How fungi keep time: circadian system in *Neurospora* and other fungi

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The circadian system in *Neurospora* remains a premier model system for understanding circadian rhythms, and evidence has now begun to accumulate suggesting broad conservation of rhythmicity amongst the filamentous fungi. A well-described transcription–translation-based negative feedback loop involving the FREQUENCY, WHITE COLLAR-1 and WHITE COLLAR-2 proteins is integral to the *Neurospora* system. Recent advances include descriptions of the surprisingly complex *frequency* transcription unit, an enhanced appreciation of the roles of kinases and their regulation in the generation of the circadian rhythm and their links to the cell cycle, and strong evidence for an additional WHITE COLLAR-associated feedback loop. Documentation of sequence homologs of integral circadian and photoresponsive proteins amongst the 42 available sequenced fungal genomes suggests unexpected roles for circadian timing among both pathogens and saprophytes.

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Introduction

Fungi employ a variety of lifestyles from saprophytic to pathogenic in order to occupy an extremely wide variety of habitats. Most fungi however, whether free-living or residing in a plant or animal, share a dimension of their environment that is so pervasive that it is easy to take it for granted: this dimension is time, and for most organisms the dominant aspect of time is imposed by the earth's rotation, the daily cycles of light and dark and of warm and cool that influence activity or rest in animals, and photosynthesis and carbon utilization in plants. Like most organisms and nearly all eukaryotes, fungi express single cell-based biological clocks that enable them to track internal time and to anticipate dependable environmental changes. The timing mechanism that facilitates this is known as the circadian system. It is comprised of a circadian oscillator and its associated inputs and outputs, and its product is a circadian rhythm.

Stepping back for a moment, it should be acknowledged that fungi can express a variety of rhythms with various period lengths and characteristics. Glycolytic oscillations, with a period length of minutes and based on the feedback of metabolic products of glycolysis on enzymes, were described decades ago (reviewed in [1]) and, more recently, metabolic rhythms in yeast with period lengths of hours have been generated by careful control of growth conditions [2]. Such cycles are of interest in understanding the greater principles that govern cellular metabolism. On a longer time scale, rhythms in growth and sporulation that have period lengths in excess of 100 h have been produced in filamentous fungi including *Aspergillus*, *Leptosphaeria* and *Neurospora*, by manipulation of genetic background and nutrition [3,4], and lichens can display growth rings with period lengths exceeding decades. All these cycles share some traits in that they are not generally responsive to environmental signals such as light and temperature changes and, importantly, their period lengths are highly dependent on ambient temperature and nutrition. Whereas the biology of such cycles is interesting, in most cases they almost certainly reflect some aspect of biology that is peculiar to the organism and not shared widely with other living things [5]. Between these extremes are circadian rhythms, all of which by definition share characteristics that aid in their utility to the host organism: circadian rhythms have a period length close to 24 h, can be reset ('entrained' in circadian terminology) to exactly 24 h through periodic light or temperature cues, and have period lengths that are relatively insensitive to nutrition or ambient temperature within the physiological range ('temperature compensation'). In the fungi, true circadian rhythms have been described in growth rate, aerial hyphal formation and sporulation, sexual development, CO_2 evolution and in expression or activity of a host of genes and enzymes (reviewed in [6,7].

By far the best understood circadian system in fungi is that of *Neurospora*, in which clock-mutant strains were identified concurrently with those in *Drosophila* [1]. Molecular analysis of the *Neurospora* circadian system was initiated shortly after that of *Drosophila* in the mid 1980s and resulted in the cloning of the clock gene *frequency* (*frq*) [8] and the completion of global screens for clock-controlled genes called *ccg*s [9]. In the 1990s, molecular manipulations of *frq* expression showed *frq* to encode a central component of the core oscillator itself,

and many additional clock genes (now more than 10) have since been characterized and cloned. With a genetic framework in place, steady progress has been made in understanding the molecular bases for sustainability of the rhythm, period length, resetting of the circadian system by light and temperature cues, and for gating of input cues (reviewed in [10–13]). Recently, *Neurospora* has been suggested as a useful model for understanding photoperiodism [14], and is proving to be a valuable system for examining the role of coupled feedback loops in clocks, and for defining global features of circadian output. The presently advanced state of understanding of the *Neurospora* clock system provides a base from which to view clocks in other fungal systems. This review presents an overview of the known components of the *Neurospora* circadian oscillatory system, and looks at how some of these components are conserved amongst other fungi. From this perspective it is possible to step back and look at the entire circadian system in *Neurospora*.

How the molecular elements in the circadian oscillator of *Neurospora* keep time

The *Neurospora* clock includes a genuine timekeeper in which a slow molecular negative feedback loop based on transcription and translation operates in real time over the course of approximately a day. The principal components of this loop are two proteins, WHITE COLLAR-1 (WC-1) and WHITE COLLAR-2 (WC-2), that act together as a transcription factor (the WCC) in order to drive expression of another protein, FREQUENCY (FRQ) that, once made, acts to repress the activity of WC-1 and WC-2. Very recently, a second feedback loop also using the WCC but not FRQ has been observed [15••] and is discussed below, following description of the better-understood mechanisms. These core elements operate in the context of and are supported by many other proteins, and most investigators believe that this feedback loop actually generates time information. In this way, the state of the various molecular components, as seen in Figure 1, provides a glimpse of the subjective time in the cell.

