Published in final edited form as:

Microbiol Spectr. 2017 May ; 5(3): . doi:10.1128/microbiolspec.FUNK-0039-2016.

Making time: conservation of biological clocks from fungi to animals

Jay C. Dunlap¹ and Jennifer J. Loros^{1,2}

¹Department of Molecular and Systems Biology, Geisel School of Medicine at Dartmouth, Hanover, NH 03755, U.S.A

²Department of Biochemistry and Cell Biology, Geisel School of Medicine at Dartmouth, Hanover, NH 03755, U.S.A

SUMMARY

The capacity for biological timekeeping arose at least three times through evolution, in prokaryotic cyanobacteria, in cells that evolved into higher plants, and within the group of organisms that eventually became the Fungi and the Animals. *Neurospora* is a tractable model system for understanding the molecular bases of circadian rhythms in the last of these groups, and is perhaps the most intensively studied circadian cell type. Rhythmic processes described in fungi include growth rate, stress responses, developmental capacity and sporulation as well as much of metabolism; fungi use clocks to anticipate daily environmental changes. A negative feedback loop comprises the core of the circadian system in fungi and animals. In *Neurospora*, the best studied fungal model, it is driven by two transcription factors, WC-1 and WC-2, that form the White Collar Complex (WCC). WCC elicits expression of the *frq* gene. FRQ complexes with other proteins, physically interacts with the WCC, and reduces its activity; the kinetics of these processes is strongly influenced by progressive phosphorylation of FRQ. When FRQ become sufficiently phosphorylated that it loses the ability to influence WCC activity the circadian cycle starts again. Environmental cycles of light and temperature influence *frq* and FRQ expression and thereby reset the internal circadian clocks. The molecular basis of circadian output is also becoming understood. Taken together, molecular explanations are emerging for all the canonical circadian properties, providing a molecular and regulatory framework that may be extended to many members of the Fungal and Animal kingdoms, including humans.

Keywords

Neurospora crassa; circadian clock; frequency; White Collar Complex; FLO; circadian Output

INTRODUCTION

The Biology of Circadian Rhythms

What are circadian rhythms?—Despite years of study and a great deal of exposure in the scientific press, there is still sometimes confusion as regards what are the defining characteristics of a rhythmic process that makes it truly circadian. The essence of this is that the rhythm must be shown not to depend in any way on continued environmental cycles, and to have a period of about a day. So in this way it is obvious that in petri dish cultures grown

under a light-dark cycle, a rhythmic change in orange color in *Neurospora crassa*, or in melanin in *Aspergillus fumigatus*, could not be categorized as circadian because the change in pigmentation would be seen as the result of light-induction of, respectively, carotenoid or polyketide synthesis. As humans, our pupils constrict in proportion to the amount of light to which the eye is exposed, so a daily rhythm in pupil size would not be circadian but would instead reflect a diurnal cycle in ambient light. This part of the definition identifies the rhythm as truly endogenous in nature, in this way being the overt expression of an internal circadian oscillator. Beyond this, the definition is more subtle and reflects the biology of rhythms.

What does it benefit a cell to have a circadian oscillator and not simply an hourglass timer that is set at environmental transitions such as dusk or dawn? There are at least two answers. One is that an oscillator doesn't need to be restarted every day; it will continue even without a time cue, for instance even if the organism (like a fungus) is growing underground. Another answer is that it provides an additional level of flexibility in responding to a changing environment. In this context we should briefly talk about phase and phase angle (Figure 1). Every rhythm performs has a peak and a trough as well as other defining point (e.g. point of sharpest decline, mid-way to peak, etc.) and in general such definable points are called phase reference points. In circadian biology phase or phase angle is defined as the difference in time between the phase reference points of the environmental cycle and the circadian cycle. For instance, in *Neurospora* growing on an agar surface or a piece of bread in a light cycle consisting of 12 hrs light and 12 hrs dark, the number of aerial hyphae and conidia peak about 2 hrs before dawn, so if the phase reference point of the environmental cycle is dawn and the phase reference point of the circadian cycle is the peak of conidiation then the phase of conidiation is about -2 hrs (i.e. before dawn) which is the same as +22 hrs (after dawn) on a 24 hr cycle. When free-running under constant conditions, two rhythms can have the same period length but have different phases because the peaks occur at different times with respect to the last light-to-dark transition. For a rhythm to be useful to the organism it must be able to control activities so that they happen at appropriate times of day, and appropriate means not just a given number of hours after dawn or dusk but more often, for example, the middle of the day period or night period, a point that can be a different number of hours after dawn or dusk depending on the season. To achieve this circadian rhythms are entrained to environmental cycles as distinct from synchronized with them. Synchronized would mean that the rhythm tracks the external cycle and assumes a fixed phase and period length of the external cycle whereas entrained means that the rhythm assumes the period but the phase reflects interplay between the circadian oscillator and the environmental cycle. The value of this is that the circadian system can (and has) evolved to preserve phase across the spectrum of light dark cycles seen in a year. At the temperate latitude in Vermont where we live, daylight can range from 8 to 16 hrs depending on the season. The circadian system can keep an important biological activity (say conidiation in *Neurospora* or sleep in people) occurring at roughly the same point in the cycle, such as late at night, despite changes in the light-dark cycle. As described in more detail later in the chapter, in *Neurospora* a large variety of processes ranging from gross characteristics such as sexual and asexual developmental potential, growth rate and the cell cycle, to gene expression and fluxes through metabolic pathways, are all coordinately regulated by the

circadian system. This coordination is adaptive because it helps the organism to organize its growth and metabolism in anticipation of dependable environmental changes such as temperature, ambient light, and UV stress.

Circadian homeostasis—Another environmental variable that has profound impact on biological systems is temperature. For a clock to be useful in the context described above, namely being sure to keep important processes happening at the right time(s) of day, it would not do for the rate of progression of the clock to be strongly influenced by ambient temperature. Physiologists and biochemists often frame general discussions of temperature effects on rates of processes in terms of Q_{10} , the rate of a process at one temperature divided by the rate at 10°C lower temperature. A typical enzymatic reaction will have a Q_{10} of 2, and a complex set of reactions such as mitochondrial respiration may have a Q_{10} of 4. Imagine the effect on phase angle if the clock ran three times faster during a 30°C day in July as on a 15°C night. Circadian rhythms are special because they have evolved to have a Q_{10} of close to 1. A process with a Q_{10} of exactly 1 would be temperature independent; circadian period lengths are not temperature independent, and even in the same organism across some ranges the Q_{10} can be slightly less than 1 and across other ranges slightly greater than 1. For this reason circadian period lengths are said to be temperature compensated.

Another aspect of circadian homeostasis is that circadian period length is resilient to the effects of changing nutrition. This again makes sense ecologically, especially for a fungus that can encounter enormously variable nutritional conditions over the course of weeks as it moves from fresh to rotting to desiccated substrates. Temperature and nutritional compensation distinguishes circadian rhythms from other circa-24 hr rhythms such as the mammalian cell cycle.

Time cues for keeping internal time in sync with external time—While circadian period length needs to be resilient to the effects of ambient temperature, it is immediately plain that the clock needs to be sensitive to daily changes in a variety of environmental variables so that internal time can be a proxy for external time. In most organisms daily changes in light intensity set the phase of the internal clock, with lights-on corresponding to dawn and lights-off to dusk. All circadian organisms including fungi have evolved ways to detect even low levels of light and to use changes in intensity to set their clocks. Indeed humans and fungi use light in the same way, and the understanding of how light is used to reset the circadian clock was first elucidated in *Neurospora* as discussed later. Clocks are sensitive to temperature changes to the extent that they can also be used as time cues for day and night. Indeed in many organisms (including *Neurospora*) temperature can be a stronger time cue than light.

The Definition of Circadian Rhythm

To paraphrase, we have defined a true circadian rhythm in this way: a circadian rhythm is an oscillation in a biochemical, physiological, or behavioral function that persists under conditions of constant light or darkness, temperature, and other environmental variables with a period length of about a day, whose phase can be reset by a brief interruption in the

constant regimen, and whose period length is relatively independent of temperature and nutrition within the physiological range of normal growth (1). Thus, these rhythms are not a simple response to the environment but are phased by it. Overall it is these few characteristics – period length of about a day, persistence under constant conditions, resettable by changes in this constant regimen, and compensated against ambient changes in temperature or nutrition – that define a rhythm as being circadian. Rhythms having these characteristics, whether in fungi or animals, are united by a common biology and, as it turns out, by a common molecular mechanism. These are also the characteristics that molecular models for the circadian oscillators have sought to explain. Specifically then there have been three salient questions: How does the oscillator work? (Mechanism) How do environmental cues impart time-of-day information on the oscillator? (Input) How does the oscillator control the behavior and metabolic potential of the cell? (Output) Work on *Neurospora* played a, or in some cases *the*, leading role in answering the first two of these and remains at the forefront of answering the third.

Circadian Rhythms in Fungi

Many fungi can express one (or even more than one) kind of rhythm - growth, morphology, or even in gene expression - if the medium and environmental conditions are just right (2–4). In the vast majority of cases these are not truly circadian by the definition described above, and in fact true circadian rhythms in fungi have been described in only a few organisms including *Neurospora* spp (4). and the closely related *Sordaria fimicola* (5), *Aspergillus flavus* (6), *Cercospora kikuchii* (7), *Botrytis cinerea* (8, 9), *Pyronema confluens* (10), and the basidiomycete *Neonothopanus gardneri* (11) where the circadian clock controls fungal bioluminescence and is thought to help with spore dispersal. Extensive effort has failed to reveal convincingly a rhythm in *Aspergillus nidulans* or *Aspergillus fumigatus* (although a rhythm in *gapdh* mRNA has been reported in *Aspergillus flavus* (6)), and reports of circadian rhythms in the yeasts *Schizosaccharomyces* (12) and *Saccharomyces* (13) have been questioned or not been reproducible. Given the extensive conservation of the core clock elements FRQ, WC-1 and WC-2 within the Ascomycota in general and the Sordariaceae in particular it seems likely that rhythms could be identified in many additional organisms. A wonderful example of this is the recent description of a canonical FRQ/WCC based clock in the pathogen *Botrytis cinerea* that influences its pathogenesis (8, 9).

The field of rhythms in fungi other than *Neurospora* has been excellently reviewed elsewhere recently and interested readers may want additional perspectives (8, 14–20). While rhythms can include both circadian and infradian non-circadian cycles, more attention will be paid to the relatively extensive literature describing non-circadian rhythms in fungi near the end of this chapter. Before that the focus will be on the true circadian rhythm in *Neurospora* and what it has shown about circadian rhythms in general.

