then it would yield a shorter period and would not be a good pacemaker. While there are numerous biological oscillations in organisms, only those rhythms that meet these four criteria are considered circadian (Johnson et al. 2004).

As circadian clocks appear to have evolved independently in only a few cases, there is a high degree of similarity between the mechanisms that time the clocks of fungi and other higher eukaryotes, e.g., mammals and the fruit fly *Drosophila*, making *N. crassa* an excellent model system in which to study these clock mechanisms (Crosthwaite et al. 1997; Dunlap 1999; Bell-Pedersen et al. 2005; Dunlap and Loros 2017). Much of the foundational research into the molecular mechanism underlying circadian clocks in higher eukaryotes occurred in the filamentous fungus *N. crassa* (Dunlap 2008; Dunlap and Loros 2017; Loros 2019). A mutant in *N. crassa*, which displayed a "banding" developmental growth pattern on **race tubes** was

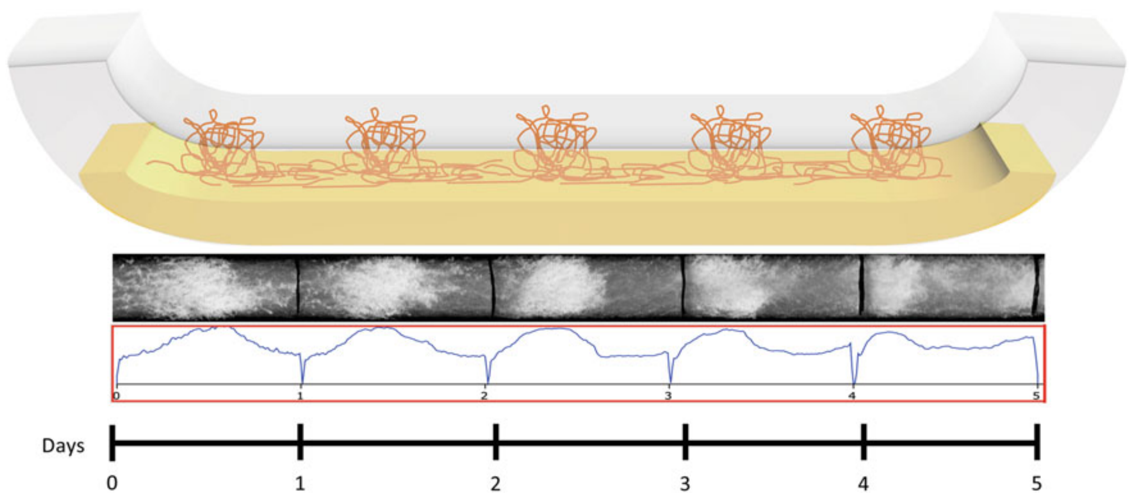

Fig. 4.1 Race tubes and the *bd* mutation enable period and phase analysis in *Neurospora crassa*. A race tube is a tool used to measure the overt phenotypic rhythm of banding strains of *N. crassa*. A hollow glass tube is bent upward at both ends and filled with molten agar growth media, as shown at top. Once the media has cooled and solidified, an *N. crassa* strain with a band (*bd*) background is inoculated at one end and then grows laterally down the tube. As the *bd* strain grows, it will periodically (in time with the circadian cycle) form aerial hyphae and conidia, shown here as the orange clumps. The race tube is marked once every 24 h at the growth front. The periodicity of the aerial hyphal formations is directly tied to the period of the clock. Race tubes can be scanned and densitometric analysis of these race tube images can be used to measure the time between successive bands to predict the period of the strain, as shown at bottom. Image courtesy of Joshua Thomas

among the earliest observable evidence that organisms have an internal circadian clock (Fig. 4.1) (Brandt 1953; Pittendrigh et al. 1959). This **band (*bd*)** strain is characterized by zones of hyphal growth alternating with daily “bands” or clumps of conidia during constant dark conditions. We now know that this banding phenotype is related to an increase in the expression of some genes (e.g., the conidial regulation protein *fluffy*) in response to an increase in reactive oxygen species (ROS), due to a mutation in the gene *ras-1* (Fig. 4.1) (Belden et al. 2007a).

A rapidly growing and non-pathogenic fungus that is easy to genetically manipulate, *N. crassa* has been a strong genetic model system since the work of George W. Beadle and Edward Tatum on metabolic mutants (Beadle and Tatum 1941). *N. crassa* can reproduce both sexually and asexually, depending on environmental cues such as light and nutrient availability, allowing for its efficient use in genetic research (Tan et al. 2004; Filippovich et al. 2015). With its strength as a genetic system, as well as the *bd* mutant phenotype allowing for easy detection of rhythms, *N. crassa* has served as an important model organism in the clocks field for over half a century (Dunlap et al. 2007; Dunlap and Loros 2017). In more recent years, technological advancements have yielded a fully sequenced genome and the development of further molecular tools will ensure that *N. crassa* remains an important model organism in the clocks field for years to come (Galagan et al. 2003; McCluskey et al. 2010).

The conserved circadian timekeeping apparatus in higher eukaryotes, uncovered in large part due to research in *N. crassa*, appears to be a transcriptional-translational negative feedback loop (TTFL) that forms a molecular oscillator or **core clock** (Dunlap and Loros 2018; Hurley et al. 2016a). At subjective dawn, the transcriptional apparatus of the TTFL, the positive arm protein complex (in *N. crassa* **White Collar-1 (WC-1)** and **White Collar-2 (WC-2)**), binds to the promoter region of one of the genes involved in the repressive part of the TTFL, the negative arm protein complex (in *N. crassa* **Frequency (FRQ)** and **Frequency-interacting RNA helicase (FRH)**), and activates its transcription (Fig. 4.2). The positive arm also activates a host of other gene promoters that are not involved in the regulation of the TTFL, generating oscillations in 10–40% or more of transcripts to effect the coordination of many cellular processes (Hurley et al. 2014, 2018; Sancar et al. 2015; Mure et al. 2018). Once translated, the negative arm enters the nucleus and interacts with the positive arm proteins to repress their transcriptional activity. As the day progresses, the negative arm proteins are phosphorylated by many different **kinases** (e.g., **Casein Kinase-1a (CK-1a)** and Casein Kinase-2 (CK-2)), which leads to their inactivation and **degradation**, allowing the positive arm proteins to resume transcriptional activity, starting the cycle anew. The clock is also able to incorporate environmental inputs (such as light, temperature, and nutrients) into the core oscillator to optimally time the circadian cycle, with the earliest examples of this integration in higher eukaryotes first demonstrated in *N. crassa* (Garceau et al. 1997; Crosthwaite et al. 1997; Liu et al. 1998; Sancar et al. 2012; Loros 2019).

In this review, we summarize the key investigations that use the genetic model organism *N. crassa* to create an understanding of the molecular underpinnings of circadian regulation. As the research in the clocks’ field from *N. crassa* is substantial, and our space short, we focus our review into five broad contributions to clock research. The first three sections will describe what is known about *N. crassa* circadian timekeeping at the three basic levels of a molecular clock, the input, the core oscillator, and the output, and how these discoveries have informed an understanding of clocks in higher eukaryotes. The fourth section covers how circadian oscillations are measured in *N. crassa*, and the final section will address *N. crassa* as a model organism for clocks in other fungi. Where important research has occurred beyond the scope of these contributions, or at a depth our mandate did not allow for, we point the reader to previously published reviews focusing on that topic specifically.

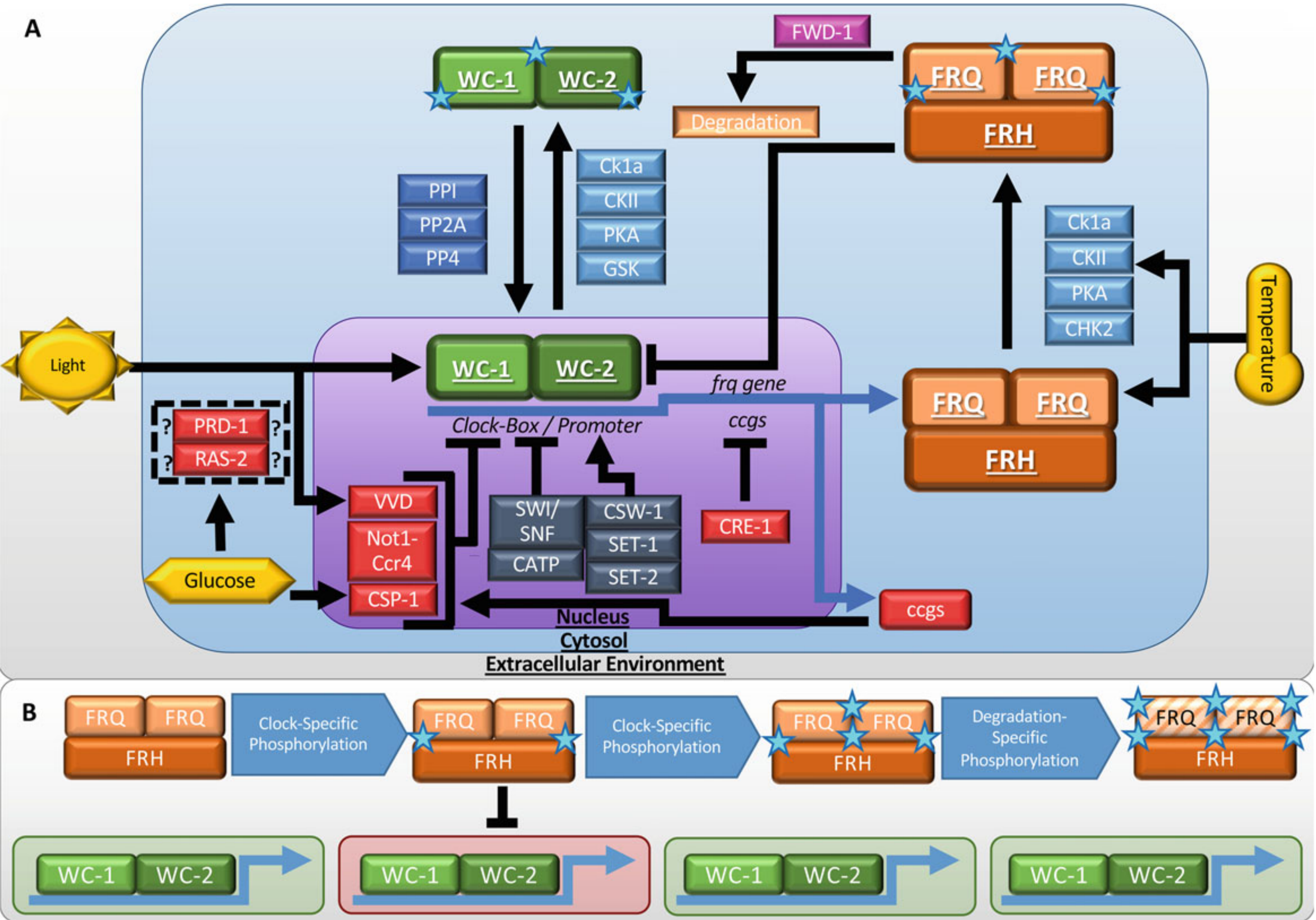

Fig. 4.2 The molecular components of the *Neurospora crassa* circadian clock. (**a**) The circadian cycle begins in the nucleus with the positive arm, made up of White Collar 1 (WC-1, light green) and White Collar 2 (WC-2, dark green), which form the White Collar Complex (WCC). The positive arm transcriptionally activates

II. Input: Various Inputs to the Core Clock Allow Phase Entrainment to the External Environment

One of the tenets of circadian rhythms is that their period is buffered against different constant conditions but is still able to incorporate signals from the environment, allowing the clock phase to align, or "entrain," to changes in the external environment. The most obvious of these signals is the light from the sun, a cue to which circadian rhythms were adapted (Hut and Beersma 2011). However, in recent years, the circadian field has discovered that the clock is responsive to many other environmental cues, such as temperature and glucose (Garceau et al. 1997; Liu et al. 1998; Sancar et al. 2012). For this review, we have grouped these signals, and the pathways that respond to them, into three basic categories: light, temperature, and nutrients.

A. *Neurospora crassa*, the Circadian Clock, and Photoreception

While fungi are not photosynthetic organisms, the ability to sense different colors and intensities of light still contributes to their overall fitness (Idnurm et al. 2010). *N. crassa* has conserved proteins that are essential for the detection of red light, green light, and blue light, though not all of these proteins are functional **photoreceptors** (for more complete reviews, see Fuller et al. 2015; Yu and Fischer 2019). The primary external timing cue for the clock in *N. crassa* is blue light, which is sensed through the positive arm protein WC-1 (Fig. 4.2a). WC-1 is a transcription factor that utilizes **Per-Arnt-Sim (PAS) domains** for transcriptional activation. The PAS domain located toward the protein's N-terminal end is of a special class called a **"light oxygen voltage" (LOV) domain** (Ballario and Macino 1997; Ballario et al. 1998). The LOV domain is able to form a transient cysteinyl adduct with a blue light-absorbing chromophore flavin adenine dinucleotide (FAD) when exposed to blue light (Ballario et al. 1998; Linden et al. 1997). Along with its partner protein, WC-2, WC-1 hetero-dimerizes to form the white collar complex (WCC) (Cheng et al. 2002). When the WCC is exposed to blue light, a conformational change occurs which creates a transcriptionally active WCC (Froehlich et al. 2002; He et al. 2002; Zoltowski et al. 2007; Malzahn et al. 2010). The WCC transcriptionally activates genes that have a **light-responsive element (LRE)** in their promoters, with hundreds of genes affected, either directly or indirectly, by WCC binding (Chen et al. 2009; Collett et al. 2002; Froehlich et al. 2003; Hurley et al. 2014; Smith et al. 2010; Wu et al. 2014). As the WCC contains both a light-sensitive element and forms the positive arm of the circadian clock (see Sect. III.A), research in *N. crassa* was foun-

Fig. 4.2 (continued) numerous genes, including *frequency* (*frq*). In order to do this, the promoter regions of these genes must be accessible and numerous chromatin remodeling factors (dark blue) work with the WCC to effect remodeling at these promoters. The transcriptional activation of *frq* leads to the transcription and translation of FRQ (light orange), which then dimerizes and associates with Frequency-interacting RNA helicase (FRH, dark orange), forming the FFC. FRQ is then phosphorylated (cyan stars) by numerous kinases (light blue) until the FFC can interact with the WCC, repressing WCC activation. The WCC is phosphorylated by several kinases, decreasing transcription at the *frq* locus and other loci regulated by the WCC, and then potentially removed from the nucleus. FRQ undergoes further phosphorylation until it can no longer interact with the WCC and it is recognized by FWD-1 (pink) for degradation. Without the FFC, the WCC is dephosphorylated by phosphatases (blue) and returns to the nucleus to restart the cycle. The genes under the influence of the WCC that do not play a role in the core clock are called clock-controlled genes (*ccgs*, red) and are the drivers of circadian behavior. Some of these *ccgs* can act back on the core circadian loop. In addition, inputs from the environment (yellow) are able to entrain the clock, such as light, temperature, and nutrients (e.g., glucose). (**b**) Negative arm turnover via phosphorylation triggered degradation was previously believed to relieve positive arm (WCC, green) repression. However, new evidence suggests that the negative arm (FFC, orange) undergoes two types of phosphorylation, a clock-specific phosphorylation, which alters the ability of the FFC to suppress WCC activity, and degradation-specific phosphorylation, which targets the negative arm proteins for degradation. This degradation occurs only after the FFC has lost its ability to repress the WCC, and this highly phosphorylated FFC does not impact the clock

dational in demonstrating that the positive arm serves the crucial function of integrating light cues into the clock.