THE *NEUROSPORA* CIRCADIAN OSCILLATOR

The historical development of this field has been quite interesting but has been adequately reviewed elsewhere (21–29) and will not be re-developed in depth here. Instead we will aim

for a succinct and comprehensible (at the expense of comprehensive) overview of how the circadian system works.

WCC Drives *frq*

The White Collar Complex or WCC is comprised of WC-1 and WC-2. WC-1 is a GATA type transcription factor having a Zn finger DNA binding domain and three PAS-like domains, amino acid sequences often associated with protein-protein interaction or with ligand binding (30). One of these is a specialized PAS domain called a LOV domain, a subtype associated with Light, Oxygen and Voltage regulation. As discussed below under Input, WC-1 is the founding member of the principal class of fungal blue light photoreceptors, an activity mediated by the LOV domain although for the circadian oscillator itself the LOV domain is dispensable (31). The other PAS domains, however, are essential in that they mediate the interaction between WC-1 and its partner protein in the WCC, WC-2. Figure 2a shows a cartoon of the domains of WC-1 and WC-2 as well as describing the activities associated with these domains.

The WCC binds to two places in the promoter of the *frequency (frq)* gene (32) (Figure 2b). One is the Proximal Light Regulatory Element (PLRE) that is very near to the transcription start site (TSS) and that is required for the acute induction of *frq* expression by light that forms the basis of light resetting of the clock (see Input, below). Binding to another element more than a kilobase distal (5') from the TSS, the Clock Box (C-box) is required for circadian activation of *frq* expression as a part of the circadian oscillator. Binding of WCC to the C-box displaces local nucleosomes (33) and requires the Zn Fingers of both WC-1 and WC-2 as well as a basic region of WC-1 just N-terminal from the WC-1 Zn finger, as region called the DBD because proteins lacking it are Defective in DNA Binding (34). One might reasonably ask how binding of WCC to the C-box can effect transcriptional activation if the site is more than a thousand bases away from the TSS. The answer is that an N-terminal region of WC-1 serves to attract the SWI-SNF chromatin remodeling complex (31), a group of proteins known to be involved in nucleosome removal and chromatin remodeling as well as in bending of DNA (35). Thus, the model is that SWI-SNF promotes bending of the DNA in the *frq* promoter to bring the WCC bound to the C-box into proximity with the TSS where it can attract the general transcription factors and Mediator complex involved in initiation of transcription (31).

In addition to the transcription of *frq* in the sense (coding) direction there is also a *frq* antisense transcript (36) named, appropriately *qrf* (*frq* spelled backwards) (Figure 2b). The WCC binds downstream of the *frq* coding region where it serves to mediate light-induction of this antisense transcript; the exact location of the promoter driving constitutive *qrf* expression has not been mapped. Xue et al. have recently provided a nice rationale for the expression of the antisense *qrf* transcript: even in the absence of WCC binding to the C-box there is a low level of sense strand *frq* expression, and the resulting low level of FRQ expression causes the oscillator to dampen (31). Constant low level *qrf* expression runs counter to the sense strand expression and serves to reduce this background to near zero thereby keeping the oscillator from damping (37). *qrf* expression also provides antisense transcripts that may elicit dicer-independent siRNA (disiRNA)-mediated facultative

heterochromatin formation at *frq* and thereby aid in shutting down *frq* expression (38). WCC binding within the *qrf* promoter promotes light-induction of *qrf*, and the spike of antisense transcript coincident with the spike of light-induced sense *frq* transcript aids in light-resetting of the clock by making sure that FRQ appears as a pulse and not a tonic increase. Light-resetting is compromised in the absence of *qrf* expression (36).

FRQ Is Made and Acts

frq mRNA moves to the cytoplasm for translation although there is a surprisingly long lag between when it first appears and when FRQ protein finally turns up several hours later (Figure 3). The cause of this long lag is unknown but it is likely a result of explicit regulation because after acute light-induction of *frq* the FRQ protein appears well within an hour (39). The translation of FRQ has received some attention recently with interesting results. It's been known for a long time that the codon bias of *frq* is strongly skewed towards rarely used codons, those that require low abundance tRNAs. Zhou et al examined the importance of this by changing the codon bias in favor of those codons serviced by abundant tRNAs and the result was more FRQ, as expected, but less functional FRQ: The FRQ produced by the codon-bias-“improved” gene was less stable, less able to enter into productive complexes, and less able to support its role in the oscillator (40). The model is that FRQ folds co-translationally, perhaps promoted by interaction with other protein(s) as described below, and that this co-translational folding and interaction requires some pausing of translation as helped by the codon choice in the wild type gene (41).

Upon translation, FRQ dimerizes and forms a complex with another protein, FRH. Dimerization of FRQ is essential (42) as FRQ's immediate interaction with its partner protein FRH. FRQ is an Intrinsically Disordered Protein (IDP) meaning that unlike more typical proteins it has no inherent structure. Most proteins fold up into specific structures and can be thought of as oil droplets where hydrophobic residues are pushed away from water and into the center of the protein allowing hydrophilic amino acid residues to be on the outside. For proteins such as these the structure of the protein plays an essential role in its function and also contributes to its stability. When such proteins are heated so that the structure is disrupted and the hydrophobic residues face outside, the protein becomes denatured and falls out of solution; think of the albumin of a cooked egg. IDPs, however, have no inherent structure so when heated they remain in solution, but they are also subject to rapid proteolysis in the cell unless they are protected by another protein – enter FRH.

Two Cellular Roles for FRH

FRH, the FRQ Interacting RNA Helicase, was identified in 2004 as an essential protein that interacts stoichiometrically with FRQ (43). As its name implies FRH has a “normal function” in a subcellular structure called the exosome that plays a role in the turnover of RNA (44), specifically in unwinding the RNA in a process driven by the hydrolysis of ATP. FRH belongs to a well studied family of such ATP-dependent RNA helicases, several of which have crystal structures and all of which bear conserved sequences that are essential for their enzymatic functions including binding and hydrolyzing ATP. With all of this evidence pointing to an enzymatic function for FRH it was natural to assume that the role of FRH in the clock was enzymatic; surprisingly, however, this is not so. In a startling turn of

events, mutants of FRH that have lost their required catalytic residues have been shown still to function normally in the clock (45). FRH is thus understood to play a purely structural role, stabilizing the IDP FRQ and protecting it from proteolytic turnover.

Processive and Choreographed Phosphorylation of FRQ

The observant reader will be asking how FRQ can do what it does if it has no structure and is just a random coil of amino acids in a chain floating in the cytoplasm. The answer is that FRQ is one of the most heavily post-translationally modified proteins known, and it is almost certainly these modifications that lead FRQ to participate in the diverse time-of-day-specific interactions that lead to a functional clock. A cartoon structure of the functional domains of FRQ is shown in Figure 4. FRQ is phosphorylated at over 100 serine and threonine residues across much of the protein, but this happens very gradually over the course of the circadian day and in a highly choreographed fashion (Figure 4) such that some regions of the protein become highly phosphorylated before other regions are significantly modified (46–48). It is very likely that the addition of the highly charged phosphate groups changes the local charge of the protein, and this suggests that each wave of phosphorylations elicits changes in the local structure of FRQ and in its ability to interact with other proteins. Consistent with this mutation of phosphorylation sites within FRQ can speed up the cycle or slow it down depending on where the sites are and when in the cycle they are phosphorylated (Figure 4). Indeed this is seen when one looks at the spectrum of proteins with which FRQ interacts (Figure 3). With the exception of FRH these change in a time-of-day specific manner. Early in the day FRQ interacts strongly with WC-1 and WC-2 and as the day goes on this interaction decreases until by the end of the day there is no strength to the interaction above background. This seems to fit well with the net action of FRQ in inhibiting the transcriptional activity of the WCC: As soon as FRQ appears WCC activity plummets with the result that *frq* mRNA is elevated for only a relatively short period of time whereas FRQ protein is elevated for most of the day.

A bit later in the day the interaction of FRQ and FRH with casein kinase I (CK1) increases, and after this CK1 is also a part of the complex containing FRQ, FRH, WC-1 and WC-2. These are the strongest interactions, strong enough to survive purification of FRQ through multiple steps in preparation for the mass spectrometry in which the FRQ-interacting partners were identified and the sites of phosphorylation determined (47). There are, however, very likely a number of additional kinases that interact more transiently with FRQ as these have been identified explicitly with various functions or activities (24, 49–51). For instance, in addition to the data showing physical interactions, *Neurospora* strains bearing mutations in CK1 show abnormal period lengths (52). Likewise casein kinase 2 (CK2) weakly interacts with FRQ and importantly CK2 has been shown to play a role in temperature compensation as described below (51, 53). PRD-4 in *Neurospora* is the ortholog of checkpoint kinase-2, a kinase activated by DNA damage. In *Neurospora* the clock controls the cell cycle and DNA damage arrests the cell cycle in part through the action of CHK2, and also resets the circadian clock again through the action of CHK2 (i.e. PRD-4) (54). Protein kinase A (PKA) and Calcium Calmodulin kinase-1 (CAMK-1) have been implicated in FRQ phosphorylation and clock function because mutants in them affect circadian period length and because they can phosphorylate FRQ in vitro (55). Relatedly,

three protein phosphatases (proteins that remove phosphates from proteins) have been implicated in clock function (56). These are PP1, PP2A and PP4; however, these may be indirect effects because in vivo analysis of FRQ phosphorylation revealed no strong evidence for dephosphorylation of any residues. Instead the progression appeared to be unidirectional. Many of these protein modifying enzymes have conserved roles in the circadian oscillators of animals as described later on.

FRQ Becomes Inactive, the Cycle Begins Again, and FRQ is Turned Over

The function of the extensive post-translational modifications of FRQ is plainly to regulate its activity, but a separate question concerns how the daily cycle is terminated. One of the first and most durable hallmarks of all circadian oscillators in the fungal and animal lineages is the observation of a daily cycle in the mRNA encoding the negative elements, accompanied by a daily cycle in their abundance (57–59). In addition in both *Neurospora* and *Drosophila* there is an excellent correlation between the measured half-life of the negative elements (FRQ and PER respectively) and circadian period such that longer half-life (more stable proteins) corresponds to longer period lengths (60, 61). This gave rise to the very reasonable assertion that the circadian cycle ends when the negative element is degraded, thereby releasing the positive element to begin the cycle of transcription anew. Despite the common restatement of this is nearly every clock review, recent work in *Neurospora* (62), while validating the correlation, have shown it to be just that, only a correlation, and that the underlying causal connection between clock protein stability and circadian period length does not exist.