While the WCC is the point protein for clock entrainment to light cues, the activity of another protein, **Vivid (VVD)**, is also crucial for proper clock phase timing based on light in the environment (Fig. 4.2a). A small protein, only containing an N-terminal cap and a LOV domain, VVD plays an important role in light integration into the clock by altering the activity of the WCC (Zoltowski et al. 2007; Hunt et al. 2010; Chen et al. 2010). The WCC induces *vvd* expression in response to light, after which VVD interacts with WC-1's LOV domain and blocks the transcriptional capabilities of WC-1 (Zoltowski et al. 2007; Hunt et al. 2010; Malzahn et al. 2010; Chen et al. 2010; Dasgupta et al. 2015). The action of VVD on the WCC is an important example of **photoadaptation**, ensuring that light-induced genes are only transiently upregulated (Hunt et al. 2010; Chen et al. 2010). VVD's photoadaptive activities are not only important for keeping the clock from resetting inappropriately, such as during nights with bright moonlight, but also in supporting the optimal timing of light responses by the WCC (Heintzen et al. 2001; Malzahn et al. 2010).

B. The Effect of Temperature on the Negative Arm of the Clock

Though it is logical to think of light as the primary environmental input for the clock, surprisingly many other factors, such as **temperature**, can strongly impact the circadian mechanism. For example, temperature changes can reset the phase of the clock, such as an increase from a low to a high temperature. While light input is chiefly transduced via the positive arm (see Sects. II.A and III.A), evidence from *N. crassa* shows that temperature input is primarily conveyed by changes in the molecular dynamics of the negative arm of the clock (Liu et al. 1997; Pregueiro et al. 2005, 2006; Diernfellner et al. 2005), particularly the dynamics of the levels of the core negative arm protein frequency (FRQ) (see Sect. III.B.1 and Fig. 4.2a). The current understanding of the response to temperature shifts is that while the level of *frq* mRNA remains stable at different temperatures, FRQ protein levels oscillate around a higher midline at higher temperatures, with the peak and trough of FRQ levels falling above the peak level of FRQ in a strain grown at a low enough temperature (Liu et al. 1997). Due to the difference in FRQ levels at different temperatures, the comparatively low levels of FRQ at a lower temperature will create an artificial "trough" from which FRQ levels rise when the organism is shifted to a higher temperature, thereby resetting the phase of the clock (Liu et al. 1998). A decrease in temperature can have the same resetting effect, as FRQ levels decrease from an artificial "peak" time in higher temperatures to start oscillating around a lower midline of protein levels (Liu et al. 1998). Extreme temperature changes ($\pm 10\ ^{\circ}C$) have a strong effect on setting the phase of the clock, so much so that it supersedes the effects of light, re-entraining the molecular clock even in a conflicting light-dark regime (Liu et al. 1998). This research paved the way for similar discoveries in higher eukaryotes. Temperature has subsequently been shown to be an important phase resetting cue in mammal peripheral tissues, such that daily temperature changes within physiological ranges (36–37.5 °C) can entrain mouse peripheral tissues (Buhr et al. 2010). Moreover, within *Drosophila*, the mutation of all detected phosphorylation sites to alanine on the positive arm clock protein dCLOCK led to compromised temperature resetting, but unaffected light resetting, suggesting that temperature is more important than light as a timing cue for *Drosophila* tissues as well (Lee et al. 2014).

Though temperature changes can shift the phase of the clock, one of the key tenets of a circadian rhythm is that its period is compensated and regulated at different growing temperatures. However, the underlying mechanism for temperature regulation in the clock is poorly understood. What is known is that temperature impacts the choice of FRQ start codons during translation (Colot et al. 2005; Diernfellner et al. 2007; Liu et al. 1997). A temperature-sensitive **alternate splicing** mechanism occurs in *frq* mRNA, leading to different

ratios of the two isoforms of FRQ: long FRQ (L-FRQ; 1–989 aa) and short FRQ (S-FRQ; 100–989 aa). At higher temperatures, L-FRQ levels increase relative to S-FRQ (Liu et al. 1997; Colot et al. 2005; Diernfellner et al. 2007). L-FRQ-only strains have a shorter clock period than S-FRQ-only strains (Liu et al. 1997; Diernfellner et al. 2007). Therefore, the temperature-based ratio of L- to S-FRQ levels is believed to contribute to the robustness of the clock and the fine-tuning of the clock period at different ambient temperatures (Liu et al. 1997; Diernfellner et al. 2005, 2007). Evidence also suggests that post-translational mechanisms are involved in temperature compensation, e.g., the phosphorylation of FRQ. The mutation of **phosphorylation sites** that exist only on L-FRQ yield a strain with a longer period, similar to the period of a S-FRQ-only strain (Diernfellner et al. 2007; Baker et al. 2009). In addition, mutations in kinases that phosphorylate FRQ (i.e., CK2) have been shown to impact temperature compensation (Mehra et al. 2009b). While different FRQ isoform ratios and changes in FRQ phosphorylation contribute to temperature regulation, further work is necessary to uncover the underlying mechanisms that provide temperature compensation to the clock.

C. The Impact of Nutrient Sensing on the Circadian Clock

Though a great deal of research has gone into the understanding of how light and temperature align the molecular clock, recent data from *N. crassa* has shown that levels of **nutrients**, such as glucose and lipids, can also affect the clock and must be compensated for, termed metabolic or nutritional compensation (Hurley et al. 2016b; Sancar et al. 2012; Starkey et al. 2013). The best studied type of nutritional compensation is glucose compensation, which includes a known circadian-auxiliary loop that utilizes **Conidial Separation Protein 1 (CSP-1)** (Fig. 4.2a). When glucose levels are high, the core clock proteins and many of their downstream targets, including CSP-1, are translated at an increased rate (Sancar et al. 2012). To compensate for the increased clock protein levels, the resulting higher levels of CSP-1 interact with additional clock-regulated proteins, Regulation of Conidiation 1 (RCO-1) and Regulation of Conidiation and Morphology-1 (RCM-1), to repress *wc-1* expression and complete a negative feedback loop that provides glucose compensation (Sancar et al. 2012; Dovzhenok et al. 2015; Olivares-Yañez et al. 2016). Other proteins are also recognized for their impact on glucose compensation, including Period-1 (PRD-1/DBP-2) and Ras-like-2 (RAS-2). PRD-1 is an ATP-dependent DEAD-box RNA helicase, and the clock in *prd-1* mutants is not compensated against glucose changes, leading to longer periods when glucose is not limited (Starkey et al. 2013; Emerson et al. 2015; Adhvaryu et al. 2016). Like *prd-1* mutants, the clock in a Δ*ras2* strain displays longer periods and is not glucose-compensated (Gyöngyösi et al. 2017). Further work is needed to fully understand how glucose compensation is imparted, either by multiple feedback loops including CSP-1, RCO-1, RCM-1, PRD-1, and RAS-2, or through an underlying mechanism yet to be discovered. Nutrient compensation has long been shown to be an important element of the mammalian circadian clock and the research into the mechanisms that regulate nutrient compensation in *N. crassa* has enabled an appreciation of how these might work in higher eukaryotes (Peek et al. 2012).

Beyond glucose compensation, there is evidence that the clock can be affected by lipid levels. Longevity assurance gene (LAG-1) is thought to be part of a ceramide synthase complex and likely plays a role in metabolic clock regulation through stress responses involving sphingolipid metabolism (Case et al. 2014). In a *lag-1* mutant, the clock period lengthens to 41 h, suggesting that changes in sphingolipid levels can affect clock period. Further evidence that the clock is sensitive and responsive to lipid levels includes the relationship between high levels of DAG (sn-1,2-diacylglycerol) and longer clock periods (Ramsdale and Lakin-Thomas 2000; Case et al. 2014). With the extent of reported metabolic effects on the clock through glucose and lipid levels, it is likely that other cell components that play a role in

clock nutritional compensation are still to be determined.

III. Core Oscillator: The *N. crassa* Core Circadian Timekeeping Mechanism

The circadian inputs described above feed into the architecturally-conserved circadian **transcriptional-translational negative feedback loop (TTFL)** (Hurley et al. 2016a; Dunlap and Loros 2017). In addition to the conservation of the underlying architecture of the TTFL across higher eukaryotes, there is sequence homology between WC-1 and WC-2, the positive arm **transcription factors** in *N. crassa*, and transcription factors in other fungal and mammalian systems (Dunlap and Loros 2017). Furthermore, conformational heterogeneity and post-translational regulation of the clock proteins by phosphorylation, especially of negative arm proteins such as FRQ, are also conserved (Lee et al. 2009; Chiu et al. 2011; Hurley et al. 2016a; Pelham et al. 2018). Finally, phosphorylation-dependent ubiquitination and degradation of the negative components supports the functioning of most eukaryotic clocks (Grima et al. 2002; Ko et al. 2002; He et al. 2003; Eide et al. 2005). All of these conserved features of the clock were, at least in part, discovered in *N. crassa*. In this section, we review the abovementioned key elements of the *N. crassa* core clock mechanism, which have contributed greatly to the understanding of the TTFL in other fungi and higher eukaryotes.

A. The Transcriptionally Active Positive Arm of the Clock

In *N. crassa*, the circadian cycle is initiated by the positive arm of the clock, a heterodimeric GATA-like transcription factor complex termed the White Collar Complex (WCC) comprising two proteins, WC-1 and WC-2 (Belden et al. 2007b; Hurley et al. 2014). WCC transcriptional activity peaks around subjective dawn, commencing the circadian cycle (Belden et al. 2007b; Hurley et al. 2014). WC-1 is a 1167 amino acid (aa) long protein that contains a circadian transactivation domain, three Per-Arnt-Sim (PAS) domains (including the light-responsive LOV domain and a "defective in binding" (DBD) region involved in DNA binding and mediating interactions with the negative arm), and a single zinc finger domain involved in DNA binding (Ballario et al. 1996; Lee et al. 2000; Cheng et al. 2002; Wang et al. 2014, 2016). As mentioned previously in Sect. II.A, WC-1 is the primary blue-light photoreceptor in *N. crassa* and is responsible for the integration of light signaling into the circadian clock.

WC-2 is a 530 aa-long protein that contains a single zinc finger domain, a single PAS domain and, unlike WC-1, is unable to sense light (Linden and Macino 1997; Collett et al. 2002). WC-1 and WC-2 exhibit strong oscillations at the transcriptional level, but neither show strong oscillations at the protein level (Hurley et al. 2018). Interestingly, the negative arm protein, FRQ, is needed for proper levels of WC-1 to accumulate (Schafmeier et al. 2006). WC-1 and WC-2 interact with each other through their PAS domains to form the White-Collar Complex (WCC), which promotes the expression of genes in the negative arm as well as other genes not involved in maintaining the core clock, termed **clock-controlled genes (*ccgs*)** (Fig. 4.2a) (Linden and Macino 1997; Crosthwaite et al. 1997; Cheng et al. 2002; Hurley et al. 2014; Wu et al. 2014).

The WCC exists in two conformations, a light-activated conformation and a dark conformation, which serve to control the DNA binding of the WCC. The light-activated complex is composed of two WC-1s and one WC-2, binding to a subset of genes that are considered to be light-responsive (Froehlich et al. 2002; Cheng et al. 2003). In contrast, the dark conformation comprises a single WC-1 and WC-2 and is essential for the expression of *frq* and other *ccgs* under "free-running" or constant dark conditions (Froehlich et al. 2002; Cheng et al. 2003). The zinc fingers of both WC-1 and WC-2 are essential for binding DNA in the dark, aided by the DBD motif on WC-1 (Wang et al.

2016). The ability of the WCC to bind DNA is also dependent on its phosphorylation state, as has been shown for *Drosophila* and mouse positive arm CLOCK protein, with daily rhythms in phosphorylation occurring (He et al. 2005; Baker et al. 2009; Yoshitane et al. 2009; Lee et al. 2014; Wang et al. 2019). There are 80 phosphorylation sites on WC-1, with phosphorylations near its zinc-finger domain playing an important role in closing the negative feedback loop (He et al. 2005; Baker et al. 2009; Sancar et al. 2009; Wang et al. 2019). WC-2 phosphorylation peaks around late subjective day, with 15 phosphorylation sites detected in 2 different clusters, with 1 cluster near its zinc finger domain, as in WC-1 (Schafmeier et al. 2005; Wang et al. 2019). While earlier work suggested that the mutation of 5 phosphorylation sites in WC-1 leads to arrhythmicity, it is now evident that phosphorylations on both WC-1 and WC-2 are needed to close the clock's negative feedback loop and support circadian rhythmicity (He et al. 2005; Wang et al. 2019). Plainly, both the light-dependent change in WCC structure and the overall phosphorylation state of the WCC play an important role in delineating the WCC's function in the light response pathway from its role in the positive arm of the circadian clock (Wang et al. 2016, 2019).

While the WCC is the primary transcriptional activator of the negative arm gene *frq*, there are other proteins that influence *frq* transcription through modifications of the chromatin structure at the *frq* locus. The *frq* promoter has two unique elements, the **proximal LRE (pLRE)** and the **Clock Box (c-box)** (Froehlich et al. 2003). The heterotrimeric, light-activated WCC binds to the pLRE in response to light, but the heterodimeric, dark-active WCC binds to the c-box to regulate the rhythmic expression of *frq* that is necessary for proper clock function in continual darkness (Froehlich et al. 2003; Gooch et al. 2014). This binding is facilitated by **chromatin remodelers**, including the positive regulators Clock ATPase (CATP), SWItch/SucroseNonFermentable (SWI-SNF), Chromodomain helicase DNA-binding (CHD-1), and Defective in methylation (DIM-2), and the negative regulators Clockswitch (CSW-1) and Su(var)3–9-enhancer-of-zeste-trithorax-1 and 2 (SET-1 and SET-2) (Fig. 4.2a). CATP positively regulates *frq* expression via its promotion of the removal of histones at the *frq* locus, allowing for WCC binding (Cha et al. 2013). Once the WCC binds to the c-box, it recruits the SWI-SNF complex, which removes a nucleosome that partially covers the c-box and likely loops the DNA, bringing the WCC closer to the transcription start site (Zhang et al. 2006; Wang et al. 2014). CHD-1 also contributes to necessary changes in *frq* chromatin structure to aid transcription, and DIM-2-catalyzes the removal of transient DNA methylation that helps set clock phase timing (Belden et al. 2011). The protein CSW-1 is involved in the final stages of closing the clock's feedback loop, compacting the chromatin structure at the c-box to down-regulate *frq* expression (Belden et al. 2007b). SET-1 and SET-2 further this process by methylating histones H3K4 and H3K36, respectively, making the chromatin less accessible (Raduwan et al. 2013; Sun et al. 2016). Only when these supporting proteins properly maintain the chromatin architecture of the *frq* locus can the WCC complete its positive activation of *frq* transcription to maintain rhythmicity. Circadian rhythms in histone modifications have also been detected in mammal systems, and chromatin-modifying proteins are important for the repression of positive arm clock proteins (Koike et al. 2012; Takahashi 2017).

B. The Negative Arm of the Clock

1. Oscillations in FRQ Protein and Phosphorylation Levels Determine Clock Period

Frequency's role in determining clock period was originally discovered through the isolation of "banding" (*bd*) mutants with different period lengths (Aronson et al. 1994; Feldman and Hoyle 1973; Gardner and Feldman 1980; Loros et al. 1986). In constant conditions (25 °C and constant darkness), the *bd* strain displays a conidial band once every 21.6 (± 0.5) h (Fig. 4.1). However, mutants in the *bd* strain were discovered to have periods ranging from

16.5–19.0 h (short-period mutants) to 24.0–29.0 h (long-period mutants) or to be completely arrhythmic (Feldman and Hoyle 1973; Gardner and Feldman 1980; Aronson et al. 1994). Many of the mutations in these short- and long-period strains mapped to a single locus, which was dubbed *frequency* (*frq*) (Feldman and Hoyle 1973; Gardner and Feldman 1980, 1981; Loros et al. 1986).

Full-length FRQ is a largely disordered 989 aa long protein containing several smaller potentially well-ordered domains that are involved in the formation of the negative arm protein complex. Due to the low proportion of in silico predicted structure, FRQ was believed to be an **intrinsically disordered protein (IDP)**, a protein that lacks a fixed or ordered three-dimensional structure instead sampling a hetergenous ensemble of conformations. This prediction of disorder was demonstrated biochemically as FRQ remained soluble after heat treatment and was more quickly digested by proteases (Hurley et al. 2013). While FRQ does not have sequence homologs amongst all higher eukaryotes, intrinsic disorder does appear to be conserved among negative arm proteins, suggesting that disorder is a key feature of negative arm proteins (Hurley et al. 2013).

There are two isoforms of FRQ that are the products of alternate translation start sites, Long-FRQ (989 aa) and Short-FRQ (889 aa, missing the first 100 amino acids), which are thought to contribute to temperature compensation of the clock (Garceau et al. 1997), as previously discussed in Sect. II.B. Both FRQ isoforms encompass several key protein-protein interaction domains, including the coiled-coil interaction domain that allows for the dimerization of FRQ (necessary for rhythmicity; Cheng et al. 2001a), the nuclear localization region (Luo et al. 1998), and the FRQ-CK1 interacting domains FCD1 and FCD2 (Querfurth et al. 2011). In addition, both isoforms contain two predicted proline, glutamic acid, serine, threonine (PEST) domains that may be important for protein turnover, PEST-1 and PEST-2 (Merrow and Dunlap 1994). Thus far, the only in vivo evidence that these domains act in protein turnover is that the PEST-1 region is phosphorylated before FRQ is degraded (Liu et al. 2000; Görl et al. 2001; Baker et al. 2009). Finally, the isoforms include an FRQ-FRH interacting domain called the FFD (Guo et al. 2010). These interaction domains contribute to the formation of the larger negative arm complex, the Frequency-FRH Complex (FFC) (Baker et al. 2009; Guo et al. 2010; Querfurth et al. 2011; Hurley et al. 2013).

FRQ transcript and protein levels oscillate on a daily basis as a result of the activity of the WCC. At the beginning of the circadian cycle, WCC activates *frq* expression, and approximately 4 h later there is a peak in translated FRQ levels (Garceau et al. 1997; Merrow et al. 1997). Shortly after translation, FRQ dimerizes with itself and binds to FRQ-interacting RNA Helicase (FRH) to form the FRQ/FRH complex (FFC); all FRQ is found in complex with FRH in a two to one ratio (Cheng et al. 2001a, 2005; Baker et al. 2009). This interaction helps to stabilize FRQ and is discussed further in Sect. III.B.2 (Cheng et al. 2005; Hurley et al. 2013). Many kinases act to phosphorylate FRQ (see Sect. III.D for more details) and this phosphorylation allows the FFC to enter the nucleus early on in the cycle to repress WCC activation of *frq* transcription, closing the negative feedback loop (Fig. 4.2) (Cheng et al. 2005; Hong et al. 2008; Baker et al. 2009; Cha et al. 2011). Phosphorylation eventually leads to FRQ degradation via the ubiquitin-proteasome pathway (Fig. 4.2), and a new wave of FRQ protein is translated as the circadian cycle continues (He and Liu 2005a; Larrondo et al. 2015).

In addition to direct WCC regulation, further transcriptional regulation supports robust oscillations in *frq* mRNA levels. The WCC activates transcription of *frq*'s long non-coding **antisense transcript, *qrf*** (*frq* spelled backwards) in response to light by utilizing a promoter in the 3′ UTR of *frq*, and this aids in light resetting of the clock (Kramer et al. 2003). In constant darkness, *qrf* expression is also regulated by another unknown mechanism (Xue et al. 2014). Data suggests that *qrf* silences *frq* expression to tune the timing and period of the clock, yielding more robust oscillations of *frq* by counteracting any "leaky" *frq* expression that would lead to dampening of the central oscillator. However, two competing models suggest two distinct mechanisms as to how this occurs. These include either the stalling/collision of the RNA polymerases transcribing

frq and *qrf* (Xue et al. 2014) or transcriptional permissiveness at the *frq* locus (Li et al. 2015). As the mammalian clock also contains an antisense RNA to the negative arm proteins, the answer to this debate has implications for clock regulation in higher eukaryotes (Li et al. 2015; Xue et al. 2014).

In addition to the transcriptional regulation of *frq*, there is also regulation that occurs at the level of translation. One mechanism arises from the presence of non-optimal codons within *frq*'s open reading frame. Among the codons used to code for the same amino acid, some codons occur less often than others genome-wide and these are termed **non-optimal codons.** Non-optimal codons correlate to low copy numbers of a given tRNA and therefore are thought to result in slower translation elongation rates (Zhou et al. 2013). The sequence of *frq* is biased toward more non-optimal codons and evidence suggests that this codon bias is important for the stability of the resulting FRQ protein. When more frequently occurring codons were substituted into the N-terminus of the *frq* open reading frame, the clock no longer functioned (Zhou et al. 2013). As more optimal codons are thought to lead to faster rates of translation elongation, the loss of rhythmicity was attributed to the improper folding of FRQ, similar to results obtained from the codon optimization of the negative arm protein PER in *Drosophila* (Zhou et al. 2013; Fu et al. 2016). Alternatively, and to include the more recent data showing that FRQ is an IDP, if FRQ is translated too quickly, it may not have a chance to bind with its stabilizing "Nanny" FRH (see also Sect. III.B.2), which could lead to rapid degradation (Hurley et al. 2013). Regardless of the mechanism, the conservation of non-optimal codons within *frq* appears to be essential for circadian regulation in *N. crassa* and other organisms (Xu et al. 2013; Fu et al. 2016).

A second mechanism of FRQ translational regulation lies in the many **upstream Open Reading Frames (uORFs)** of *frq*. The *frq* transcript contains an approximate 1.5 kb untranslated region at its 5′ end (5′ UTR) that includes six uORFs (Garceau et al. 1997; Liu et al. 1997). When most of the 5′ UTR was deleted, FRQ levels increased, suggesting that ribosome scanning through uORFs could decrease levels of FRQ under some conditions (Liu et al. 1997). However, unpublished experiments suggest that the deletion of those six uORFs had no effect on the clock's overall rhythmicity and did not shorten the 4 h lag between peak *frq* mRNA and peak FRQ protein levels (Garceau et al. 1997). Interestingly, uORFs are also conserved in multiple clock proteins as a regulatory mechanism in mammals (Janich et al. 2015).

2. FRQ-Interacting RNA Helicase (FRH) Supports FRQ as a "Nanny" Protein

While all FRQ is associated with FRH, only ~40% of FRH within the cell is bound to FRQ, with the other ~60% presumably carrying out other functions within the cell (Cheng et al. 2005). These functions are likely related to RNA surveillance, as FRH is a DEAD-box-type RNA Helicase protein and is homologous to Mtr4p in *Saccharomyces cerevisiae,* which assists in RNA metabolism via its interaction with the exosome (Lykke-Andersen et al. 2011). FRH interacts with exosome proteins in *N. crassa* (e.g., RRP44), has been found to bind *frq* mRNA and influence its stability, and is also a functional RNA helicase (Guo et al. 2009; Morales et al. 2018).

FRH has many homologs among fungal and animal species, including some involved in the clock complex (Cheng et al. 2005; Padmanabhan et al. 2012), and has a well-characterized and conserved overall structure (Conrad et al. 2016; Morales et al. 2018). Four domains form a ring-like structure (two canonical RecA-like domains, a small winged helix domain, and a helical DSHCT domain) and a fifth domain forms an arch domain or "arm" that spans across one side of the ring and ends in a KOW or "fist" module (Conrad et al. 2016; Morales et al. 2018). A known binding region for FRQ is found in an additional domain (aa 100–150) within the largely unstructured N-terminus, and the KOW/fist domain includes a binding site for WCC interaction that can be further modulated by an interaction with VVD (Hunt et al. 2010; Shi et al. 2010; Hurley et al. 2013; Conrad et al. 2016; Morales et al. 2018).

Given the clear homology in structure with other RNA helicases and the demonstrated ATPase RNA Helicase function, it was surprising when a strain with a point mutation (R806H) in the KOW domain of FRH had healthy growth but an arrhythmic clock (Shi et al. 2010). This suggested that FRH's ATPase function did not explain its role in the core clock. Further support for FRH playing dual roles within the cell came from an additional mutant strain where FRH lost its ATPase function yet still supported a functional clock (Hurley et al. 2013). Moreover, the downregulation of FRH caused an ~80% reduction in FRQ protein levels (Cheng et al. 2005). In total, this suggested that FRH plays a structural function to support or act as a **"Nanny" protein** for the intrinsically disordered protein FRQ and protect it from premature turnover (Hurley et al. 2013). Without the stabilizing influence of FRH, FRQ phosphorylation profiles are impacted and FRQ becomes more prevalent in the nucleus rather than the cytoplasm, highlighting the importance of FRH's Nanny role for FRQ to function in the clock (Cheng et al. 2005; Guo et al. 2010; Cha et al. 2011; Hurley et al. 2013).

C. The Negative Arm Represses the Positive Arm to Close the TTFL

While a great deal is known about the activation that drives the transcriptional portion of the TTFL, the mechanistic underpinnings of WCC repression, the "closing of the loop," is not as well understood. What is known is that the FFC represses WCC transcriptional activity at the *frq* locus via a direct interaction which involves at least three key regions of FRQ (Aronson et al. 1994; Cheng et al. 2005; He and Liu 2005a; He et al. 2006; Guo et al. 2010). Within the clock complex, WC-2 can interact independently with WC-1 and FRQ and is thought to be required for the interaction between WC-1 and FRQ (Denault et al. 2001). In addition, recent evidence shows that in the dark, the "DBD" motif in WC-1 is also necessary for the interaction with the FFC (Wang et al. 2016). Finally, FRH has also been shown to interact independently with the WCC (Hunt et al. 2010), highlighting the complexity of the interactions within and between the core clock complexes.