This surprising turn of events began with the study of FRQ turnover which identified a ubiquitin ligase, FWD-1, as the enzyme responsible for ubiquitylating FRQ, the signal modification that targets it for protein turnover in the proteasome (63). In a very large number of proteins, FRQ included, phosphorylation of groups of amino acids in domains of the protein is the signal that targets a proteins for ubiquitylation and turnover, so the extensive phosphorylation of FRQ fit this pattern well. A prediction, however, is plain, namely that mutations in FWD-1 that increase the half-life of FRQ must increase period length in a predictable manner even yielding arrhythmicity and indeed, when examined on race tubes, strains lacking FWD-1 do not display a rhythm (63). However, surprisingly when the clocks of such strains are examined using a luciferase reporter an underlying rhythm is revealed (62). At the level of the oscillator itself the rhythm is of normal amplitude, and moreover the canonical allelic series of short period and long period mutations in FRQ has the same effect on the oscillator, indicating that the clock in these strains is the same clock as is normally seen on race tubes. The feedback loop is being closed, determining period length, without a concomitant turnover of FRQ protein. How can this be?

The model proposed to explain these data (62) is that as FRQ (and by extension the other salient negative element clock proteins in animals such as PER in mammals) undergoes its unidirectional daily cycle in phosphorylation, its changes in charge allows it to participate in sequential structures and complexes that impact the activity of the WCC, and perhaps other enzymes. The cycle ends when FRQ becomes so phosphorylated that it no longer interacts with the WCC or other relevant proteins; indeed by days end there is no longer any

statistically significant interaction between FRQ and the WCC (47). Beyond this point it does not matter if it is there, and in fact in the FWD-1 knockout strains it is still there: Total cellular FRQ can have a very long half life but the clock as measured by luciferase runs along with little change, the small period differences being explained by effects of loss of FWD-1 on other substrates.

In wild type strains once FRQ has no further function and no further interactions with other relevant proteins it is degraded; however, this act is viewed as only cellular housekeeping. By the time FRQ is being turned over the WCC has already begun a new cycle of FRQ expression and a new circadian day as started.

Temperature and Metabolic Compensation

As described above in the section on **Circadian Homeostasis**, for a circadian clock to be truly useful to the organism it needs to be compensated against changes in the ambient environment. This is known as metabolic/nutritional and temperature compensation and is a core property of clocks as reflected by the fact that even isolated mammalian tissues and cells in culture display temperature compensation (64, 65). The mechanism for compensation is not known and indeed even few clues in part because the experimental analysis is challenging. Rather than just determining the period length of mutant strains, to see if compensation is altered the period length must be determined by growing organisms at several different temperatures across the physiological range. Moreover partial loss of compensation is commonly associated with period defects suggesting that it may not be informative to mechanism if only because mutant proteins may have altered stability. In this case a temperature gradient turns into a gradient in activity or effective dosage. However in the study of compensation *Neurospora* again led the way. Because loss of compensation mutations might not be informative, mutations yielding enhanced compensation were targeted for study. The *Neurospora* clock is normally modestly undercompensated meaning that the period length becomes slightly shorter as the temperature increases from 20°C to 30°C. Two mutants, *prd-3* and *chr*, reverse this and instead show over- or extended compensation (66). Cloning of these genes revealed them to encode separate subunits of casein kinase 2 (CK2): *chr* encodes the regulatory β subunit that also contributes to substrate recognition and *prd-3* is mutated within the catalytic core of the α subunit (50). The finding that the only two mutants known to cause overcompensation are subunits of the same enzyme is strong circumstantial evidence pointing to a role for CK2 in temperature compensation.

In many circadian systems CK2 is known to play a circadian role. In *Arabidopsis* the β subunit CKB3 associates with CCA1 and overexpression of CKB3 leads to a shorter period (67) and in *Drosophila*, CK2 β and CK2 α are defined respectively by *Andante* and *Timekeeper*, two long period clock mutants. In *Neurospora*, FRQ is phosphorylated in vitro by CK2 and period lengthens with reduced dosage (53); although CK2 α null mutants will not germinate *ckb-1* is viable but arrhythmic, the effect of CK2 dosage on compensation can be assessed in this background. When the regulatable *quinic acid-2 (qa-2)* promoter (e.g. 68, 69) was used to drive *ckb-1* expression, leaky expression absent inducer causes a long 28 to 30 hour period at 25°C (50). Interestingly though, as CK2 β 1 levels are increased the

period drops to within the wild type range, and strains expressing high levels of CK2 β 1 are slightly undercompensated like the wild type clock, and with less inducer as CK2 β 1 levels decrease, compensation passes through a range of better than normal compensation, finally changing to over-compensated mimicking the *ckb-1^{chr}* overcompensation. Only the dose of WT proteins is being controlled in these experiments. The straightforward interpretation is that higher temperature results in more kinase activity (as confirmed in 50), and this acts to slow the pace of the clock at higher temperatures. These data establish a clear role for CK2 in effecting compensation.

INPUT TO THE CLOCK

Although much emphasis historically has been placed on the mechanism of the oscillator and the precision of the clock, it is the ability to be reset every day that may really be most important. Even a bad watch is still useful if it is corrected twice a day at dawn and dusk, and in fact this makes the clock useful in many different photoperiods spanning the seasonal differences from a winter of 6 hrs of light and 16 hrs of dark to the summer of long days and short nights. Clocks use available dependable environmental cues for clues as to time of day and these are known as zeitgebers from the German for time-givers. The most dependable environmental cues are light and temperature and these act on all known circadian clocks, although more complicated organisms such as people can also use derived cues such as feeding time or even social interactions.

Light

The mechanism through which fungal and mammalian circadian oscillators are reset by light was first elucidated in *Neurospora*. The key to understanding how this works is to understand the task in resetting. A single unidirectional environmental cue, the presence of light, must result in a bi-directional shift in the phase of the rhythm: if light is seen in the latter part of the night it means that dawn has arrived and so the light signal should act to shift the phase of the clock forward to morning, whereas if light is seen in the early part of the night it means that the sun has not yet set so the light signal should delay the clock back to the previous dusk. And of course, if light is perceived during the middle of the day it should have little effect on the clock.

This is achieved through the simple existence of a daily cycle in FRQ abundance and the fact that light induces the expression of *frq* mRNA and FRQ protein (Figure 5) (70). Succinctly, in the daily cycle of *frq* mRNA abundance it takes about 8–9 hrs to go from trough to peak and about 12–14 hrs to go from peak to trough. If mRNA is rising slowly and the organism sees light, then *frq* is rapidly induced and rises; what would have taken hours now happens within minutes, essentially an immediate phase advance. Conversely, if *frq* mRNA levels are falling and the organism sees light, then *frq* is rapidly induced and the clock is set back to the time of peak. In constant conditions peak *frq* mRNA levels happen in the subjective morning (the phase that would be morning on a light-dark cycle) so the effect of resetting is always to push the clock to subjective morning.

In *Neurospora* the photoreceptor that brings about this acute light induction is WC-1, the same transcription factor protein that heterodimerizes with WC-2 to make the positive

element in the core feedback loop. There is a specific protein domain that senses light, the LOV domain, which is a specialized kind of PAS domain. The LOV domain binds a chromophore, FAD (flavin adenine dinucleotide) that strongly absorbs blue light (32, 71). When it absorbs blue light it becomes chemically excited and makes a covalent adduct with a cysteine in the binding pocket of WC-1. By analogy to the VVD protein that also possesses a LOV domain and is a blue light photoreceptor (72), this event causes structural changes in the WC-1 protein that propagate to the surface of the protein where a helix is released exposing an interaction surface. WC-1 interacts with itself (multimerizing, 32) and exposing a transcriptional activation domain(s) that interact with general transcription machinery to drive the acute induction of the *frq* gene, events that all happen within minutes of light exposure. These events happen at the pLRE in the *frq* promoter, a region that is close upon the transcription start site and, as described earlier, well separated from the Clock-box where WC-1/WC-2 (the WCC) mediate activation of *frq* as a part of the circadian cycle that happens in constant darkness.

Interestingly, clocks in mammals are reset in very much the same way although they use a different means to detect light (73). As we all know, in mammals photoreceptors for light are present only in the eyes, so light perception elicits neuronal signals from each eye that travel down the optic tracks to the brain; the tracks cross at the optic chiasm. There in the hypothalamus, and directly above the optic chiasm, lie two bundles of neurons called the suprachiasmatic nuclei (SCN), about 10,000 each, that comprise the central pacemaker that controls locomotor activity and sleep wake cycles. The light signal goes to a part of the SCN where it elicits release of the neurotransmitter glutamate, which elicits intracellular release of calcium ions, activating PKA and other kinases which phosphorylate and activate CREB (cAMP response element binding protein) and CRTC1 (CREB-regulated transcription coactivator 1), which bind to the *Per1* and *Per2* promoters and induce their expression, thus resetting the mammalian core clock (73, 74). Thus, although the means to achieve clock gene induction are different and reflect the biology of the cell types involved, the overall mechanism, acute light-induction of negative elements in the core oscillator, is the same in *Neurospora* and in mammals.

Interestingly, the regulatory parallels between fungi and mammalian clocks do not end with the mechanism of light resetting; light also induces an acute repressor of the light response. This was also first elucidated in *Neurospora* where light acutely induces *vivid* which encodes VVD, another photoreceptor protein that acts as a negative regulator of the WCC (75). VVD physically interacts with WC-1 (76–78) and dampens its activity, and this has two effects. First, VVD turns what would be a step increase in WCC activity into a pulsatile increase followed by a return to background levels of activity. Second, VVD facilitates a graded response to successively higher light responses; this is because VVD binds to light activated WC-1 and sequesters it, and the next higher intensity light signal then releases some of the sequestered WCC to allow re-induction of light-regulated genes (79). In mammals, light-driven CREB activity induces Sik1 (salt-inducible kinase1), which inactivates CREB through phosphorylation (80). Thus, as with acute light induction of circadian negative elements, there is conservation of the regulatory architecture of the light response in mammals to achieve the same end, turning what would be a step increase into a pulsatile increase.