However, direct interactions do not explain the functional mechanism of repression, and until recently there were two competing theories of how repression occurs. The first potential mechanism involves the deactivation of the WCC via phosphorylation by FRQ-associated kinases, which is thought to lead to the repression of WCC transcriptional activation of *frq* (Cheng et al. 2001b; Froehlich et al. 2003; Schafmeier et al. 2005; He et al. 2006; Wang et al. 2019). It is known that hyper-phosphorylated WCC has a weaker affinity for DNA and that the WCC is not phosphorylated unless it interacts with FRQ (Schafmeier et al. 2005; He et al. 2006; Wang et al. 2019). Recently, the specific clusters of phosphorylation sites that are important for decreased DNA binding have been identified, with phosphorylations near the zinc-finger binding domains of both WC-1 and WC-2 being important for repression of WCC activity (Wang et al. 2019). Additionally, there is some support for a second, "clearance-based" model of repression. Research has shown that oscillations in WCC phosphorylation and de-phosphorylation may also be important for the localization of the WCC complex (Schafmeier et al. 2008; Wang et al. 2016). There is evidence that FFC binding to the WCC in the nucleus leads to the export of the WCC to the cytoplasm (Hong et al. 2008). This would suggest that repression could occur via degradation or nuclear exclusion of the WCC due to the physical interaction between the WCC and the FFC (Hong et al. 2008). In the end, neither mechanism is mutually exclusive, and a two-step process has been suggested where the WCC is removed from the DNA (by phosphorylation) and then exported to the cytoplasm and thereby sequestered (Fig. 4.2) (Cha et al. 2008). Whether closure of the clock's negative feedback loop is restricted to WCC phosphorylation, or also includes nuclear export, the study of these repression mechanisms in *N. crassa* has, and will continue, to inform the understanding of

similar mechanisms in higher eukaryotic clock systems (Yoshitane et al. 2009; Mahesh et al. 2014).

D. Post-translational Modifications in Clock Regulation

1. Kinases Play an Essential Role in Circadian Timing

Post-translational modifications, such as phosphorylation, are central to the functioning of the positive and negative arm of the core clock in *N. crassa* and other clock systems (for more complete reviews of the role of post-translational modifications in higher eukaryotic systems, see Mehra et al. 2009a; Weber et al. 2011; Hirano et al. 2016). The addition of phosphate groups by numerous kinases has been shown to affect circadian timing by altering the stability of the protein and its ability to form protein-protein interactions. In *N. crassa*, these modifications are carried out by the kinases CAMK1, CHK2 (PRD-4), CK1a, CK1b, CK2, GSK, and PKA, which act on the core proteins of the negative and positive arms of the clock (Fig. 4.2a).

As mentioned previously, FRQ is progressively phosphorylated in a circadian manner with over 85 different residues phosphorylated, making FRQ one of the most highly phosphorylated proteins ever documented (Baker et al. 2009; Tang et al. 2009). CK1a has been suggested to carry out much of this phosphorylation, as CK1a is found in complex with FRQ and FRH throughout a large proportion of the circadian day (Baker et al. 2009). Moreover, when FRQ is not able to interact with CK1a, FRQ is in a hypo-phosphorylated state and shows increased stability (He et al. 2006; Querfurth et al. 2011). In addition, other kinases such as CK2, PKA, CHK2 (PRD-4), CK1b, and CAMK-1 interact more transiently with FRQ and likely play a smaller role in its phosphorylation and circadian regulation (Yang et al. 2001, 2002; Pregueiro et al. 2006; He et al. 2006; Huang et al. 2007).

Throughout the daily cycle, different regions of FRQ are phosphorylated in a clustered manner by interacting kinases, and the phosphorylation of these different FRQ segments can variously stabilize or de-stabilize this protein (Baker et al. 2009). The kinase PKA increases FRQ stability, while CK1a, CK2, and CHK2 decrease FRQ stability over a circadian cycle (Pregueiro et al. 2006; Huang et al. 2007). Phosphorylation also regulates FRQ's ability to interact with other proteins. The extensive phosphorylation of FRQ affects its half-life through increased affinity with ubiquitin ligases, such as FWD-1 (He et al. 2003; also see Sect. III.D). In addition, FRQ's phosphorylation by CK2 decreases its ability to interact with the WCC, necessary for the start of another round of transcription in the circadian loop (Cheng et al. 2001b; Yang et al. 2002). Besides targeting FRQ for eventual degradation via the ubiquitin-proteasome pathway (Fig. 4.2a), these phosphorylations may affect FRQ's IDP conformation to regulate the time-specific binding of its different interaction partners to complete the negative feedback loop that determines clock period (He et al. 2006; Baker et al. 2009; Querfurth et al. 2011; Hurley et al. 2013; Pelham et al. 2018).

There is also evidence that phosphorylation regulates the positive arm of the clock, influencing the activity, interactions, and degradation of the WCC. The WCC is able to bind to the *frq* c-box promoter when hypo-phosphorylated, but its transcriptional activity is reduced when the WCC is phosphorylated first by PKA, which acts as a priming kinase, followed by CK1a and CK2 in a FRQ-dependent manner (He et al. 2006; Huang et al. 2007; Wang et al. 2019). Beyond the canonical circadian kinases, Glycogen synthase kinase (GSK) phosphorylates both WC-1 and WC-2, presumably promoting WCC degradation via phosphorylation, and the resultant decrease in WCC levels lengthens the circadian period (Tataroğlu et al. 2012). Like WC-1, WC-2 shows a circadian oscillation in phosphorylation (Schafmeier et al. 2005), with increasing WC-2 phosphorylation leading to a decrease in overall WCC activity.

2. Phosphatases Support the Proper Timing of Circadian Period and Phase

Phosphatases are a group of widely conserved post-transcriptional modifiers that remove phosphate groups and have demonstrated importance for proper clock function. Three protein phosphatases, PP1, PP2A, and PP4, all play a role in supporting the core clock (Cha et al. 2008; Yang et al. 2004). PP1 and PP4 support FRQ stability and therefore proper phase timing and period length, and PP2A is important for maintaining the proper *frq* mRNA level (Yang et al. 2004; Cha et al. 2008). However, the most detailed study of FRQ phosphorylation showed only progressive phosphorylation. Without in vivo evidence for de-phosphorylation, the effects of phosphatases on FRQ may thus be indirect (Baker et al. 2009). Indeed, some of these phosphatases also act on the WCC and thereby may affect *frq* levels and FRQ protein via the core feedback loop. For example, PP2A and PP4 de-phosphorylate the WCC, while it is in the cytosol. This is important as this de-phosphorylation potentially impacts the ability of the WCC to re-enter the nucleus and start a new round of transcription, suggesting that the major impact of phosphatases on FRQ is via the positive arm (Fig. 4.2a) (Cha et al. 2008; Schafmeier et al. 2005, 2008). Phosphatases also play an important role in the circadian clocks of *Drosophila* and mammals, modulating the phosphorylation state of clock proteins such that an approximately 24 h period is maintained (Reischl and Kramer 2011).

E. Ubiquitination and Degradation Contribute to a Robust Circadian Clock

While not all phosphorylation leads to degradation, the overall progressive phosphorylation of FRQ is associated with its regulated turnover by the **ubiquitin-proteasome pathway** (Garceau et al. 1997; Liu et al. 2000; Baker et al. 2009; Guo et al. 2010). Following the phosphorylation of FRQ, particularly in the PEST-1 region, FRQ is ubiquitinated by the F-box/WD40 repeat-containing protein FWD-1, which promotes FRQ's degradation via the proteasome (Fig. 4.2a) (He et al. 2003). FWD-1 is part of an SKP/Cullin/F-box (SCF)-type E3 ubiquitin ligase complex that can bind phosphorylated FRQ motifs, with more FRQ phosphorylation presenting more potential FWD-1 binding sites (He et al. 2003; He and Liu 2005a). When *fwd-1* is knocked out, the resulting strain is overtly arrhythmic; hyperphosphorylated FRQ accumulates to high levels and conidial banding is lost (He et al. 2003; He and Liu 2005a). However, recent work using a **luciferase reporter** system has shown that FRQ degradation is not strictly required for the completion of the circadian feedback loop and the maintenance of period length (Fig. 4.2b) (Larrondo et al. 2015). By measuring in vivo luminescence from a strain expressing luciferase under the control of a segment from the promoter of *frq* that contains the c-box, circadian rhythms in *frq* transcriptional activation were found even when *fwd-1* was knocked out (Larrondo et al. 2015). This suggests that negative arm protein degradation is not required for the core circadian oscillator and is not actually a determinant of clock period, and has since been confirmed in a mammalian system (Larrondo et al. 2015; Ode et al. 2017).

Even though FRQ degradation may not be required for clock functionality, premature FRQ degradation still leads to arrhythmic strains (Guo et al. 2010; Hurley et al. 2013). As an intrinsically disordered protein (IDP), without its stabilizing FRH "Nanny," FRQ is rapidly degraded within 3 h through a passive "degradation by default" pathway that does not involve ubiquitination (Tsvetkov et al. 2009; Guo et al. 2010; Hurley et al. 2013). Only when protected by FRH can FRQ have a chance to mature and carry out its circadian functions before undergoing ubiquitination and targeted degradation. This degradation by default pathway also has implications for mammal clock systems, since the negative arm PER proteins are also predicted to be IDPs (Hurley et al. 2013).

IV. Output: Clock Output Regulates Cellular Physiology at the Transcriptional and Post-transcriptional Levels

The core circadian oscillator affects many processes within the cell via transcriptional and post-transcriptional regulation, termed the clock's "output" (Vitalini et al. 2006; Hurley et al. 2014, 2018; Sancar et al. 2015). Regulation occurs at multiple levels, including the regulation of transcriptional cascades by the positive arm of the clock and the more recent evidence of post-transcriptional regulation of protein levels (Smith et al. 2010; Hurley et al. 2014, 2018). For the purposes of this review, we will discuss output in two sections: transcriptional output and post-transcriptional output.

A. Transcriptional Regulation of Cellular Output via the Circadian Clock

1. Circadian Transcriptional Activation of Clock-Controlled Genes (*ccgs*)

The positive arm of the TTFL in animal and fungal clocks acts as a "pioneer-like" transcription factor to remodel chromatin and activate the transcription of the negative arm as well as a host of other genes, including other transcription factors (Hurley et al. 2014; Menet et al. 2014; Smith et al. 2010). Chromatin-immunoprecipitation (ChIP) sequencing identified that the WCC directly targets ~300 genes near circadian dawn (Smith et al. 2010; Hurley et al. 2014). Included in these WCC targets are additional transcription factors that regulate further groups of genes, creating a circadian transcription factor cascade. Among the detected WCC targets, a total of 28 transcription factors were found, including *ccg-9*, *sah-1*, *nit-2*, *hsf-2*, *adv-1*, *bek-1*, *sub-1*, *sah-2*, *sre*, *mip-1*, and *vad-2* (Smith et al. 2010). The binding motif for the WCC is "GATCGA," a sequence that is also found in the c-box and LRE of the *frq* promoter (Froehlich et al. 2002; Pavesi et al. 2004; He and Liu 2005b; Carlson et al. 2007; Smith et al. 2010).

Deep sequencing has shown that the transcriptional regulation stemming from the positive arm leads to up to 40% of genes within *N. crassa* undergoing rhythmic changes in transcript levels under constant conditions; these genes are termed clock-controlled genes or *ccgs* (Fig. 4.2a) (Hurley et al. 2014; Loros et al. 1989; Sancar et al. 2015). When the promoter sequences of all detected *ccgs* were analyzed, four motifs were found to be significantly enriched at certain times of day: STACASTA, GCRCTAAC, GRCGGGA, and GVCAGCCA, with each motif associated with essential cellular processes (Hurley et al. 2014). It is important to note that the set of *ccgs* varies to some extent depending on what growth media is used, showing that the clock can adjust its output to current metabolic conditions (Hurley et al. 2014). The extent and diversity of *ccgs* highlights the broad effect of circadian regulation in the cell and how the clock can fine-tune cellular output based on its metabolic environment. The diversity of output is also seen in higher eukaryotes, where as much as 80% of genes fell under circadian regulation when a wide variety of tissues were analyzed (Mure et al. 2018).

2. Circadian Transcriptional Repression of *ccgs* Is Vital to Clock Output

While the positive arm is generally considered a transcriptional activator, some of its direct or indirect targets are known transcriptional repressors. We have already reviewed some of these transcriptional repressors in the context of input signals to the clock (see Sect. II for discussions of VVD, CSP-1, RCO-1, and RCM-1). In addition to being a clock nutrient sensor and WC-1 transcriptional repressor, CSP-1 is also a direct target of the WCC and is expressed in a rhythmic fashion (Lambreghts et al. 2009; Smith et al. 2010). Forming a complex with RCO-1 and RCM-1, it modulates the expression of ~800, mostly evening-specific, genes in *N. crassa* that are predominantly involved in the metabolism of lipids and glucose (Sancar et al. 2011; see Sect. II). It is predicted that CSP-1 regulates evening-specific gene expression by

morning-specific repression of CSP-1 target genes (Sancar et al. 2011).

Carbon catabolite regulation-1 (CRE-1) is also a WCC-regulated repressor that interacts with RCO-1 and RCM-1 to inhibit the transcription of genes related to the use of alternative carbon sources when glucose is present, otherwise known as **carbon catabolite repression** (Bailey and Arst 1975; Hurley et al. 2014; Cupertino et al. 2015; Adnan et al. 2017). Among other things, CRE-1 is an inhibitor of glycogen synthesis and represses glycogenic genes, such as *gsn*, *gbn*, and *gnn* (Cupertino et al. 2015). Interestingly, the WCC and other WCC-target transcription factors, such as Viability Of Spores-1 (VOS-1) and CSP-1, also target these glycogenic genes, highlighting the complexity of clock regulation of *ccgs* (Smith et al. 2010; Baek et al. 2019). An additional example, the NOT1-CCR4 (negative on TATA 1-carbon catabolite repression 4) complex regulates WCC stability and is important for circadian phase determination (Fig. 4.2a) (Huang et al. 2013). NOT1 acts directly on the WCC and its mRNA displays circadian oscillations in a similar phase to *frq* (Huang et al. 2013). When NOT1 or CCR4 levels are reduced, the clock's phase is delayed by 3–4 h (Huang et al. 2013). These examples show that not all clock output involves transcriptional activation but that transcriptional repressors are also important for the clock, including some clock-regulated repressors that feedback onto the core clock itself.

B. Post-transcriptional Regulation via the Circadian Clock

While transcriptional regulation has long been considered the primary output of the circadian clock, recent research suggests that clock output is also regulated post-transcriptionally. The initial evidence for this **post-transcriptional regulation** is the mismatch between promoter activation and steady state mRNA levels for a given gene (Fig. 4.3a). There are many genes whose promoters are rhythmically activated but whose steady-state mRNA levels remain constant and vice versa (Hurley et al. 2014). This post-transcriptional regulation occurs at the protein level as well, as approximately one quarter of reliably detected proteins oscillate with a circadian period but 40% of these arise from transcripts that are not oscillating (Fig. 4.3a) (Hurley et al. 2018). Moreover, post-transcriptional regulation appears to be circadianly directed. For example, differential peak phase timing of enzymes that are rhythmic only at the protein level within central metabolic pathways yields a coordination of peak timing within pathways such as glycolysis and the TCA cycle that peak anti-phase to the alternate pentose-phosphate pathway (Hurley et al. 2018). Many similar studies in higher eukaryotes demonstrate that circadian post-transcriptional regulation is conserved (Beckwith and Yanovsky 2014; Koike et al. 2012; Kojima et al. 2011).

There are several circadianly regulated mechanisms that impart circadian post-transcriptional output, including mRNA degradation, translation rates, and protein degradation. The FFC interacts with components of the exosome, which facilitate a circadian influence over mRNA degradation (Guo et al. 2009). The clock also regulates **MAPK pathways** that impact the phosphorylation state of translation factors, such as translation elongation factor eEF-2 (Lamb et al. 2011, 2012; Bennett et al. 2013; Caster et al. 2016). eEF-2 has lower phosphorylation levels and therefore higher activity near subjective dusk, a time when there seems to be more energy available for translation, as well as more translation initiation factors and ribosomes (Fig. 4.3b) (Caster et al. 2016; Hurley et al. 2018; Kafri et al. 2016). Finally, circadian regulation of E3 ubiquitin ligases could yield rhythms in protein degradation (Lück et al. 2014; Hurley et al. 2018). As clock regulation affects numerous additional cell regulatory systems, there may be many other avenues through which the clock can direct post-transcriptional regulation (Hurley et al. 2014, 2018; Sancar et al. 2015).

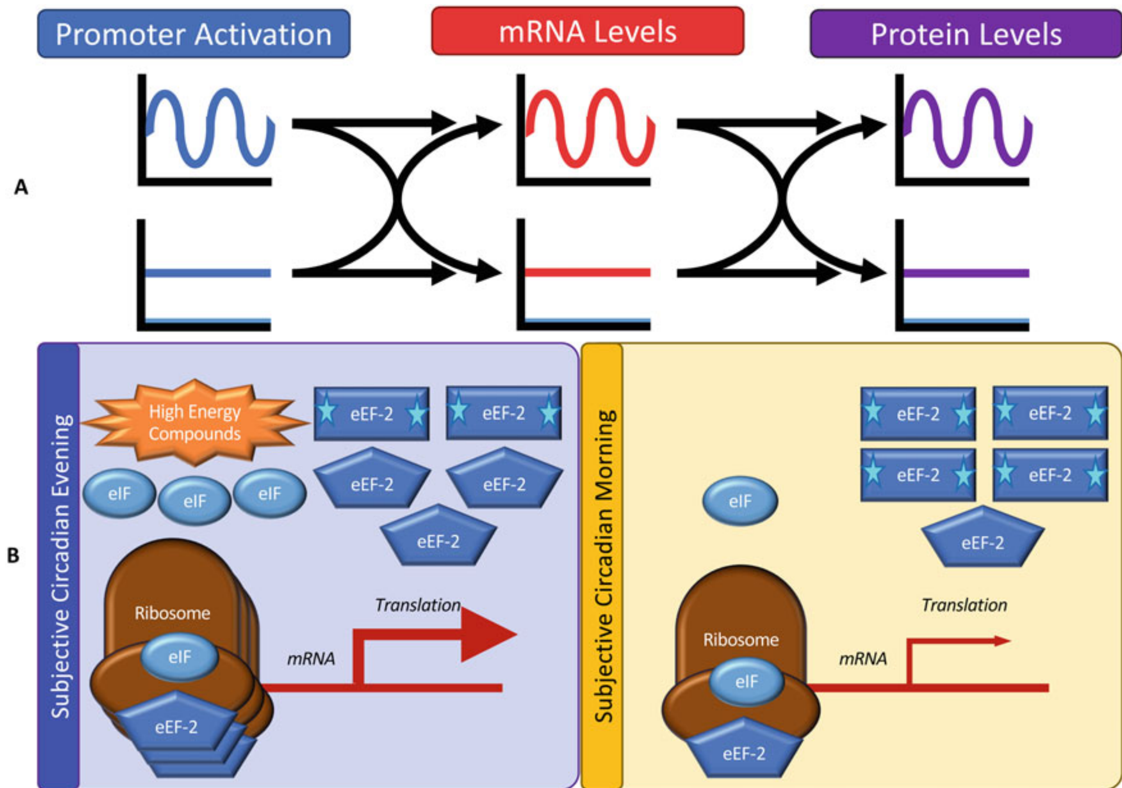

Fig. 4.3 Circadian output is highly impacted by post-transcriptional regulation. (**a**) Circadianly regulated/rhythmic promoter activity or steady-state mRNA levels do not necessarily lead to oscillations at the level of the mRNA or protein. Similarly, oscillations at the level of mRNA or protein can happen despite constitutive promoter activity or steady-state mRNA levels. This suggests there is a significant amount of post-transcriptional regulation on circadian output. (**b**) There are some proposed mechanisms that may underlie circadian post-transcriptional regulation. First, excess amounts of high energy compounds, active ribosomes, and eukaryotic initiation factors (eIF) are available in the subjective circadian evening in *Neurospora crassa*, when rhythmic proteins reach peak levels. Second, the *N. crassa* translation factor eukaryotic elongation factor 2 (eEF-2) is less phosphorylated (cyan stars) in the circadian evening, which increases the translation elongation rate, likely yielding more proteins reaching peak levels at this time

C. Circadian Output Interfaces with Many Cellular Systems

The output generated from the circadian oscillator regulates many vital processes within the cell, the foremost of which is cellular **metabolism**. There are connections between the clock and metabolism that result in changes in clock output depending on the type of growth media, as discussed in Sect. IV.A.1, as well as some ion cycles that may in fact be circadianly-regulated (Hurley et al. 2014, 2016b; Feeney et al. 2016; Dunlap and Loros 2016). Overall, gene ontologies related to metabolic functions are highly enriched for *ccgs* at both the transcript and protein levels (Hurley et al. 2014, 2018; Sancar et al. 2015). These include, at the transcriptional level, the division between anabolic and catabolic pathways between subjective night and subjective day, while at the protein level, there is an overall peak in enzymes in many energy pathways near circadian dusk (Hurley et al. 2014, 2018; Sancar et al. 2015). Other studies have looked at oscillations within the cell that may occur outside the circadian feedback loop involving the WCC and the FFC, though many of these oscillations are not in fact compensated and so cannot be considered

truly circadian (see Dunlap and Loros 2017 for a recent review). While there is much more to learn about these cellular oscillations, it is interesting that studies continue to find previously unknown links between these oscillations and the circadian clock, such as a widely conserved daily oscillation in Mg^{2+}, which can now be explained by clock regulation of genes involved in both active ion transport (via pumps) and passive ion channels (Feeney et al. 2016; Dunlap and Loros 2016).

In addition, the clock plays a role in the regulation of the cell cycle. Hong et al. (2014) found that there was an overall circadian rhythm in **mitosis**, with most cells dividing in the evening, while morning is the peak time for cells to be in interphase. The circadian clock rhythmically regulates the kinase STK-29 and the G1 and G2 cyclins, CLN-1 and CLB-1, which in turn regulate entry into mitosis (Hong et al. 2014). The clock and cell cycle are also interconnected by Chk2 (PRD-4 in *N. crassa*), a key kinase in both processes, and it is through this kinase that the cell cycle can impact the clock (Pregueiro et al. 2006). Chk2 binds to FRQ and phosphorylates it in a DNA damage-dependent manner, triggering destabilization and leading to FRQ degradation, shifting the phase of the clock and enabling circadian responsiveness to DNA damage (Gamsby et al. 2009; Pregueiro et al. 2006). This research in *N. crassa* demonstrates that the cross-talk between the circadian clock and cellular systems contributes to the optimal timing of cellular growth and development, important relationships that are conserved and are now being elucidated in higher eukaryotes (e.g., Gaucher et al. 2018; Reinke and Asher 2019).

V. Methods for Detecting Circadian Rhythms

As noted earlier (see Sect. I), for something to be defined as a circadian rhythm, the period must be free running and at or near 24 h. Therefore, to identify circadian rhythms in *Neurospora crassa*, it is standard procedure to collect a time series of regularly recorded observations under constant conditions, after a strong resetting cue is given. From this time series, the period, amplitude, and phase can be calculated, as it can be for any oscillatory wave. To look at rhythms in vivo in *N. crassa*, two common methods are utilized: the “band” or *bd* mutant strain background and a luciferase reporter system. To track the period of the clock using the *bd* mutant background, the strain of interest with a *bd* background is grown in constant conditions on a race-tube, a long glass tube with agar media in the bottom (Fig. 4.1). Bands of conidia and aerial hyphae form at regular intervals as the strain grows and the periodicity of this banding is linked to the circadian clock (Brandt 1953; Pittendrigh et al. 1959). This banding is used as a proxy for the clock but in reality is a measure of the periodicity of the clock’s output.

To look more directly at the core clock, a luciferase reporter system was developed using an *N. crassa* codon-optimized luciferase gene from fireflies fused to a promoter region or gene of interest. When fused to the core clock gene *frq* or its promoter, this system allows for the tracking of changes in steady-state protein levels or transcriptional activity, respectively, over the course of multiple days (Gooch et al. 2008; Larrondo et al. 2012; Morgan et al. 2003). With the advent of RNA microarrays, RNA-sequencing and mass spectrometry, ex vivo methods of tracking rhythms in mRNA or protein levels have allowed further investigation of circadian regulation within the cell. Samples are extracted from tissue grown under constant conditions, which has been shown to preserve the oscillations in clock genes and proteins in *N. crassa* (Loros et al. 1989). From the extracted samples, a variety of assays and computational approaches can be performed to measure rhythms in the core clock and identify clock output, including Western blots, qPCR, RNA-seq, and proteomics (e.g., Hurley et al. 2014, 2018). Novel techniques and technologies are developed in *N. crassa* on a regular basis to allow circadian biologists to learn more about the core clock and its regulation of cellular output (e.g., De los Santos et al. 2017; Pelham et al. 2018).

VI. Clock Conservation in Fungi

Neurospora crassa is not only a model for clocks in higher eukaryotes but is a model for clocks in other fungi. An analysis of 64 fungal genomes based on the *N. crassa* circadian genes *frq*, *wc-1*, *wc-2*, *frh*, and *fwd-1* (Salichos and Rokas 2010) showed that FRH and FWD-1 were the earliest conserved clock proteins, not surprising based on their other essential functions in the organism (Jonkers and Rep 2009; Putnam and Jankowsky 2013). WC-1 and WC-2 are further along the evolutionary tree and FRQ is the most recently evolved protein, with sequential homologs appearing in only three classes within the Ascomycetes: Sordariomycetes, Leotiomycetes, and Dothideomycetes (Salichos and Rokas 2010). However, as FRQ is a known IDP, and it has been documented that there is little conservation of sequence among IDPs, it is likely that the functional homologs of FRQ may not have sequence homology and that other negative arm proteins exist in the wider fungal families (Hurley et al. 2013). Though many species contain homologs of clock proteins, and some even display oscillatory phenotypes (e.g., *Podospora anserina*), so far only a few fungi that display measurable phenotypic or molecular rhythms have been shown to be truly circadian, such as the rhythms in Ascomycetes *Sordaria fimicola*, *Pyronema confluens*, *Botrytis cinerea*, *Aspergillus flavus*, and *Cercospora kikuchii* and the Basidiomycete *Neonothopanus gardneri* (Brandt 1953; Austin 1968; Lysek and Esser 1970; Greene et al. 2003; Bluhm et al. 2010; Hevia et al. 2015, 2016; Traeger and Nowrousian 2015).

B. cinerea has the best molecular and phenotypic evidence for a fungal circadian clock outside of *N. crassa* (Montenegro-Montero et al. 2015). It has orthologues of *frq*, *wc*-1, and *wc*-2 (*bcfrq1*, *bcwcl1*, and *bcwcl2*, respectively), and the clock has been shown to maximize virulence through the control of its sexual and asexual cycle (Schumacher and Tudzynski 2012; Canessa et al. 2013; Hevia et al. 2015). In contrast, while the *Aspergillus* family does not contain a recognized *frq* homolog, they do have *wc-1* and *wc-2* homologs and evidence of circadian regulation. The level of the *gpdA* gene may oscillate under free running conditions in *A. nidulans*, and *A. flavus* has rhythms in sclerotia formation (Greene et al. 2003). *Cercospora kikuchii*, of the Dothideomycetes subclass, displays phenotypic rhythms through concentric hyphal rings which persist on Petri dishes under free-running conditions for several days that are both temperature-compensated and dependent on a white collar-like gene (Bluhm et al. 2010). Through these examples it can be seen that the clock of *N. crassa* can be used to gain further insights into the clocks of other fungi.

VII. Summary

Neurospora crassa is a key model organism for research into the molecular mechanism of the circadian clock. All of the above discussed elements of clock function were, at least in part, discovered through the use of molecular and genetic techniques in *N. crassa*. The more that is understood about the clock in *N. crassa*, including the similarity of its basic architecture and circadian output to the clocks in higher eukaryotes, the more we understand what this organism has to contribute as a model system to studies of clocks in other eukaryotes. It is therefore likely that *N. crassa* will continue to contribute as a model for circadian and fungal research for years to come.