Temperature

Temperature is the environmental variable other than light that is most dependable on planet Earth and as a result it influences phase of all known circadian clocks. Interestingly, and despite our well described mechanisms for whole body temperature homeostasis, this even extends to clocks in isolated human cells. There is a reliable daily rhythm in body temperature with an amplitude of greater than one degree Centigrade; cycles of this magnitude are sufficient to entrain the clocks of cells in culture (81) and probably contributes to keeping cells in synchrony within our bodies.

The molecular mechanisms underlying temperature entrainment were first examined in *Neurospora* and the molecular response appears distinct from that seen for light (Figure 6). In *Neurospora* the amount of *frq* mRNA is not regulated by temperature and remains fairly constant across the physiological range; however, post-transcriptional mechanisms govern the amount of total FRQ made such that FRQ cycles around a higher mean level at higher temperatures (82). Even the lowest amount of total cellular FRQ seen at 30°C is higher than the highest amount seen at 20°C, and this fact drives the model for resetting based on temperature steps (83). When there is a step from high to low temperature, no matter at what time of day the shift happens, there is so much FRQ that the clock is set to the time corresponding to peak FRQ levels, around subjective dusk. Conversely, following a shift from low to high temperatures there is so little FRQ that the clock is set to the time of day corresponding to low FRQ levels, around subjective dawn (84).

OUTPUT FROM THE CLOCK

Circadian clocks are important because they control and coordinate the activities of cells so that appropriate biochemical activities happen at appropriate times of day. There are numerous examples from the circadian literature demonstrating the advantage of circadian regulation, chiefly by showing that organisms with clocks whose period lengths correspond with environmental cycles outcompete organisms with clocks whose period lengths are different from the environment: for instance, wt clocks do better than long period clocks in a 24 hr environment, but long period clocks do better than wt clocks in a long period environment (e.g. 85). There are a variety of reasons why this might be the case. For instance, organization and partition of metabolism by time of day would prevent futile cycles and ensure that necessary precursors are available for metabolic needs.

In *Neurospora*, as has been described earlier, there is a very obvious rhythm in growth and development. This is seen under light dark cycles but not in constant darkness unless carbon sources are limited or efforts are made to reduce ambient CO₂ or to enhance levels of reactive oxygen species (86, 87). It follows that there should be a rhythm in gene expression that supports these visible outputs from the clock, and indeed there is, but it should be noted that this plain quest for clock-regulated genes was first formulated and pursued in *Neurospora* (88). In the late 1980s efforts were undertaken to use subtractive hybridization to enrich for genes whose expression peaked in a time-of-day specific manner and this resulted in the identification of *clock-controlled gene-1* (*cgg-1*) and *cgg-2* (later shown to be allelic to *eas*, 89). This work also introduced the term “clock-controlled gene” into the circadian lexicon where it is now commonly used: clock-controlled genes are genes whose

daily expression is controlled in a circadian fashion by the clock, but whose gene products do not generally impact the operation of the clock (Figure 7A). This distinction, although it seems obvious now, was truly novel at the time and laid the foundation for studying output as a distinct sub-field with circadian research.

Circadian Control of Transcription

It is now widely recognized that the principal means of output from the clock in all organisms from bacteria through humans is through daily control of transcription and the generation of *ccgs*. Efforts to identify *ccgs* have tracked the available technologies through the years, beginning with subtractive hybridization, and then differential hybridization, EST sequencing, microarrays, and most recently (and successfully) RNA-seq which is now taken as the state of the art. This technology has been applied to identify the *ccgs* in a wide variety of organisms including *Neurospora*.

Some technical issues surrounding the identification of *ccgs* should be mentioned. Overall gene expression will change in response to time-of-day in constant conditions (true circadianly regulated genes), but will also change in response to age of the culture, ambient temperature, changes in nutrition (e.g. a shift in nitrogen sources from ammonium to nitrate, or from glucose to cellulose, or a variety of other factors). Because of this efforts must be made to avoid such changes, beginning with the use of a medium such as Bird (90) that contains only one nitrogen source. Cultures must be as close as possible to the same age (hours since germination) as is feasible, and to address this strict schedules have been developed to adjust the times of transfer from light to dark (the cue that sets the clock running) of sets of cultures so that when all are harvested they can be the same developmental age but represent different circadian clock times (88, 91). There have been steady developments in software to extract rhythmic signals from the non-rhythmic background; these are now highly developed and in passing a few technical issues should be noted. There are more stringent (JTK, 92, 93) and less stringent (ARSER, 94)) software and algorithms available for calling rhythmic genes. To be dependably able to call rhythmic genes sampling density must be high; samples taken at least every 2 hrs over 2 days are needed to reliably identify even 80% of rhythmic genes, and hourly sampling is better (92). Lastly, time series data can be highly influenced by initial conditions and in *Neurospora* the first hours are still strongly influenced by the previous light dark cycle; in no case can data from a single day be concatenated to make an imaginary two day time series unless the goal is imaginary data.

Early studies (e.g., 88, 95) identified *ccgs* but due to their inherent limitations tended to identify only the most abundantly expressed genes, and these were numbered in the order they were identified from *ccg*-1 to *ccg*-16. At this point there was the realization that there would be potentially thousands of *ccgs* (as there now are) and so this method of naming was discontinued (96, 97). The most recent data that adheres to the standards of the previous paragraph as shown in Figure 7B. These data were collected from cultures growing in liquid medium with glucose and gentle shaking in constant darkness. Under these conditions there will be only very limited development (no aerial hyphae, for instance) so the spectrum of *ccgs* might be greatly expanded if cultures were collected from solid medium. Nonetheless

and despite the relatively restricted overt development, the data show that as much as 40% of the genome is clock controlled (false discovery rate of 0.05) and roughly 10% is strongly and dependably clock controlled (91). This is likely to be an underestimate because when random promoters were assayed for their ability to drive circadian gene expression by fusion with a luciferase reporter, 75% were shown to be rhythmic only by luciferase; these were predominantly low abundance genes, suggesting that the depth of sequencing was underpowered to detect rhythms at a 0.05 confidence level when there were simply too few transcripts present. Another interesting conclusion is that the spectrum of *ccgs* changes with the composition of the growth medium. It is plain that circadian regulation exerts a profound influence on the genome, and in fact if the criteria for choosing *ccgs* is relaxed just a bit the majority of expressed genes will show some influence of time of day.

This finding has several important implications. First, it is plain that culture conditions matter a great deal in all experiments and not just if one is looking for *ccgs*. Prior light-dark treatment, as well as light-dark conditions during an experiment can manifestly influence what is and is not seen. The level of expression of a gene might appear to be 10 fold higher or lower depending on when tissue is collected from an incubator that excludes light. Sometime effects are due to circadian regulation but sometimes it is just light. An interesting example is the identification of DNA methylation at several places in the genome including the *frq* locus; methylation is plainly seen if cultures have been exposed to light but not if they have been in extended darkness or at elevated temperatures (33). There are also implications simply for choosing invariant “control genes” for normalization of RT-QPCR data because many genes, including commonly used as “invariant controls” such as *gapdh* (a.k.a. *ccg-7*), are in fact clock controlled. In a useful spin off from this study, mathematical tools were developed and used to identify truly invariant genes whose expression levels span the range of normal gene expression (98). The genes listed in this study provide truly valid control genes for normalization of gene expression studies.

The data of Hurley *et al.* ((91) (and to some extent Sancar *et al.* although because of the experimental design these data are strongly influenced by the prior light-dark cycle, 99)) reveal significant metabolic partitioning as a function of time of day that extends to control of major aspects of cell biology including growth, protein synthesis, stress responses, and intermediary metabolism. The greatest influence of circadian control on gene expression is seen around subjective dawn and dusk and the fewest relative number of *ccgs* peak in the middle of the day and night. While a great amount of detail is known and could be written about this, it suffices to say here that, in broad outline, morning and daytime gene expression tends to favor catabolism, energy production, and precursor assembly whereas dusk and nighttime gene expression tends to favor genes whose products are involved in biosynthesis of cellular components and biomass accumulation (91).

The timing of transcriptional regulation itself provides abundant means for regulation. Whenever the WCC drives expression of another transcription factor (TF), regulation of that TF can provide a means for altering phase because it can feed back or forward to influence other TFs and co-regulators, and in addition the TF itself can be posttranslational modified. A current edge of research is the description of this network of TFs, who drives their expression and which other TFs they in turn regulate.

After the first tier of transcriptional regulation, of course, there are a number of additional steps involved in filtering the output from the clock. This is most clearly understood when one realizes that the circadian signal begins with the rhythmic activation of the WCC that, based on the expression of *frq*, its most salient output, peaks in the morning. It follows that in order to obtain the spectrum of peak output phases shown in Figure 7B there must be many additional levels of regulation. We can begin with the act of transcription yielding a nascent transcript. Splicing of the transcript can be regulated followed by transcript stability. As a rule of thumb, as the stability of a transcript increases its peak phase will become more and more delayed as compared to the peak time of its transcription. Hurley et al attempted to estimate regulation at the post-transcriptional level by comparing the peak times of *ccgs* from RNA-seq (i.e. total amounts) with the peak times of expression based on promoter luciferase fusions for around 300 genes (91). The two did not match well for a large number of genes, indicating that additional levels of post-transcriptional regulation of transcripts are not uncommon.

Moving down the Central Dogma from mRNA there can be regulation at the level of translation, post-translational modification of the protein to influence its binding partners, activity, or intracellular localization, and finally to regulate its stability. Many of these levels of regulation also have the capacity for global effects. For instance, another edge of research in *Neurospora* is regulation of kinase activity. Roles for MAP kinases in cellular responses to stress or in mating are well known (e.g., 100) and they activate a wide variety of proteins, but interestingly the clock has been shown to regulate basal MAPK signaling (101, 102). In *Neurospora* OS-4 is the MAPKKK that initiates signaling through the cascade in response to stress and OS-2 is the terminal effector kinase in the MAPK cascade. In the subjective morning OS-2 activity is high both because the WCC directly drives high OS-4 levels and because the activity of the OS-4 inhibitor RRG-1 is low, again due to circadian regulation. In the evening the reverse is true. Circadian regulation of MAPK signaling is another aspect of regulation conserved between *Neurospora* and mammals (103).