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References

Adhvaryu K, Firoozi G, Motavaze K, Lakin-Thomas P (2016) PRD-1, a component of the circadian system of *Neurospora crassa*, is a member of the DEAD-box RNA helicase family. J Biol Rhythm 31:258–271. https://doi.org/10.1177/0748730416639717

Adnan M, Zheng W, Islam W et al (2017) Carbon catabolite repression in filamentous Fungi. Int J Mol Sci 19:48. https://doi.org/10.3390/ijms19010048

Aronson BD, Johnson KA, Dunlap JC (1994) Circadian clock locus frequency: protein encoded by a single open reading frame defines period length and temperature compensation. Proc Natl Acad Sci U S A 91:7683–7687

Austin B (1968) An endogenous rhythm of spore discharge in *Sordaria fimicola*. Ann Bot 32:262–278

Baek M, Virgilio S, Lamb TM et al (2019) Circadian clock regulation of the glycogen synthase (*gsn*) gene by WCC is critical for rhythmic glycogen metabolism in *Neurospora crassa*. Proc Natl Acad Sci 116:10435–10440. https://doi.org/10.1073/pnas.1815360116

Bailey C, Arst HNJ (1975) Carbon catabolite repression in *Aspergillus nidulans*. Eur J Biochem 51:573–577. https://doi.org/10.1111/j.1432-1033.1975.tb03958.x

Baker CL, Kettenbach AN, Loros JJ et al (2009) Quantitative proteomics reveals a dynamic Interactome and phase-specific phosphorylation in the *Neurospora* circadian clock. Mol Cell 34:354–363. https://doi.org/10.1016/j.molcel.2009.04.023

Ballario P, Macino G (1997) White collar proteins: PASsing the light signal in *Neurospora crassa*. Trends Microbiol 5:458–462. https://doi.org/10.1016/S0966-842X(97)01144-X

Ballario P, Vittorioso P, Magrelli A et al (1996) White collar-1, a central regulator of blue light responses in *Neurospora*, is a zinc finger protein. EMBO J 15:1650–1657. https://doi.org/10.1002/j.1460-2075.1996.tb00510.x

Ballario P, Talora C, Galli D et al (1998) Roles in dimerization and blue light photoresponse of the PAS and LOV domains of *Neurospora crassa* white collar proteins. Mol Microbiol 29:719–729. https://doi.org/10.1046/j.1365-2958.1998.00955.x

Beadle GW, Tatum EL (1941) Genetic control of biochemical reactions in *Neurospora*. Proc Natl Acad Sci 27:499

Beckwith EJ, Yanovsky MJ (2014) Circadian regulation of gene expression: at the crossroads of transcriptional and post-transcriptional regulatory networks. Curr Opin Genet Dev 27:35–42. https://doi.org/10.1016/j.gde.2014.03.007

Belden WJ, Larrondo LF, Froehlich AC et al (2007a) The band mutation in *Neurospora crassa* is a dominant allele of *ras-1* implicating RAS signaling in circadian output. Genes Dev 21:1494–1505. https://doi.org/10.1101/gad.1551707

Belden WJ, Loros JJ, Dunlap JC (2007b) Execution of the circadian negative feedback loop in *Neurospora* requires the ATP-dependent chromatin-remodeling enzyme CLOCKSWITCH. Mol Cell 25:587–600. https://doi.org/10.1016/j.molcel.2007.01.010

Belden WJ, Lewis ZA, Selker EU et al (2011) CHD1 remodels chromatin and influences transient DNA methylation at the clock gene frequency. PLoS Genet 7:e1002166. https://doi.org/10.1371/journal.pgen.1002166

Bell-Pedersen D, Cassone VM, Earnest DJ et al (2005) Circadian rhythms from multiple oscillators: lessons from diverse organisms. Nat Rev Genet 6:544–556. https://doi.org/10.1038/nrg1633

Bennett LD, Beremand P, Thomas TL, Bell-Pedersen D (2013) Circadian activation of the mitogen-activated protein kinase MAK-1 facilitates rhythms in clock-controlled genes in *Neurospora crassa*. Eukaryot Cell 12:59–69. https://doi.org/10.1128/EC.00207-12

Bluhm BH, Burnham AM, Dunkle LD (2010) A circadian rhythm regulating hyphal melanization in *Cercospora kikuchii*. Mycologia 102:1221–1228. https://doi.org/10.3852/09-041

Brandt WH (1953) Zonation in a prolineless strain of *Neurospora*. Mycologia 45:194–208

Buhr ED, Yoo S-H, Takahashi JS (2010) Temperature as a universal resetting cue for mammalian circadian oscillators. Science 330:379–385. https://doi.org/10.1126/science.1195262

Canessa P, Schumacher J, Hevia MA et al (2013) Assessing the effects of light on differentiation and virulence of the plant pathogen *Botrytis cinerea*: characterization of the white collar complex. PLoS One 8:e84223. https://doi.org/10.1371/journal.pone.0084223

Carlson JM, Chakravarty A, DeZiel CE, Gross RH (2007) SCOPE: a web server for practical de novo motif discovery. Nucleic Acids Res 35:W259–W264

Case ME, Griffith J, Dong W et al (2014) The aging biological clock in *Neurospora crassa*. Ecol Evol 4:3494–3507. https://doi.org/10.1002/ece3.1202

Caster SZ, Castillo K, Sachs MS, Bell-Pedersen D (2016) Circadian clock regulation of mRNA translation through eukaryotic elongation factor eEF-2. Proc Natl Acad Sci 113:9605–9610. https://doi.org/10.1073/pnas.1525268113

Cha J, Chang SS, Huang G et al (2008) Control of WHITE COLLAR localization by phosphorylation is a critical step in the circadian negative feedback process. EMBO J 27:3246–3255. https://doi.org/10.1038/emboj.2008.245

Cha J, Yuan H, Liu Y (2011) Regulation of the activity and cellular localization of the circadian clock protein FRQ. J Biol Chem 286:11469–11478. https://doi.org/10.1074/jbc.M111.219782

Cha J, Zhou M, Liu Y (2013) CATP is a critical component of the *Neurospora* circadian clock by regulating the nucleosome occupancy rhythm at the frequency locus. EMBO Rep 14:923–930. https://doi.org/10.1038/embor.2013.131

Chen CH, Ringelberg CS, Gross RH et al (2009) Genome-wide analysis of light-inducible responses reveals hierarchical light signalling in

Neurospora. EMBO J 28:1029–1042. https://doi.org/10.1038/emboj.2009.54

Chen C-H, DeMay BS, Gladfelter AS et al (2010) Physical interaction between VIVID and white collar complex regulates photoadaptation in *Neurospora*. Proc Natl Acad Sci 107:16715–16720

Cheng P, Yang Y, Heintzen C, Liu Y (2001a) Coiled-coil domain-mediated FRQ-FRQ interaction is essential for its circadian clock function in *Neurospora*. EMBO J 20:101–108. https://doi.org/10.1093/emboj/20.1.101

Cheng P, Yang Y, Liu Y (2001b) Interlocked feedback loops contribute to the robustness of the *Neurospora* circadian clock. Proc Natl Acad Sci U S A 98:7408–7413. https://doi.org/10.1073/pnas.121170298

Cheng P, Yang Y, Gardner KH, Liu Y (2002) PAS domain-mediated WC-1/WC-2 interaction is essential for maintaining the steady-state level of WC-1 and the function of both proteins in circadian clock and light responses of *Neurospora*. Mol Cell Biol 22:517–524. https://doi.org/10.1128/MCB.22.2.517-524.2002

Cheng P, Yang Y, Wang L et al (2003) WHITE COLLAR-1, a multifunctional *Neurospora* protein involved in the circadian feedback loops, light sensing, and transcription repression of *wc-2*. J Biol Chem 278:3801–3808. https://doi.org/10.1074/jbc.M209592200

Cheng P, He Q, He Q et al (2005) Regulation of the *Neurospora* circadian clock by an RNA helicase. Genes Dev 19:234–241. https://doi.org/10.1101/gad.1266805.234

Chiu JC, Ko HW, Edery I (2011) NEMO/NLK phosphorylates PERIOD to initiate a time-delay phosphorylation circuit that sets circadian clock speed. Cell 145:357–370. https://doi.org/10.1016/j.cell.2011.04.002

Collett MA, Garceau N, Dunlap JC, Loros JJ (2002) Light and clock expression of the *Neurospora* clock gene frequency is differentially driven by but dependent on WHITE COLLAR-2. Genetics 160:149–158

Colot HV, Loros JJ, Dunlap JC (2005) Temperature-modulated alternative splicing and promoter use in the circadian clock gene frequency. Mol Biol Cell 16:5563–5571. https://doi.org/10.1091/mbc.E05-08-0756

Conrad KS, Hurley JM, Widom J et al (2016) Structure of the frequency – interacting RNA helicase : a protein interaction hub for the circadian clock. EMBO J 35:4–7. https://doi.org/10.15252/embj.201694327

Crosthwaite SK, Dunlap JC, Loros JJ (1997) *Neurospora wc-1* and *wc-2*: transcription, Photoresponses, and the origins of circadian rhythmicity. Science 276 (80):763–769

Cupertino FB, Virgilio S, Freitas FZ et al (2015) Regulation of glycogen metabolism by the CRE-1, RCO-1 and RCM-1 proteins in *Neurospora crassa*. The role of CRE-1 as the central transcriptional regulator. Fungal Genet Biol 77:82–94. https://doi.org/10.1016/j.fgb.2015.03.011

Dasgupta A, Chen C-H, Lee C et al (2015) Biological significance of photoreceptor photocycle length: VIVID photocycle governs the dynamic VIVID-white collar complex pool mediating photo-adaptation and response to changes in light intensity. PLoS Genet 11:e1005215

De los Santos H, Collins EJ, Hurley JM, Bennett KP (2017) Circadian rhythms in *Neurospora* exhibit biologically relevant driven and damped harmonic oscillations. In: Proceedings of the 8th ACM international conference on bioinformatics, computational biology, and health informatics. ACM, New York, NY, pp 455–463

DeCoursey PJ (2014) Survival value of suprachiasmatic nuclei (SCN) in four wild sciurid rodents. Behav Neurosci 128:240–249

DeCoursey PJ, Krulas JR, Mele G, Holley DC (1997) Circadian performance of suprachiasmatic nuclei (SCN)-lesioned antelope ground squirrels in a desert enclosure. Physiol Behav 62:1099–1108. https://doi.org/10.1016/S0031-9384(97)00263-1

DeCoursey PJ, Walker JK, Smith SA (2000) A circadian pacemaker in free-living chipmunks: essential for survival? J Comp Physiol A 186:169–180. https://doi.org/10.1007/s003590050017

Denault DL, Loros JJ, Dunlap JC (2001) WC-2 mediates WC-1–FRQ interaction within the PAS protein-linked circadian feedback loop of *Neurospora*. EMBO J 20:109

Diernfellner ACR, Schafmeier T, Merrow MW, Brunner M (2005) Molecular mechanism of temperature sensing by the circadian clock of *Neurospora crassa*. Genes Dev 19:1968–1973. https://doi.org/10.1101/gad.345905

Diernfellner A, Colot HV, Dintsis O et al (2007) Long and short isoforms of *Neurospora* clock protein FRQ support temperature-compensated circadian rhythms. FEBS Lett 581:5759–5764. https://doi.org/10.1016/j.febslet.2007.11.043

Dovzhenok AA, Baek M, Lim S, Hong CI (2015) Mathematical modeling and validation of glucose compensation of the *Neurospora* circadian clock. Biophys J 108:1830–1839. https://doi.org/10.1016/j.bpj.2015.01.043

Dunlap JC (1999) Molecular bases for circadian clocks. Cell 96:271–290. https://doi.org/10.1016/S0092-8674(00)80566-8

Dunlap JC (2008) Salad days in the rhythms trade. Genetics 178:1–13

Dunlap JC, Loros JJ (2016) Yes, circadian rhythms actually do affect almost everything. Cell Res 26:759–760. https://doi.org/10.1038/cr.2016.65

Dunlap JC, Loros JJ (2017) Making time: conservation of biological clocks from fungi to animals. Microbiol Spectr 5(3). https://doi.org/10.1128/microbiolspec.FUNK-0039-2016

Dunlap JC, Loros JJ (2018) Just-so stories and origin myths: phosphorylation and structural disorder in circadian clock proteins. Mol Cell 69:165–168. https://doi.org/10.1016/j.molcel.2017.11.028

Dunlap JC, Borkovich KA, Henn MR et al (2007) Enabling a community to dissect an organism: overview of the *Neurospora* functional genomics project. Adv Genet 57:49–96. https://doi.org/10.1016/S0065-2660(06)57002-6

Eide EJ, Woolf MF, Kang H et al (2005) Control of mammalian circadian rhythm by CKI-regulated proteasome-mediated PER2 degradation. Mol Cell Biol 25:2795–2807. https://doi.org/10.1128/MCB.25.7.2795-2807.2005

Emerson JM, Bartholomai BM, Ringelberg CS et al (2015) Period-1 encodes an ATP-dependent RNA helicase that influences nutritional compensation of the *Neurospora* circadian clock. Proc Natl Acad Sci U S A 112:15707–15712. https://doi.org/10.1073/pnas.1521918112

Feeney KA, Hansen LL, Putker M et al (2016) Daily magnesium fluxes regulate cellular timekeeping and energy balance. Nature 532:375

Feldman JF, Hoyle MN (1973) Isolation of circadian clock mutants of *Neurospora crassa*. Genetics 75:605–613

Filippovich SY, Bachurina GP, Shcherbakov DL (2015) Quantitative assessment of the combined effect of the nitrogen status, light and dehydration of mycelium on conidiation in *Neurospora crassa*. Appl Biochem Microbiol 51:342–349. https://doi.org/10.1134/S0003683815030060

Froehlich AC, Liu Y, Loros JJ, Dunlap JC (2002) White Collar-1, a circadian blue light photoreceptor, binding to the frequency promoter. Science 297 (80):815–819

Froehlich AC, Loros JJ, Dunlap JC (2003) Rhythmic binding of a WHITE COLLAR-containing complex to the frequency promoter is inhibited by FREQUENCY. Proc Natl Acad Sci U S A 100:5914–5919. https://doi.org/10.1073/pnas.1030057100

Fu J, Murphy KA, Zhou M et al (2016) Codon usage affects the structure and function of the *Drosophila* circadian clock protein PERIOD. Genes Dev 30:1761–1775. https://doi.org/10.1101/gad.281030.116

Fuller KK, Loros JJ, Dunlap JC (2015) Fungal photobiology: visible light as a signal for stress, space and time. Curr Genet 61:275. https://doi.org/10.1007/s00294-014-0451-0

Galagan JE, Calvo SE, Borkovich KA et al (2003) The genome sequence of the filamentous fungus *Neurospora crassa*. Nature 422:859

Gamsby JJ, Loros JJ, Dunlap JC (2009) A phylogenetically conserved DNA damage response resets the circadian clock. J Biol Rhythm 24:193–202. https://doi.org/10.1177/0748730409334748

Garceau NY, Liu Y, Loros JJ, Dunlap JC (1997) Alternative initiation of translation and time-specific phosphorylation yield multiple forms of the essential clock protein FREQUENCY. Cell 89:469. https://doi.org/10.1016/S0092-8674(00)80227-5

Gardner GF, Feldman JF (1980) The frq locus in *Neurospora crassa*: a key element in circadian clock organization. Genetics 96:877–886

Gardner GF, Feldman JF (1981) Temperature compensation of circadian period length in clock mutants of *Neurospora crassa*. Plant Physiol 68:1244–1248

Gaucher J, Montellier E, Sassone-Corsi P (2018) Molecular cogs: interplay between circadian clock and cell cycle. Trends Cell Biol 28:368–379. https://doi.org/10.1016/j.tcb.2018.01.006

Gooch V, Mehra A, Larrondo L et al (2008) Fully codon-optimized luciferase uncovers novel temperature characteristics of the *Neurospora* clock. Eukaryot Cell 7(1):28–37

Gooch VD, Johnson AE, Bourne BJ et al (2014) A kinetic study of the effects of light on circadian rhythmicity of the frq promoter of *Neurospora crassa*. J Biol Rhythm 29:38–48. https://doi.org/10.1177/0748730413517981

Görl M, Merrow M, Huttner B et al (2001) A PEST-like element in FREQUENCY determines the length of the circadian period in *Neurospora crassa*. EMBO J 20:7074–7084. https://doi.org/10.1093/emboj/20.24.7074

Greene AV, Keller N, Haas H, Bell-Pedersen D (2003) A circadian oscillator in *Aspergillus* spp. regulates daily development and gene expression. Eukaryot Cell 2:231–237. https://doi.org/10.1128/EC.2.2.231-237.2003

Grima B, Lamouroux A, Chélot E et al (2002) The F-box protein Slimb controls the levels of clock proteins period and timeless. Nature 420:178

Guo J, Cheng P, Yuan H, Liu Y (2009) The exosome regulates circadian gene expression in a posttranscriptional negative feedback loop. Cell 138:1236–1246. https://doi.org/10.1016/j.cell.2009.06.043

Guo J, Cheng P, Liu Y (2010) Functional significance of FRH in regulating the phosphorylation and stability of *Neurospora* circadian clock protein FRQ. J Biol Chem 285:11508–11515. https://doi.org/10.1074/jbc.M109.071688

Gyöngyösi N, Szőke A, Ella K, Káldi K (2017) The small G protein RAS2 is involved in the metabolic compensation of the circadian clock in the circadian model *Neurospora crassa*. J Biol Chem 292:14929–14939. https://doi.org/10.1074/jbc.M117.804922

He Q, Liu Y (2005a) Degradation of the *Neurospora* circadian clock protein FREQUENCY through the ubiquitin–proteasome pathway. Biochem Soc Trans 33:953

He Q, Liu Y (2005b) Molecular mechanism of light responses in *Neurospora*: from light-induced transcription to photoadaptation. Genes Dev 19:2888–2899. https://doi.org/10.1101/gad.1369605

He Q, Cheng P, Yang Y et al (2002) White Collar-1, a DNA binding transcription factor and a light sensor. Science 297(80):840–843

He Q, Cheng P, Yang Y et al (2003) FWD1-mediated degradation of FREQUENCY in *Neurospora* estab-

lishes a conserved mechanism for circadian clock regulation. EMBO J 22:4421–4430. https://doi.org/10.1093/emboj/cdg425

He Q, Shu H, Cheng P et al (2005) Light-independent phosphorylation of WHITE COLLAR-1 regulates its function in the *Neurospora* circadian negative feedback loop. J Biol Chem 280:17526–17532. https://doi.org/10.1074/jbc.M414010200

He Q, Cha J, He Q et al (2006) CKI and CKII mediate the FREQUENCY-dependent phosphorylation of the WHITE COLLAR complex to close the *Neurospora* circadian negative feedback loop. Genes Dev 20:2552–2565. https://doi.org/10.1101/gad.1463506

Heintzen C, Loros JJ, Dunlap JC (2001) The PAS protein VIVID defines a clock-associated feedback loop that represses light input, modulates gating, and regulates clock resetting. Cell 104:453–464. https://doi.org/10.1016/S0092-8674(01)00232-X

Hevia MA, Canessa P, Müller-Esparza H, Larrondo LF (2015) A circadian oscillator in the fungus Botrytis cinerea regulates virulence when infecting *Arabidopsis thaliana*. Proc Natl Acad Sci 112:8744–8749

Hevia MA, Canessa P, Larrondo LF (2016) Circadian clocks and the regulation of virulence in fungi: getting up to speed. Semin Cell Dev Biol 57:147–155. https://doi.org/10.1016/j.semcdb.2016.03.021

Hirano A, Fu Y-H, Ptáček LJ (2016) The intricate dance of post-translational modifications in the rhythm of life. Nat Struct Mol Biol 23:1053

Hong CI, Ruoff P, Loros JJ, Dunlap JC (2008) Closing the circadian negative feedback loop: FRQ-dependent clearance of WC-1 from the nucleus. Genes Dev 22:3196–3204. https://doi.org/10.1101/gad.1706908

Hong CI, Zamborszky J, Baek M et al (2014) Circadian rhythms synchronize mitosis in *Neurospora crassa*. Proc Natl Acad Sci 111:1397–1402. https://doi.org/10.1073/pnas.1319399111

Huang G, Chen S, Li S et al (2007) Protein kinase a and casein kinases mediate sequential phosphorylation events in the circadian negative feedback loop. Genes Dev 21:3283–3295. https://doi.org/10.1101/gad.1610207

Huang G, He Q, Guo J et al (2013) The Ccr4-not protein complex regulates the phase of the *Neurospora* circadian clock by controlling WHITE COLLAR protein stability and activity. J Biol Chem 288:31002–31009. https://doi.org/10.1074/jbc.M113.494120

Hunt SM, Thompson S, Elvin M, Heintzen C (2010) VIVID interacts with the WHITE COLLAR complex and FREQUENCY-interacting RNA helicase to alter light and clock responses in *Neurospora*. Proc Natl Acad Sci 107:16709–16714. https://doi.org/10.1073/pnas.1009474107

Hurley JM, Larrondo LF, Loros JJ, Dunlap JC (2013) Conserved RNA helicase FRH acts nonenzymatically to support the intrinsically disordered *Neurospora* clock protein FRQ. Mol Cell 52:832–843. https://doi.org/10.1016/j.molcel.2013.11.005

Hurley JM, Dasgupta A, Emerson JM et al (2014) Analysis of clock-regulated genes in *Neurospora* reveals widespread posttranscriptional control of metabolic potential. Proc Natl Acad Sci 111:16995–17002. https://doi.org/10.1073/pnas.1418963111

Hurley JM, Loros JJ, Dunlap JC (2016a) Circadian oscillators: around the transcription–translation feedback loop and on to output. Trends Biochem Sci 41:834–846. https://doi.org/10.1016/j.tibs.2016.07.009

Hurley JM, Loros JJ, Dunlap JC (2016b) The circadian system as an organizer of metabolism. Fungal Genet Biol 90:39–43. https://doi.org/10.1016/j.fgb.2015.10.002

Hurley JM, Jankowski MS, De los Santos H et al (2018) Circadian proteomic analysis uncovers mechanisms of post-transcriptional regulation in metabolic pathways. Cell Syst 7:613. https://doi.org/10.1016/j.cels.2018.10.014

Hut RA, Beersma DGM (2011) Evolution of time-keeping mechanisms: early emergence and adaptation to photoperiod. Philos Trans R Soc Lond Ser B Biol Sci 366:2141–2154. https://doi.org/10.1098/rstb.2010.0409

Idnurm A, Verma S, Corrochano LM (2010) A glimpse into the basis of vision in the kingdom Mycota. Fungal Genet Biol 47:881–892. https://doi.org/10.1016/j.fgb.2010.04.009

Janich P, Arpat AB, Castelo-Szekely V et al (2015) Ribosome profiling reveals the rhythmic liver translatome and circadian clock regulation by upstream open reading frames. Genome Res 25:1848–1859. https://doi.org/10.1101/gr.195404.115

Johnson CH, Elliott J, Russell F et al (2004) Fundamental properties of circadian rhythms. In: Dunlap JC, Loros JJ, DeCoursey PJ (eds) Chronobiology: biological timekeeping. Sinauer Associates, Sunderland, MA, pp 67–106

Jonkers W, Rep M (2009) Lessons from fungal F-box proteins. Eukaryot Cell 8:677–695. https://doi.org/10.1128/EC.00386-08

Kafri M, Metzl-Raz E, Jona G, Barkai N (2016) The cost of protein production. Cell Rep 14:22–31. https://doi.org/10.1016/j.celrep.2015.12.015

Ko HW, Jiang J, Edery I (2002) Role for Slimb in the degradation of *Drosophila* period protein phosphorylated by Doubletime. Nature 420:673

Koike N, Yoo S-H, Huang H-C et al (2012) Transcriptional architecture and chromatin landscape of the core circadian clock in mammals. Science 338:349–354. https://doi.org/10.1126/science.1226339

Kojima S, Shingle DL, Green CB (2011) Post-transcriptional control of circadian rhythms. J Cell Sci 124:311–320. https://doi.org/10.1242/jcs.065771

Kramer C, Loros JJ, Dunlap JC, Crosthwaite SK (2003) Role for antisense RNA in regulating circadian clock function in *Neurospora crassa*. Nature 421:948

Lamb TM, Goldsmith CS, Bennett L et al (2011) Direct transcriptional control of a p38 MAPK pathway by the circadian clock in *Neurospora crassa*. PLoS One 6:e27149–e27149. https://doi.org/10.1371/journal.pone.0027149

Lamb TM, Finch KE, Bell-Pedersen D (2012) The *Neurospora crassa* OS MAPK pathway-activated transcription factor ASL-1 contributes to circadian rhythms in pathway responsive clock-controlled genes. Fungal Genet Biol 49:180–188. https://doi.org/10.1016/j.fgb.2011.12.006

Lambreghts R, Shi M, Belden WJ et al (2009) A high-density single nucleotide polymorphism map for *Neurospora crassa*. Genetics 181:767–781. https://doi.org/10.1534/genetics.108.089292

Larrondo LF, Loros JJ, Dunlap JC (2012) High-resolution spatiotemporal analysis of gene expression in real time: in vivo analysis of circadian rhythms in *Neurospora crassa* using a FREQUENCY-luciferase translational reporter. Fungal Genet Biol 49:681–683. https://doi.org/10.1016/j.fgb.2012.06.001

Larrondo LF, Olivares-Yañez C, Baker CL et al (2015) Decoupling circadian clock protein turnover from circadian period determination. Science 347:1257277. https://doi.org/10.1126/science.1257277

Lee K, Loros JJ, Dunlap JC (2000) Interconnected feedback loops in the *Neurospora* circadian system. Science 289(80):107–110. https://doi.org/10.1126/science.289.5476.107

Lee H, Chen R, Lee Y et al (2009) Essential roles of CKIδ and CKIε in the mammalian circadian clock. Proc Natl Acad Sci 106:21359. https://doi.org/10.1073/pnas.0906651106

Lee E, Jeong EH, Jeong H-J et al (2014) Phosphorylation of a central clock transcription factor is required for thermal but not photic entrainment. PLoS Genet 10:e1004545–e1004545. https://doi.org/10.1371/journal.pgen.1004545

Li N, Joska TM, Ruesch CE, Coster SJ, Belden WJ (2015) The natural antisense transcript first promotes, then represses, gene expression via facultative heterochromatin. Proc Natl Acad Sci USA 112(14):4357–4362. https://doi.org/10.1073/pnas.1406130112

Linden H, Macino G (1997) White collar 2, a partner in blue-light signal transduction, controlling expression of light–regulated genes in *Neurospora crassa*. EMBO J 16:98–109. https://doi.org/10.1093/emboj/16.1.98

Linden H, Ballario P, Macino G (1997) Blue light regulation in *Neurospora crassa*. Fungal Genet Biol 22:141–150. https://doi.org/10.1006/fgbi.1997.1013

Liu Y, Garceau NY, Loros JJ, Dunlap JC (1997) Thermally regulated translational control of FRQ mediates aspects of temperature responses in the *Neurospora* circadian clock. Cell 89:477–486. https://doi.org/10.1016/S0092-8674(00)80228-7

Liu Y, Merrow M, Loros JJ, Dunlap JC (1998) How temperature changes reset a circadian oscillator. Science 281(80):825

Liu Y, Loros J, Dunlap JC (2000) Phosphorylation of the *Neurospora* clock protein FREQUENCY determines its degradation rate and strongly influences the period length of the circadian clock. Proc Natl Acad Sci U S A 97:234–239

Loros JJ (2019) Principles of the animal molecular clock learned from *Neurospora*. Eur J Neurosci 51:19. https://doi.org/10.1111/ejn.14354

Loros JJ, Richman A, Feldman J (1986) A recessive circadian clock mutation at the *frq* locus of *Neurospora crassa*. Genetics 114(4):1095–1110

Loros JJ, Denome SA, Dunlap JC (1989) Molecular cloning of genes under control of the circadian clock in *Neurospora*. Science 243(80):385–388. https://doi.org/10.1126/science.2563175

Lück S, Thurley K, Thaben PF, Westermark PO (2014) Rhythmic degradation explains and unifies circadian transcriptome and proteome data. Cell Rep 9:741–751. https://doi.org/10.1016/j.celrep.2014.09.021

Luo C, Loros JJ, Dunlap JC (1998) Nuclear localization is required for function of the essential clock protein FRQ. Embo 17:1228–1235

Lykke-Andersen S, Tomecki R, Jensen TH, Dziembowski A (2011) The eukaryotic RNA exosome: same scaffold but variable catalytic subunits. RNA Biol 8:61–66

Lysek G, Esser K (1970) Rhythmic mycelial growth in *Podospora anserina*. Arch Mikrobiol 73:224–230. https://doi.org/10.1007/BF00410623

Mahesh G, Jeong E, Ng FS et al (2014) Phosphorylation of the transcription activator CLOCK regulates progression through a ~ 24-h feedback loop to influence the circadian period in *Drosophila*. J Biol Chem 289:19681–19693. https://doi.org/10.1074/jbc.M114.568493