NONCIRCADIAN OSCILLATORS IN FUNGI

Most fungi will display discernable rhythms in growth, morphology or development under light-dark and temperature cycles but only a few of these are actually circadian in nature. The “rhythms” appear because most fungi with the exception of yeasts can perceive and respond to light, as described in the chapter by Fischer *et al.* in this volume, and temperature is an all-pervasive metabolic controller. However, as noted earlier, under the right conditions – even conditions of constant nutrition, darkness, temperature and humidity – a wide variety of fungi will express rhythms in growth habit, growth rate, hyphal branching, morphology any or all of which are likely accompanied by changes in gene expression. These rhythms no doubt contribute to the vitality of the organism and they received much attention in the 1960s and 70s; however, as the definition of circadian was refined it became clear that they are not truly circadian (3). This was an important stage in the development of circadian biology as a field because it was only when such diverse systems were jettisoned that it became possible to focus on commonalities in approach and mechanism. As every geneticist knows, screens are possible only when phenotypes are robust, so it was this winnowing of noncircadian from truly circadian rhythms (e.g., 22, 104) that allowed the field to focus and

deliver a molecular answer to the question of how the circadian clock works. The first screens for clock mutants were initiated by Jerry Feldman in the early 1970s (105) and yielded the *frq* gene whose cloning in the early 1980s (106) nucleated the molecular dissection of the clock in *Neurospora*.

Leaving circadian rhythms aside for the present, there is a very rich biology describing non-circadian rhythms although much of it is now becoming dated. Noncircadian rhythms in hyphal branching or zonation are known in *Podospora anserina* (107), *Aspergillus niger* (108), *Leptosphaeria michotii* (e.g. 109), *Nectria cinnabarina* (110), along with *Penicillium* spp, *Alternaria tenuis*, and a number of additional fungi (3, 111). There is even a rhythm in the formation of nematode traps by the nematophagous fungus *Arthrobotrys oligospora* (112). The first noncircadian rhythms in *Neurospora crassa* date from the 1960s (113), and more than three decades ago, the defining FLO rhythm was described in the *frq*-null strain *frq⁰* (114, 115). When *frq* was cloned and the mutant alleles analyzed the allele was shown to encode a truncated and nonfunctional FRQ and the existence of the noncircadian oscillator was confirmed (68); it can have a period between 12 and 34 hours depending on the nutrition and temperature (that is, it is completely uncompensated) and cannot be entrained by light. Since then, in *Neurospora* where cellular rhythms have been studied more deeply than in any other cell, there are at least nine noncircadian rhythms described that can be distinguished by their period lengths or the means used to induce them (e.g. 96, 116, 117–121). Generically these are termed FLOs (for FRQ-Less Oscillators, 122). All share the property of cyclicity displaying in the aggregate periods ranging from 10 to over 100 hours, and none show the full range of defining circadian characteristics.

Much confusion in the field, and within fungal biology, has arisen from investigators confusing the output of the circadian clock with the clock itself. In *Neurospora* the clock controls conidiation as seen on race tubes. The logical fallacy has been to assume that anything that control conidiation must therefore be part of the circadian clock. This statement is obviously flawed because conidiation, like any developmental process, can be controlled by many factors and will not always happen; it is merely one obvious aspect of overt circadian control along with all of the rhythms in gene expression noted above. Although there are detailed descriptions of many FLOs, to date it is still unclear whether these serve any adaptive function to the organism or whether they are simply accidental by-products of metabolic regulation by feedback. On this point the NRO oscillator, which appears to derive from feedback loops associated with regulation of nitrate reductase, is instructive (116). In part because of the logical fallacy of equating all conidiation rhythms with circadian rhythms, some FLOs have been studied in depth in the hope that they would reveal something of use for understanding circadian rhythms, and one that has been studied most intensively is induced by choline starvation (119). This CDO (Choline Deficiency Oscillator) exerts control of overt growth and development such that conidiation rhythmicity can assume a period well in excess of 100 hrs and growth is up to 8-fold faster between conidial bands as within conidial bands. The CDO is not temperature compensated, has lost phase control, and it is light-sensitive in *wc-1⁺* genetic backgrounds and (obviously) does not require *frq* (123). The question of whether this oscillator interacts with the circadian FRQ/WC Oscillator was answered definitively in experiments using a luciferase reporter to track *frq* expression and WCC activity under conditions of choline limitation where the

CDO had taken control of conidiation. These experiments, done in *frq*⁺ and *frq*[period mutant] backgrounds clearly showed the circadian FRQ/WC Oscillator running in the background with a period appropriate to the *frq* allele (22 or 29 hrs) while the CDO ran with a circa-80 hr period length. The two cycles crossed each other at all phases more than once with no evidence for interaction (124).

Although the CDO plainly does not interact with the circadian clock other FLOs might take input from the clock, in particular those with period lengths comparable to the circadian clock, i.e. circa 24 hrs, like the NRO but unlike the CDO. In this context, one rationale for the existence of FLOs is that they might serve as slave oscillators that could under the right conditions assume proximal control of aspects of metabolism while remaining under the overall control of the circadian system. A slave oscillator could be open to evolutionary fine-tuning without altering the more finely tuned compensated/entrainable circadian clock, and the slave need not have the full complement of circadian properties; in a normal cell it would still be a part of the circadian system as it would be entrained by the circadian clock. In a *frq* or *wc* null mutant background and absent the circadian clock, each slave reverts to its inherent periodicity (28, 29, 125, 126).

CONCLUSION

Neurospora has provided a durable system for the study of biological clocks and oscillators in the broadest sense, and more specifically in circadian rhythms. Decades of analysis have shown that the *Neurospora* circadian clocks shares a common regulatory architecture as well as conserved components with circadian oscillators in animals, and many aspects of circadian biology and molecular biology in these organisms were first elucidated in *Neurospora*. These include the first systematic screens for *ccgs* and elucidation of transcriptional control as a principal means for circadian output (88), experimental proof that transcriptional negative feedback is at the core of the clock (68), elucidation of the mechanism for light-induced (70) and temperature-induced (83) clock resetting, identification of the first PAS domain-mediated heterodimer as the positive element in a circadian feedback loop (127), and closure of the loop in a single step via action of the negative element on the positive element (127). Work on *Neurospora* also led the way to understanding the cellular roles of noncircadian oscillators and documented the fact that many such oscillators can co-exist within a single mycelium. Many of the components involved in both kinds of oscillators are conserved among fungi and even more broadly.

Acknowledgments

This work was supported by grants from the National Institutes of Health to JCD (R35GM118021) and to JJJ (R35GM118022).

References

1. Dunlap, JC. Loros, JJ., Decoursey, P., editors. Chronobiology: Biological Timekeeping. Sinauer Assoc; Sunderland, MA: 2004.
2. Ingold, CT. Fungal Spores. Clarendon Press; Oxford: 1971.
3. Bünning, E. The Physiological Clock. 3. Springer-Verlag; New York: 1973.
4. Brandt WH. Zonation in a prolineless strain of *Neurospora*. Mycologia. 1953; 45:194–208.

5. Austin B. An endogenous rhythm of spore discharge in *Sordaria fimicola*. *Annals of Botany*. 1968; 32:262–278.
6. Greene AV, Keller N, Haas H, Bell-Pedersen D. A circadian oscillator in *Aspergillus spp.* regulates daily development and gene expression. *Eukaryot Cell*. 2003; 2:231237.
7. Bluhm BH, Burnham AM, Dunkle LD. A circadian rhythm regulating hyphal melanization in *Cercospora kikuchii*. *Mycologia*. 2010; 102:1221–1228. [PubMed: 20943572]
8. Hevia MA, Canessa P, Larrondo LF. Circadian clocks and the regulation of virulence in fungi: Getting up to speed. *Semin Cell Dev Biol*. 2016; 57:147–155. [PubMed: 27039027]
9. Hevia MA, Canessa P, Muller-Esparza H, Larrondo LF. A circadian oscillator in the fungus *Botrytis cinerea* regulates virulence when infecting *Arabidopsis thaliana*. *Proc Natl Acad Sci U S A*. 2015; 112:8744–8749. [PubMed: 26124115]
10. Traeger S, Nowrousian M. Analysis of circadian rhythms in the basal filamentous ascomycete *Pyronema confluens*. *G3 (Bethesda)*. 2015; 5:2061–2071. [PubMed: 26254031]
11. Oliveira AG, Stevani CV, Waldenmaier HE, Viviani V, Emerson JM, Loros JJ, Dunlap JC. Circadian control sheds light on fungal bioluminescence. *Curr Biol*. 2015; 25:964–968. [PubMed: 25802150]
12. Kippert F. Circadian control of heat tolerance in stationary phase cultures of *Schizosaccharomyces pombe*. *Arch Microbiol*. 1989; 151:177–179. [PubMed: 2719527]
13. Eelderink-Chen Z, Mazzotta G, Sturre M, Bosman J, Roenneberg T, Merrow M. A circadian clock in *Saccharomyces cerevisiae*. *Proc Natl Acad Sci U S A*. 2010; 107:2043–2047. [PubMed: 20133849]
14. Montenegro-Montero A, Canessa P, Larrondo LF. Around the fungal clock: Recent advances in the molecular study of circadian clocks in *Neurospora* and other fungi. *Adv Genet*. 2015; 92:107–184. [PubMed: 26639917]
15. Diernfellner AC, Schafmeier T. Phosphorylations: Making the *Neurospora crassa* circadian clock tick. *FEBS Lett*. 2011; 585:1461–1466. [PubMed: 21453704]
16. Crosthwaite SK. Circadian timekeeping in *Neurospora crassa* and *Synechococcus elongatus*. *Essays Biochem*. 2011; 49:37–51. [PubMed: 21819383]
17. Cha J, Zhou M, Liu Y. Mechanism of the *Neurospora* circadian clock, a FREQUENCY-centric view. *Biochemistry*. 2015; 54:150–156. [PubMed: 25302868]
18. Hurley J, Loros JJ, Dunlap JC. Dissecting the mechanisms of the clock in *Neurospora*. *Methods Enzymol*. 2015; 551:29–52. [PubMed: 25662450]
19. Hurley JM, Loros JJ, Dunlap JC. The circadian system as an organizer of metabolism. *Fungal Genet Biol*. 2016; 90:39–43. [PubMed: 26498192]
20. Lakin-Thomas PL, Bell-Pedersen D, Brody S. The genetics of circadian rhythms in *Neurospora*. *Adv Genet*. 2011; 74:55–103. [PubMed: 21924975]
21. Bell-Pedersen D, Garceau N, Loros JJ. Circadian rhythms in fungi. *J Genet*. 1996; 75:387–401.
22. Dunlap JC. Salad days in the rhythms trade. *Genetics*. 2008; 178:1–13. [PubMed: 18202353]
23. Dunlap, JC., Loros, JJ. Molecular Genetics of Circadian Rhythms in *Neurospora*. In: Takahashi, J.Turek, FW., Moore, RY., editors. *Handbook of Behavioral Neurobiology*. Kluwer; New York: 2001. p. 331–347.
24. Dunlap JC, Loros JJ, Colot HV, Mehra A, Belden WJ, Shi M, Hong CI, Larrondo LF, Baker CL, Chen CH, Schwerdtfeger C, Collopy PD, Gamsby JJ, Lambregts R. A circadian clock in *Neurospora*: how genes and proteins cooperate to produce a sustained, entrainable, and compensated biological oscillator with a period of about a day. *Cold Spring Harb Symp Quant Biol*. 2007; 72:57–68. [PubMed: 18522516]
25. Hurley JM, Loros JJ, Dunlap JC. Circadian oscillators: Around the transcription-translation feedback loop and on to output. *Trends Biochem Sci*. 2016; 41:834–846. [PubMed: 27498225]
26. Liu Y, Bell-Pedersen D. Circadian rhythms in *Neurospora crassa* and other filamentous fungi. *Eukaryot Cell*. 2006; 5:1184–1193. [PubMed: 16896204]
27. Liu Y, Heintzen C, Loros J, Dunlap JC. Regulation of clock genes. *Cellular and Molecular Life Sciences*. 1999; 55:1195–1205. [PubMed: 10487202]