Malzahn E, Ciprianidis S, Káldi K et al (2010) Photoadaptation in *Neurospora* by competitive interaction of activating and inhibitory LOV domains. Cell 142:762–772. https://doi.org/10.1016/j.cell.2010.08.010

McCluskey K, Wiest A, Plamann M (2010) The fungal genetics stock center: a repository for 50 years of fungal genetics research. J Biosci 35:119

Mehra A, Baker C, Loros J, Dunlap J (2009a) Post-translational modification in circadian rhythms. Trends Biochem Sci 34:483–490. https://doi.org/10.1016/j.tibs.2009.06.006.Post-Translational

Mehra A, Shi M, Baker CL et al (2009b) A role for casein kinase 2 in the mechanism underlying circadian temperature compensation. Cell 137:749–760. https://doi.org/10.1016/j.cell.2009.03.019

Menet JS, Pescatore S, Rosbash M (2014) CLOCK: BMAL1 is a pioneer-like transcription factor. Genes Dev 28:8–13. https://doi.org/10.1101/gad.228536.113

Merrow MW, Dunlap JC (1994) Intergeneric complementation of a circadian rhythmicity defect: phy-

logenetic conservation of structure and function of the clock gene frequency. EMBO J 13:2257–2266. https://doi.org/10.1002/j.1460-2075.1994.tb06507.x

Merrow MW, Garceau NY, Dunlap JC (1997) Dissection of a circadian oscillation into discrete domains. Proc Natl Acad Sci 94:3877–3882. https://doi.org/10.1073/pnas.94.8.3877

Montenegro-Montero A, Canessa P, Larrondo LF (2015) Chapter four – Around the fungal clock: recent advances in the molecular study of circadian clocks in *Neurospora* and other Fungi. In: Friedmann T, Dunlap JC, Goodwin SF (eds) Advances in genetics. Academic Press, Cambridge, MA, pp 107–184

Morales Y, Olsen KJ, Bulcher JM, Johnson SJ (2018) Structure of frequency-interacting RNA helicase from *Neurospora crassa* reveals high flexibility in a domain critical for circadian rhythm and RNA surveillance. PLoS One 13:e0196642

Morgan LW, Greene AV, Bell-Pedersen D (2003) Circadian and light-induced expression of luciferase in *Neurospora crassa*. Fungal Genet Biol 38:327–332. https://doi.org/10.1016/S1087-1845(02)00562-5

Mure LS, Le HD, Benegiamo G et al (2018) Diurnal transcriptome atlas of a primate across major neural and peripheral tissues. Science 359(80): eaao0318. https://doi.org/10.1126/science.aao0318

Ode KL, Ukai H, Susaki EA et al (2017) Knockout-rescue embryonic stem cell-derived mouse reveals circadian-period control by quality and quantity of CRY1. Mol Cell 65:176–190. https://doi.org/10.1016/j.molcel.2016.11.022

Olivares-Yañez C, Emerson J, Kettenbach A et al (2016) Modulation of circadian gene expression and metabolic compensation by the RCO-1 corepressor of *Neurospora crassa*. Genetics 204:163–176. https://doi.org/10.1534/genetics.116.191064

Ouyang Y, Andersson CR, Kondo T et al (1998) Resonating circadian clocks enhance fitness in cyanobacteria. Proc Natl Acad Sci 95:8660–8664. https://doi.org/10.1073/pnas.95.15.8660

Padmanabhan K, Robles MS, Westerling T, Weitz CJ (2012) Feedback regulation of transcriptional termination by the mammalian circadian clock PERIOD complex. Science 337(80):599–602. https://doi.org/10.1126/science.1221592

Panda S, Hogenesch JB, Kay SA (2002) Circadian rhythms from flies to human. Nature 417:329

Pavesi G, Mereghetti P, Mauri G, Pesole G (2004) Weeder web: discovery of transcription factor binding sites in a set of sequences from co-regulated genes. Nucleic Acids Res 32:W199–W203. https://doi.org/10.1093/nar/gkh465

Peek CB, Ramsey KM, Marcheva B, Bass J (2012) Nutrient sensing and the circadian clock. Trends Endocrinol Metab 23:312–318. https://doi.org/10.1016/j.tem.2012.02.003

Pelham JF, Mosier AE, Hurley JM (2018) Characterizing time-of-day conformational changes in the intrinsically disordered proteins of the circadian clock, 1st edn. Elsevier, Cambridge

Pittendrigh CS, Bruce VG, Rosenweig NS, Rubin ML (1959) A biological clock in *Neurospora*. Nature 184:169–170. https://doi.org/10.1038/184169a0

Pregueiro AM, Price-Lloyd N, Bell-Pedersen D et al (2005) Assignment of an essential role for the *Neurospora frequency* gene in circadian entrainment to temperature cycles. Proc Natl Acad Sci U S A 102:2210

Pregueiro AM, Liu Q, Baker CL et al (2006) The *Neurospora* checkpoint kinase 2: a regulatory link between the circadian and cell cycles. Science 313 (80):644–649

Putnam AA, Jankowsky E (2013) DEAD-box helicases as integrators of RNA, nucleotide and protein binding. Biochim Biophys Acta 1829:884–893. https://doi.org/10.1016/j.bbagrm.2013.02.002

Querfurth C, Diernfellner ACR, Gin E et al (2011) Circadian conformational change of the *Neurospora* clock protein FREQUENCY triggered by clustered hyperphosphorylation of a basic domain. Mol Cell 43:713–722. https://doi.org/10.1016/j.molcel.2011.06.033

Raduwan H, Isola AL, Belden WJ (2013) Methylation of histone H3 on lysine 4 by the lysine methyltransferase SET1 protein is needed for normal clock gene expression. J Biol Chem 288:8380–8390. https://doi.org/10.1074/jbc.M112.359935

Ramsdale M, Lakin-Thomas PL (2000) sn-1,2-diacylglycerol levels in the fungus *Neurospora crassa* display circadian rhythmicity. J Biol Chem 275 (36):27541–27550

Reinke H, Asher G (2019) Crosstalk between metabolism and circadian clocks. Nat Rev Mol Cell Biol 20:227–241. https://doi.org/10.1038/s41580-018-0096-9

Reischl S, Kramer A (2011) Kinases and phosphatases in the mammalian circadian clock. FEBS Lett 585:1393–1399. https://doi.org/10.1016/j.febslet.2011.02.038

Salichos L, Rokas A (2010) The diversity and evolution of circadian clock proteins in fungi. Mycologia 102:269–278

Sancar G, Sancar C, Brunner M, Schafmeier T (2009) Activity of the circadian transcription factor white collar complex is modulated by phosphorylation of SP-motifs. FEBS Lett 583:1833–1840. https://doi.org/10.1016/j.febslet.2009.04.042

Sancar G, Sancar C, Brügger B et al (2011) A global circadian repressor controls antiphasic expression of metabolic genes in neurospora. Mol Cell 44:687–697. https://doi.org/10.1016/j.molcel.2011.10.019

Sancar G, Sancar C, Brunner M (2012) Metabolic compensation of the *Neurospora* clock by a glucose-dependent feedback of the circadian repressor

CSP1 on the core oscillator. Genes Dev 26:2435–2442. https://doi.org/10.1101/gad.199547.112

Sancar C, Sancar G, Ha N et al (2015) Dawn- and dusk-phased circadian transcription rhythms coordinate anabolic and catabolic functions in *Neurospora*. BMC Biol 13:17–19. https://doi.org/10.1186/s12915-015-0126-4

Schafmeier T, Haase A, Káldi K et al (2005) Transcriptional feedback of *Neurospora* circadian clock gene by phosphorylation-dependent inactivation of its transcription factor. Cell 122:235–246. https://doi.org/10.1016/j.cell.2005.05.032

Schafmeier T, Káldi K, Diernfellner A et al (2006) Phosphorylation-dependent maturation of *Neurospora* circadian clock protein from a nuclear repressor toward a cytoplasmic activator. Genes Dev 20:297–306. https://doi.org/10.1101/gad.360906

Schafmeier T, Diernfellner A, Schäfer A et al (2008) Circadian activity and abundance rhythms of the *Neurospora* clock transcription factor WCC associated with rapid nucleo-cytoplasmic shuttling. Genes Dev 22:3397–3402. https://doi.org/10.1101/gad.507408

Schumacher J, Tudzynski P (2012) In: Pérez-Martín J, Di Pietro A (eds) Morphogenesis and infection in *Botrytis cinerea* BT – morphogenesis and pathogenicity in Fungi. Springer, Berlin, pp 225–241

Shi M, Collett M, Loros JJ, Dunlap JC (2010) FRQ-interacting RNA helicase mediates negative and positive feedback in the *Neurospora* circadian clock. Genetics 184:351–361. https://doi.org/10.1534/genetics.109.111393

Smith KM, Sancar G, Dekhang R et al (2010) Transcription factors in light and circadian clock signaling networks revealed by genomewide mapping of direct targets for *Neurospora* white collar complex. Eukaryot Cell 9:1549–1556. https://doi.org/10.1128/EC.00154-10

Starkey N, McDonald J, Lee K (2013) PERIOD-1 is a component of the *Neurospora* metabolic oscillator. Lett Gen Microbiol 1:13–15

Sun G, Zhou Z, Liu X et al (2016) Suppression of WHITE COLLAR-independent frequency transcription by histone H3 lysine 36 methyltransferase SET-2 is necessary for clock function in *Neurospora*. J Biol Chem 291:11055–11063. https://doi.org/10.1074/jbc.M115.711333

Takahashi JS (2017) Transcriptional architecture of the mammalian circadian clock. Nat Rev Genet 18:164–179. https://doi.org/10.1038/nrg.2016.150

Tan Y, Merrow M, Roenneberg T (2004) Photoperiodism in *Neurospora crassa*. J Biol Rhythm 19:135–143. https://doi.org/10.1177/0748730404263015

Tang C-T, Li S, Long C et al (2009) Setting the pace of the *Neurospora* circadian clock by multiple independent FRQ phosphorylation events. Proc Natl Acad Sci U S A 106:10722–10727. https://doi.org/10.1073/pnas.0904898106

Tataroğlu Ö, Lauinger L, Sancar G et al (2012) Glycogen synthase kinase is a regulator of the circadian clock of *Neurospora crassa*. J Biol Chem 287:36936–36943. https://doi.org/10.1074/jbc.M112.396622

Traeger S, Nowrousian M (2015) Analysis of circadian rhythms in the basal filamentous ascomycete *Pyronema confluens*. G3 Genes|Genomes|Genetics 5:2061–2071. https://doi.org/10.1534/g3.115.020461

Tsvetkov P, Reuven N, Shaul Y (2009) The nanny model for IDPs. Nat Chem Biol 5:778

Vitalini MW, de Paula RM, Park WD, Bell-Pedersen D (2006) The rhythms of life: circadian output pathways in *Neurospora*. J Biol Rhythm 21:432–444. https://doi.org/10.1177/0748730406294396

Wang B, Kettenbach AN, Gerber SA et al (2014) *Neurospora* WC-1 recruits SWI/SNF to remodel frequency and initiate a circadian cycle. PLoS Genet 10:e1004599

Wang B, Zhou XY, Loros JJ, Dunlap JC (2016) Alternative use of DNA binding domains by the *Neurospora* white collar complex dictates circadian regulation and light responses. Mol Cell Biol 36:781–793. https://doi.org/10.1128/MCB.00841-15

Wang B, Kettenbach AN, Zhou X et al (2019) The Phospho-code determining circadian feedback loop closure and output in *Neurospora*. Mol Cell 74:771–784.e3. https://doi.org/10.1016/j.molcel.2019.03.003

Weber F, Zorn D, Rademacher C, Hung H-C (2011) Post-translational timing mechanisms of the *Drosophila* circadian clock. FEBS Lett 585:1443–1449. https://doi.org/10.1016/j.febslet.2011.04.008

Wu C, Yang F, Smith KM et al (2014) Genome-wide characterization of light-regulated genes in *Neurospora crassa*. G3 Genes|Genomes|Genetics 4:1731–1745. https://doi.org/10.1534/g3.114.012617

Xu Y, Ma P, Shah P et al (2013) Non-optimal codon usage is a mechanism to achieve circadian clock conditionality. Nature 495:116–120. https://doi.org/10.1038/nature11942

Xue Z, Ye Q, Anson SR et al (2014) Transcriptional interference by antisense RNA is required for circadian clock function. Nature 514:650–653. https://doi.org/10.1038/nature13671

Yang Y, Cheng P, Zhi G, Liu Y (2001) Identification of a calcium/calmodulin-dependent protein kinase that phosphorylates the *Neurospora* circadian clock protein FREQUENCY. J Biol Chem 276:41064–41072. https://doi.org/10.1074/jbc.M106905200

Yang Y, Cheng P, Liu Y (2002) Regulation of the *Neurospora* circadian clock by casein kinase II. Genes Dev 16:994–1006. https://doi.org/10.1101/gad.965102

Yang Y, He Q, Cheng P (2004) Distinct roles for PP1 and PP2A in the *Neurospora* circadian clock. Genes Dev 18:255–260. https://doi.org/10.1101/gad.1152604

Yoshitane H, Takao T, Satomi Y et al (2009) Roles of CLOCK phosphorylation in suppression of E-box-dependent transcription. Mol Cell Biol 29:3675–3686. https://doi.org/10.1128/MCB.01864-08

Yu Z, Fischer R (2019) Light sensing and responses in fungi. Nat Rev Microbiol 17:25–36. https://doi.org/10.1038/s41579-018-0109-x

Zhang Y, Smith CL, Saha A et al (2006) DNA translocation and loop formation mechanism of chromatin remodeling by SWI/SNF and RSC. Mol Cell 24:559–568. https://doi.org/10.1016/j.molcel.2006.10.025

Zhou M, Guo J, Cha J et al (2013) Non-optimal codon usage affects expression, structure and function of clock protein FRQ. Nature 494:111–115. https://doi.org/10.1038/nature11833

Zoltowski BD, Schwerdtfeger C, Widom J et al (2007) Conformational switching in the fungal light sensor Vivid. Science 316:1054–1057. https://doi.org/10.1126/science.1137128