28. Loros JJ, Dunlap JC. Genetic and molecular analysis of circadian rhythms in *Neurospora*. *Annu Rev Physiol*. 2001; 63:757–794. [PubMed: 11181975]
29. Loros JJ, Dunlap JC, Larrondo LF, Shi M, Belden WJ, Gooch VD, Chen CH, Baker CL, Mehra A, Colot HV, Schwerdtfeger C, Lambregts R, Collopy PD, Gamsby JJ, Hong CI. Circadian output, input, and intracellular oscillators: insights into the circadian systems of single cells. *Cold Spring Harb Symp Quant Biol*. 2007; 72:201–214. [PubMed: 18419278]
30. Taylor BL, Zhulin IB. Pas domains: Internal sensors of oxygen, redox potential, and light. *Micro & Molec Biol Rev*. 1999; 63:479–506.
31. Wang B, Kettenbach AN, Gerber SA, Loros JJ, Dunlap JC. *Neurospora* WC-1 recruits SWI/SNF to remodel frequency and initiate a circadian cycle. *PLoS Genet*. 2014; 10:e1004599. [PubMed: 25254987]
32. Froehlich AC, Liu Y, Loros JJ, Dunlap JC. White Collar-1, a circadian blue light photoreceptor, binding to the *frequency* promoter. *Science*. 2002; 297:815–819. [PubMed: 12098706]
33. Belden WJ, Lewis ZA, Selker EU, Loros JJ, Dunlap JC. CHD1 remodels chromatin and influences transient DNA methylation at the clock gene *frequency*. *PLoS Genet*. 2011; 7:e1002166. [PubMed: 21811413]
34. Wang B, Zhou X, Loros JJ, Dunlap JC. Alternative use of DNA binding domains by the *Neurospora* White Collar Complex dictates circadian regulation and light responses. *Molecular and Cellular Biology*. 2015; 36:781–793. [PubMed: 26711258]
35. Zhang Y, Smith CL, Saha A, Grill SW, Miardja S, Smith SB, Cairns BR, Peterson CL, Bustamante C. DNA translocation and loop formation mechanism of chromatin remodeling by SWI/SNF and RSC. *Mol Cell*. 2006; 24:559–568. [PubMed: 17188033]
36. Kramer C, Loros JJ, Dunlap JC, Crosthwaite SK. Role for antisense RNA in regulating circadian clock function in *Neurospora crassa*. *Nature*. 2003; 421:948–952. [PubMed: 12607002]
37. Xue Z, Ye Q, Anson SR, Yang J, Xiao G, Kowbel D, Glass NL, Crosthwaite SK, Liu Y. Transcriptional interference by antisense RNA is required for circadian clock function. *Nature*. 2014; 514:650–653. [PubMed: 25132551]
38. Li N, Joska TM, Ruesch CE, Coster SJ, Belden WJ. The *frequency* natural antisense transcript first promotes, then represses, frequency gene expression via facultative heterochromatin. *Proc Natl Acad Sci U S A*. 2015; 112:4357–4362. [PubMed: 25831497]
39. Lee K, Dunlap JC, Loros JJ. Roles for WHITE COLLAR-1 in circadian and general photoperception in *Neurospora crassa*. *Genetics*. 2003; 163:103–114. [PubMed: 12586700]
40. Zhou M, Guo J, Cha J, Chae M, Chen S, Barral JM, Sachs MS, Liu Y. Non-optimal codon usage affects expression, structure and function of clock protein FRQ. *Nature*. 2013; 495:111–115. [PubMed: 23417067]
41. Hurley JM, Dunlap JC. Cell biology: A fable of too much too fast. *Nature*. 2013; 495:57–58. [PubMed: 23417066]
42. Cheng P, Yang Y, Heintzen C, Liu Y. Coiled coil mediated FRQ-FRQ interaction is essential for circadian clock function in *Neurospora*. *EMBO J*. 2001; 20:101–108. [PubMed: 11226160]
43. Cheng P, He Q, He Q, Wang L, Liu Y. Regulation of the *Neurospora* circadian clock by an RNA helicase. *Genes Dev*. 2005; 19:234–241. [PubMed: 15625191]
44. Lykke-Andersen S, Tomecki R, Jensen TH, Dziembowski A. The eukaryotic RNA exosome: same scaffold but variable catalytic subunits. *RNA Biol*. 2011; 8:61–66. [PubMed: 21289487]
45. Hurley JM, Larrondo LF, Loros JJ, Dunlap JC. Conserved RNA helicase FRH acts nonenzymatically to support the intrinsically disordered *neurospora* clock protein FRQ. *Mol Cell*. 2013; 52:832–843. [PubMed: 24316221]
46. Baker CL, Dunlap JC. Circadian rhythms: phosphorylating the CLOCK. *Cell Cycle*. 2010; 9:231–232. [PubMed: 20061797]
47. Baker CL, Kettenbach AN, Loros JJ, Gerber SA, Dunlap JC. Quantitative proteomics reveals a dynamic interactome and phase-specific phosphorylation in the *Neurospora* circadian clock. *Mol Cell*. 2009; 34:354–363. [PubMed: 19450533]
48. Tang CT, Li S, Long C, Cha J, Huang G, Li L, Chen S, Liu Y. Setting the pace of the *Neurospora* circadian clock by multiple independent FRQ phosphorylation events. *Proc Natl Acad Sci U S A*. 2009; 106:10722–10727. [PubMed: 19506251]

49. Baker CL, Loros JJ, C DJ. The circadian clock of *Neurospora crassa*. FEMS Microbiol Rev. 2012; 36:95–110. [PubMed: 21707668]
50. Mehra A, Baker CL, Loros JJ, Dunlap JC. Post-translational modifications in circadian rhythms. Trends Biochem Sci. 2009; 34:483–490. [PubMed: 19740663]
51. Mehra A, Shi M, Baker CL, Colot HV, Loros JJ, Dunlap JC. A role for casein kinase 2 in the mechanism underlying circadian temperature compensation. Cell. 2009; 137:749–760. [PubMed: 19450520]
52. He Q, Cha J, He Q, Lee H, Yang Y, Liu Y. CKI and CKII mediate the FREQUENCY-dependent phosphorylation of the WHITE COLLAR complex to close the Neurospora circadian negative feedback loop. Genes and Dev. 2006; 20:2552–2565. [PubMed: 16980584]
53. Yang Y, Cheng P, Liu Y. Regulation of the *Neurospora* circadian clock by casein kinase II. Genes Dev. 2002; 16:994–1006. [PubMed: 11959847]
54. Pregueiro A, Liu Q, Baker C, Dunlap JC, Loros JJ. The Neurospora checkpoint kinase 2: a regulatory link between the circadian and cell cycles. Science. 2006; 313:644–649. [PubMed: 16809488]
55. Yang Y, Cheng P, Zhi G, Liu Y. Identification of a calcium/calmodulin-dependent protein kinase that phosphorylates the *Neurospora* circadian clock protein FREQUENCY. J Biol Chem. 2001; 276:41064–41072. [PubMed: 11551951]
56. Yang Y, Cheng P, He Q, Wragg P, Yarden O, Liu Y. Distinct roles for PP1 and PP2A in the Neurospora circadian clock. Genes & Dev. 2004; 18:255–260. [PubMed: 14871927]
57. Aronson BD, Johnson KA, Loros JJ, Dunlap JC. Negative feedback defining a circadian clock - Autoregulation of the clock gene *frequency*. Science. 1994; 263:1578–1584. [PubMed: 8128244]
58. Dunlap JC. Molecular bases for circadian clocks. Cell. 1999; 96:271–290. [PubMed: 9988221]
59. Siwicki KK, Eastman C, Petersen G, Rosbash M, Hall JC. Antibodies to the *period* gene product of *Drosophila* reveal diverse tissue distribution and rhythmic changes in the visual system. Neuron. 1988; 1:141–150. [PubMed: 3152288]
60. Ruoff P, Loros JJ, Dunlap JC. The relationship between FRQ-protein stability and temperature compensation in the Neurospora circadian clock. Proc Natl Acad Sci U S A. 2005; 102:17681–17686. [PubMed: 16314576]
61. Syed S, Saez L, Young MW. Kinetics of doubletime kinase-dependent degradation of the Drosophila period protein. J Biol Chem. 2011; 286:27654–27662. [PubMed: 21659538]
62. Larrondo LF, Olivares-Yanez C, Baker CL, Loros JJ, Dunlap JC. Circadian rhythms. Decoupling circadian clock protein turnover from circadian period determination. Science. 2015; 347:1257277. [PubMed: 25635104]
63. He Q, Cheng P, Yang Y, He Q, Yu Q, Liu Y. FWD-1 mediated degradation of FREQUENCY in *Neurospora* establishes a conserved mechanism for circadian clock regulation. EMBO J. 2003; 22:4421–4430. [PubMed: 12941694]
64. Izumo M, Johnson CH, Yamazaki S. Circadian gene expression in mammalian fibroblasts revealed by real-time luminescence reporting: temperature compensation and damping. Proc Natl Acad Sci U S A. 2003; 100:16089–16094. [PubMed: 14657355]
65. Tosini G, Menaker M. Circadian rhythms in cultured mammalian retina. Science. 1996; 272:419–421. [PubMed: 8602533]
66. Feldman, JF., Gardner, GF., Dennison, RA. Genetic analysis of the circadian clock of *Neurospora*. In: Suda, M., editor. Biological Rhythms and their Central Mechanism. Elsevier; Amsterdam: 1979. p. 57-66.
67. Sugano S, Andronis C, Green R, Wang Z, Tobin E. Protein kinase CK2 interacts with and phosphorylates the *Arabidopsis* circadian clock-associated gene 1 protein. Proc Nat Acad Sci USA. 1998; 95:11020–11025. [PubMed: 9724822]
68. Aronson B, Johnson K, Loros JJ, Dunlap JC. Negative feedback defining a circadian clock: Autoregulation in the clock gene *frequency*. Science. 1994; 263:1578–1584. [PubMed: 8128244]
69. Mellow M, Garceau N, Dunlap JC. Dissection of a circadian oscillation into discrete domains. Proc Nat Acad Sci USA. 1997; 94:3877–3882. [PubMed: 9108072]
70. Crosthwaite SC, Loros JJ, Dunlap JC. Light-Induced resetting of a circadian clock is mediated by a rapid increase in *frequency* transcript. Cell. 1995; 81:1003–1012. [PubMed: 7600569]

71. He Q, Cheng P, Yang Y, Wang L, Gardner K, Liu Y. WHITE COLLAR-1, a DNA binding transcription factor and a light sensor. *Science*. 2002; 297:840–842. [PubMed: 12098705]
72. Vaidya AT, Chen C-H, Dunlap JC, Loros JJ, Crane BR. Structure of a light-activated LOV protein dimer that regulates transcription. *Science Signaling*. 2011; 4:ra50. [PubMed: 21868352]
73. Shigeyoshi Y, Taguchi K, Yamamoto S, Takeida S, Yan L, Tei H, Moriya S, Shibata S, Loros JJ, Dunlap JC, Okamura H. Light-induced resetting of a mammalian circadian clock is associated with rapid induction of the *mPer1* transcript. *Cell*. 1997; 91:10431053.
74. Albrecht U, Sun Z, Eichele G, Lee C. A differential response of two putative mammalian circadian regulators, *mper1* and *mper2*, to light. *Cell*. 1997; 91:1055–1064. [PubMed: 9428527]
75. Heintzen C, Loros JJ, Dunlap JC. The PAS protein VIVID defines a clock-associated feedback loop that represses light input, modulates gating, and regulates clock resetting. *Cell*. 2001; 104:453–464. [PubMed: 11239402]
76. Chen C-H, DeMay BS, Gladfelter AS, Dunlap JC, Loros JJ. Physical interaction between VIVID and white collar complex regulates photoadaptation in *Neurospora*. *Proc Natl Acad Sci U S A*. 2010; 107:16715–16720. [PubMed: 20733070]
77. Hunt SM, Thompson S, Elvin M, Heintzen C. VIVID interacts with the WHITE COLLAR complex and FREQUENCY-interacting RNA helicase to alter light and clock responses in *Neurospora*. *Proc Natl Acad Sci U S A*. 2010; 107:16709–16714. [PubMed: 20807745]
78. Malzahn E, Ciprianidis S, Kaldi K, Schafmeier T, Brunner M. Photoadaptation in *Neurospora* by competitive interaction of activating and inhibitory LOV domains. *Cell*. 2010; 142:762–772. [PubMed: 20813262]
79. Dasgupta A, Chen CH, Lee C, Gladfelter AS, Dunlap JC, Loros JJ. 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*. 2015; 11:e1005215. [PubMed: 25978382]
80. Jagannath A, Butler R, Godinho SI, Couch Y, Brown LA, Vasudevan SR, Flanagan KC, Anthony D, Churchill GC, Wood MJ, Steiner G, Ebeling M, Hossbach M, Wettstein JG, Duffield GE, Gatti S, Hankins MW, Foster RG, Peirson SN. The CRTC1-SIK1 pathway regulates entrainment of the circadian clock. *Cell*. 2013; 154:1100–1111. [PubMed: 23993098]
81. Saini C, Morf J, Stratmann M, Gos P, Schibler U. Simulated body temperature rhythms reveal the phase-shifting behavior and plasticity of mammalian circadian oscillators. *Genes Dev*. 2012; 26:567–580. [PubMed: 22379191]
82. Liu Y, Garceau N, Loros JJ, Dunlap JC. Thermally regulated translational control mediates an aspect of temperature compensation in the *Neurospora* circadian clock. *Cell*. 1997; 89:477–486. [PubMed: 9150147]
83. Liu Y, Mellow M, Loros JJ, Dunlap JC. How temperature changes reset a circadian oscillator. *Science*. 1998; 281:825–829. [PubMed: 9694654]
84. Liu Y. Molecular mechanisms of entrainment in the *Neurospora* circadian clock. *J Biol Rhythms*. 2003; 18:195–205. [PubMed: 12828277]
85. Ouyang Y, Andersson CR, Kondo T, Golden SS, Johnson CH. Resonating circadian clocks enhance fitness in cyanobacteria. *Proc Natl Acad Sci USA*. 1998; 95:8660–8664. [PubMed: 9671734]
86. Belden WJ, Larrondo LF, Froehlich AC, Shi M, Chen C-H, Loros JJ, Dunlap JC. The *band* mutation in *Neurospora crassa* is a dominant allele of *ras-1* implicating RAS-signaling in circadian output. *Genes Dev*. 2007; 21:1494–1505. [PubMed: 17575051]
87. Dunlap JC, Loros JJ. Analysis of circadian rhythms in *Neurospora*: overview of assays and genetic and molecular biological manipulation. *Methods Enzymol*. 2005; 393:3–22. [PubMed: 15817284]
88. Loros JJ, Denome SA, Dunlap JC. Molecular cloning of genes under the control of the circadian clock in *Neurospora*. *Science*. 1989; 243:385–388. [PubMed: 2563175]
89. Bell-Pedersen D, Dunlap JC, Loros JJ. The *Neurospora* circadian clock-controlled gene, *ccg-2*, is allelic to *eas* and encodes a fungal hydrophobin required for formation of the conidial rodlet layer. *Genes & Dev*. 1992; 6:2382–2394. [PubMed: 1459460]
90. Metzberg RL. Bird Medium: an alternative to Vogel Medium. *Fungal Genetics Newsletter*. 2004; 51:19–10.

91. Hurley JM, Dasgupta A, Emerson JM, Zhou X, Ringelberg CS, Knabe N, Lipzen AM, Lindquist EA, Daum CG, Barry KW, Grigoriev IV, Smith KM, Galagan JE, Bell-Pedersen D, Freitag M, Cheng C, Loros JJ, Dunlap JC. Analysis of clock-regulated genes in *Neurospora* reveals widespread posttranscriptional control of metabolic potential. *Proc Natl Acad Sci U S A*. 2014; 111:16995–17002. [PubMed: 25362047]
92. Hughes ME, Hogenesch JB, Kornacker K. JTK_CYCLE: an efficient nonparametric algorithm for detecting rhythmic components in genome-scale data sets. *J Biol Rhythms*. 2010; 25:372–380. [PubMed: 20876817]
93. Wu G, Anafi RC, Hughes ME, Kornacker K, Hogenesch JB. MetaCycle: an integrated R package to evaluate periodicity in large scale data. *Bioinformatics*. 2016; 32:3351–3353. [PubMed: 27378304]
94. Yang R, Su Z. Analyzing circadian expression data by harmonic regression based on autoregressive spectral estimation. *Bioinformatics*. 2010; 26:i168–174. [PubMed: 20529902]
95. Bell-Pedersen D, Shinohara M, Loros J, Dunlap JC. Circadian clock-controlled genes isolated from *Neurospora crassa* are late night to early morning specific. *Proc Nat Acad Sci USA*. 1996; 93:13096–13101. [PubMed: 8917550]
96. Correa A, Lewis ZA, Greene AV, March IJ, Gomer RH, Bell-Pedersen D. Multiple oscillators regulate circadian gene expression in *Neurospora*. *Proc Natl Acad Sci U S A*. 2003; 100:13597–13602. [PubMed: 14597725]
97. Nowrousian M, Duffield GE, Loros JJ, Dunlap JC. The *frequency* gene is required for temperature-dependent regulation of many clock-controlled genes in *Neurospora crassa*. *Genetics*. 2003; 164:922–933.
98. Hurley JH, Dasgupta A, Andrews P, Crowell AM, Ringelberg C, Loros JJ, Dunlap JC. A Tool Set for the Genome-Wide Analysis of *Neurospora crassa* by RT-PCR. *G3 (Bethesda)*. 2015; 5:2043–2049. [PubMed: 26248984]
99. Sancar C, Sancar G, Ha N, Cesbron F, Brunner M. Dawn- and dusk-phased circadian transcription rhythms coordinate anabolic and catabolic functions in *Neurospora*. *BMC Biol*. 2015; 13:17. [PubMed: 25762222]
100. Alvaro CG, Thorner J. Heterotrimeric G Protein-coupled Receptor Signaling in Yeast Mating Pheromone Response. *J Biol Chem*. 2016; 291:7788–7795. [PubMed: 26907689]
101. Bennett LD, Beremand P, Thomas TL, Bell-Pedersen D. Circadian Activation of the MAP Kinase MAK-1 Facilitates Rhythms in Clock-Controlled Genes in *Neurospora crassa*. *Eukaryot Cell*. 2012; 12:59–69. [PubMed: 23125351]
102. Lamb TM, Finch KE, Bell-Pedersen D. The *Neurospora crassa* OS MAPK pathway-activated transcription factor ASL-1 contributes to circadian rhythms in pathway responsive clock-controlled genes. *Fungal Genet Biol*. 2012; 49:180–188. [PubMed: 22240319]
103. Goldsmith CS, Bell-Pedersen D. Diverse roles for MAPK signaling in circadian clocks. *Adv Genet*. 2013; 84:1–39. [PubMed: 24262095]
104. Feldman J, Hoyle MN. A direct comparison between circadian and noncircadian rhythms in *Neurospora crassa*. *Plant Physiol*. 1974; 53:928–930. [PubMed: 16658817]
105. Feldman JF. Genetic approaches to circadian clocks. *Ann Rev Plant Physiol*. 1982; 33:583–608.
106. McClung CR, Fox BA, Dunlap JC. The *Neurospora* clock gene *frequency* shares a sequence element with the *Drosophila* clock gene *period*. *Nature*. 1989; 339:558–562. [PubMed: 2525233]
107. Lysek G, Esser K. Rhythmic mycelial growth in *Podospora anserina*. *Archiv für Mikrobiologie*. 1970; 73:224–230.
108. Jerebzoﬀ S, Jerebzoﬀ-Quintin S, Lambert E. *Aspergillus niger*: characteristics of endogenous and low frequency rhythms. *Int J Chronobiol*. 1974; 2:131–144. [PubMed: 4423773]
109. Jerebzoﬀ-Quintin S, Jerebzoﬀ S. Cyclic AMP level in relation to different conditions of sporulation rhythm regulation in *Leptosphaeria michotii* (west) Sacc. *Arch Microbiol*. 1981; 128:325–329. [PubMed: 6260051]
110. Bourret JA, Lincoln RG, Carpenter BH. Modification of the Period of a Noncircadian Rhythm in *Nectria cinnabarina*. *Plant Physiol*. 1971; 47:682–684. [PubMed: 16657684]

111. Jerebzoﬀ, S. Metabolic steps involved in periodicity. In: Hastings, JW., Schweiger, H-G., editors. Dahlem Workshop on the Molecular Basis of Circadian Rhythms. Dahlem Konferenzen; Berlin: 1976. p. 193-213.
112. Lysek G. An endogenous rhythm of trap formation in the nematophagous fungus *Arthrobotrys oligospora*. *Planta*. 1981; 152:50–53. [PubMed: 24302318]
113. Sussman AS, Lowrey RJ, Durkee T. Morphology and genetics of a periodic colonial mutant of *Neurospora crassa*. *Am J Botan*. 1964; 51:243–252.
114. Loros, JJ. PhD Thesis. PhD. U.C. Santa Cruz; 1986. Studies on *frq-9*, a recessive circadian clock mutant of *Neurospora crassa*.
115. Loros JJ, Richman A, Feldman JF. A recessive circadian clock mutant at the *frq* locus in *Neurospora crassa*. *Genetics*. 1986; 114:1095–1110. [PubMed: 2948874]
116. Christensen MK, Falkeid G, Loros JJ, Dunlap JC, Lillo C, Ruoff P. A nitrate-induced *frq*-less oscillator in *Neurospora crassa*. *J Biol Rhythms*. 2004; 19:280–286. [PubMed: 15245647]
117. de Paula RM, Vitalini MW, Gomer RH, Bell-Pedersen D. Complexity of the *Neurospora crassa* circadian clock system: multiple loops and oscillators. *Cold Spring Harb Symp Quant Biol*. 2007; 72:345–351. [PubMed: 18419292]
118. Granshaw T, Tsukamoto M, Brody S. Circadian rhythms in *Neurospora crassa*: farnesol or geraniol allow expression of rhythmicity in the otherwise arrhythmic strains *frq*[10], *wc-1*, and *wc-2*. *J Biol Rhythms*. 2003; 18:287–296. [PubMed: 12932081]
119. Lakin-Thomas P. Choline depletion, *frq* mutations, and temperature compensation of the circadian rhythm in *Neurospora crassa*. *J Biol Rhythms*. 1998; 13:268–277. [PubMed: 9711502]
120. Mattern D, Brody S. Circadian rhythms in *Neurospora crassa*: effects of unsaturated fatty acids. *J Bacteriol*. 1979; 139:977–988. [PubMed: 158008]
121. Nsa IY, Karunarathna N, Liu X, Huang H, Boettger B, Bell-Pedersen D. A novel cryptochrome-dependent oscillator in *Neurospora crassa*. *Genetics*. 2015; 199:233–245. [PubMed: 25361899]
122. Iwasaki H, Dunlap JC. Microbial circadian oscillatory systems in *Neurospora* and *Synechococcus*: models for cellular clocks. *Curr Opin Microbiol*. 2000; 3:189–196. [PubMed: 10744993]
123. Lakin-Thomas PL, Brody S. Circadian rhythms in *Neurospora crassa*: lipid deficiencies restore robust rhythmicity to null *frequency* and *white-collar* mutants. *Proc Nat Acad Sci USA*. 2000; 97:256–261. [PubMed: 10618405]
124. Shi M, Larrondo LF, Loros JJ, Dunlap JC. A developmental cycle masks output from the circadian oscillator under conditions of choline deficiency in *Neurospora*. *Proc Natl Acad Sci U S A*. 2007; 104:20102–20107. [PubMed: 18056807]
125. Dunlap JC, Loros JJ. The *Neurospora* circadian system. *J Biol Rhythms*. 2004; 19:414–424. [PubMed: 15534321]
126. Dunlap JC, Loros JJ. How fungi keep time: circadian system in *Neurospora* and other fungi. *Curr Opin Microbiol*. 2006; 9:579–587. [PubMed: 17064954]
127. Crosthwaite SC, Dunlap JC, Loros JJ. *Neurospora wc-1* and *wc-2*: Transcription, photoresponses, and the origins of circadian rhythmicity. *Science*. 1997; 276:763–769. [PubMed: 9115195]
128. Dunlap JC. Proteins in the *Neurospora* circadian clockworks. *Journal of Biological Chemistry*. 2006; 281:28489–28493. [PubMed: 16905532]

Figure 1.

Neurospora circadian output as seen by control of growth and development in a culture growing across an agar surface. Cultures are inoculated at the left and grow to the right. After a period in constant light the culture is transferred to constant darkness. The position of the growth front at the time of transfer is marked and is marked again every 24 hrs giving a measure of time since the light-to-dark transfer. Below the images of the cultures is a densitometric scan of the mycelial/conidial optical density. The peak of conidiation happens roughly 10 hrs after the light-to-dark transfer and recurs after that roughly every 22.5 hrs (one circadian day) for cultures grown at 25°C. Typically the peak in conidiation is used as a phase reference point, but any definable point in the cycle can be used. Adapted with permission from (49).

Figure 2.

The WC-1 and WC-2 proteins and the *frq* locus. A. Cartoon schematics of WC-1 and WC-2 are shown to scale. Landmarks include protein start sites (AUG) poly glutamine stretches (polyQ), the binding site for SWI/SNF (31), the LOV domain that mediates photoreception, the two PAS domains of WC-1 that mediate interaction with the PAS domain on WC-2, and the DBD domain that assists the ZN-finger in DNA binding to the C-box. The putative transcriptional activation domain (AD) of WC-2 is marked. B. Complex regulation and splicing involved in *frq* expression. The Clock Box and Proximal light regulatory element (LRE) are upstream from P_U and P_D, the upstream and downstream start sites for transcription respectively. The parts of the primary transcript left after splicing of intron are marked in green, asterisks mark upstream ORFs that influence the amount of FRQ made, and purple marks the FRQ coding region. Temperature regulated splicing of intron 2 governs whether AUG_L or AUG_S is used to initiate FRQ. The *frq* antisense transcript, *qrf*, is marked in red. Adapted from (128).

Figure 3.

Temporal outline of the *Neurospora* circadian clock. (Top) Amounts of clock-relevant RNAs and proteins cycle. (Bottom) Shown as a function of time are the locations of RNAs and proteins important for the circadian oscillator. Time goes from left to right, beginning at subjective dawn (CT 0, about 11 hrs after a light-to-dark transfer). Trash can = proteasome; PP = phosphatases. Adapted from (24).

Figure 4.

FRQ domains and their time-of-day specific modifications. A cartoon of the domains of FRQ is shown to the left: CC – coiled-coiled domain, NLS – nuclear localization signal, FCD – FRQ-CKI interacting domain, FFD – FRQ-FRH interacting domain, PEST-# - PEST domains important for protein turnover, *frq*[#] - *frq* alleles followed by period length. Moving to the right in the figure, the positions of phosphorylations are marked. The two heat maps show how these change over time with yellow marking higher phosphorylation and blue lower phosphorylation; each heat map consists of six columns corresponding to 4 hr blocks

of time in the circadian day from subjective dawn (CT0) through dusk (CT12) to the next night (CT20). The heat map to the right shows total phosphorylation and in the heat map to the left the level of phosphorylation at any time is normalized to the total phosphorylation of the protein to better highlight where changes are occurring; these areas are marked with boxes and arrows showing where they lie within FRQ. Farthest to the right are noted the effects of mutating the serines or threonines that are phosphorylated at the locations noted to alanines. Modified from (47).

Figure 5.

Light resets the clock. (Top) The daily cycle in the abundance of *frq* RNA is shown along with how a brief exposure to light at any time greatly increases this. (Middle) The cycle of *frq* transcription is shown in black, and the colored lines show the effect of light-induced *frq* expression on the steady state rhythm. (Bottom) The response of the clock to light is juxtaposed with the daily cycle of *frq* expression: When light is seen while *frq* levels are rising the phase of the cycle is advanced because *frq* rises to peak levels sooner. When light is seen while *frq* levels are falling the cycle is set back. Adapted from (24).

Figure 6.

How temperature might reset the circadian cycle. Cycles in FRQ levels at different temperatures are shown in green. A step up in temperature at any time (red arrows) results in not enough FRQ to close the feedback loop, so the clock is reset to the time corresponding to low FRQ, subjective dawn. A step down in temperature at any time leaves the clock with sufficient FRQ to close the feedback loop; the cycle is reset to the time marked by high FRQ, around subjective evening. (Adapted from 24).

Figure 7.

Output from the circadian clock. A. The relationship of output to the oscillator is shown. The circadian clock in fungi and animals can be approximated as a single step negative feedback loop in which the Positive Elements (in blue, the heterodimer of WC-1 and WC-2 or in animals BMAL1 and CLOCK) drive expression of the gene(s) encoding the Negative Elements (in red). The Negative Elements feed back, physically interact with the Positive Elements and turn down their activity. The cycle thus generates rhythmic activity of the Positive Elements. When they in turn regulate genes whose products do not participate in the

feedback loop, there is the means for transcriptional output from the clock. These are the *ccgs*. (Adapted from 58). B. The abundance of *ccgs* is shown as a function of the time of day at which their levels peak. The data are from 2 hr bins across the day, with a bimodal peak of gene expression in the late night to morning and another peak around dusk (From 91).