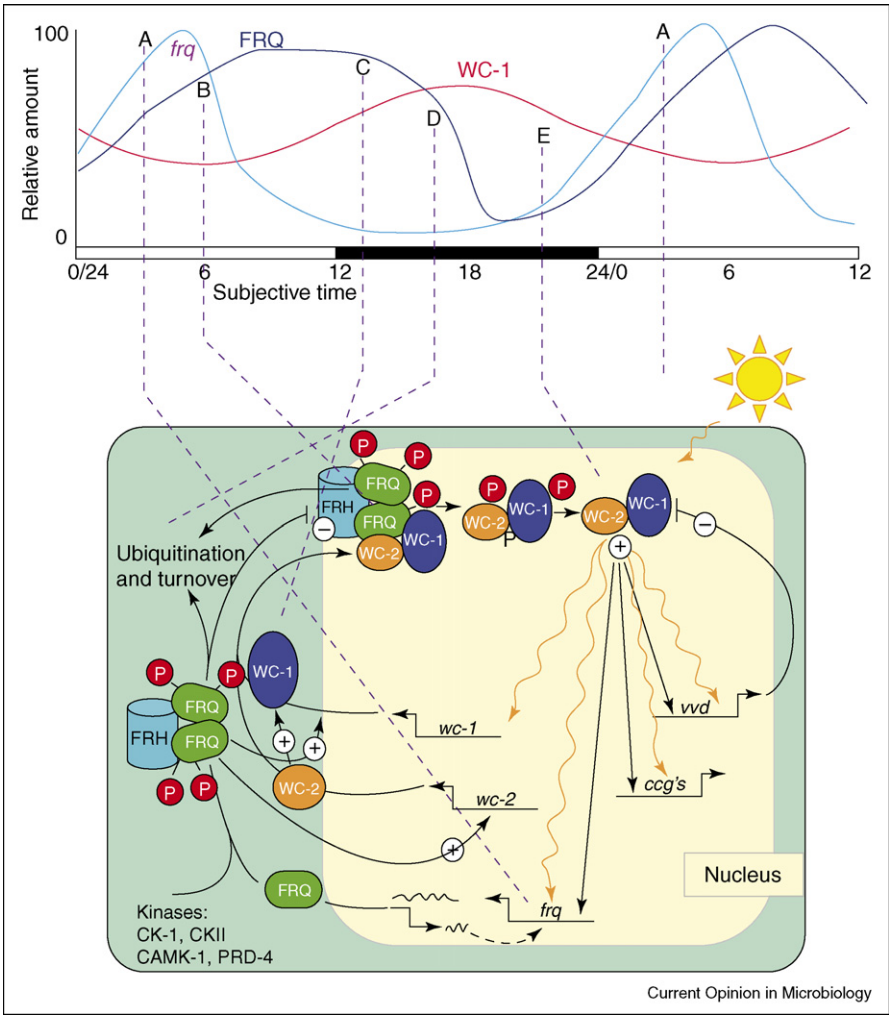
frq is transcribed

By late subjective night (Figure 1, around point D), most of the FRQ protein in the cell has become unstable and is being degraded and *frq* mRNA levels are low. WC-1 and WC-2 act together by binding to a specific sequence (the Clock Box [16]) on the *frq* promoter in order to drive transcription, and protein-encoding *frq* transcripts appear by between late night and morning (Figure 1, between E and A). The transcription and processing of the protein-encoding *frq* transcripts is itself an interesting topic because *frq* represents one of the most complex loci known in microbes. A long but non-coding antisense *frq* transcript, *qrf* (for *frq* backwards), is rhythmically expressed at low levels with a peak phase opposite to that of *frq*, is light-induced and appears to play a role in ensuring precise entrainment to light and dark cues [17]. Sense *frq* RNAs arise from two distinct start sites driven by a promoter that can be regulated both by light and time of day [16,18]. A first intron can be spliced using one of two 5′ donor sites with the same 3′ acceptor, and the second intron is alternatively spliced in a temperature-dependent manner, altogether giving rise to six major identifiable transcripts the abundance of which reflects the environmental conditions [19•,20•]. The alternatively spliced second intron contains the first AUG codon of the FRQ ORF, so this temperature-influenced splicing determines the mix of long (989 amino acids) and short (889 amino acids) FRQ proteins that are translated [19•,20•,21,22], and their amount also varies with temperature. Both long and short FRQ proteins are needed for the best robust rhythmicity, but at temperatures below 22 °C, more short FRQ is used and, overall, less FRQ is needed, whereas at temperatures above 26 °C, higher overall levels FRQ are needed, and long FRQ is predominantly used [22]. Despite this regulation, distinct molecular functions for the FRQ isoforms are not yet described. Both forms are equally stable, and modulation of translation by temperature is governed by upstream ORFs in the 5′ untranslated region of *frq* [19•,20•]. Although this complicated temperature regulation of the forms and amounts of FRQ does not play a role in temperature compensation, it does seem to help in keeping the phase of the rhythm steady across a temperature range (A Diernfellner, H Colot, J Dunlap and M Brunner, in preparation). Instead, temperature compensation appears to derive from a balance between transcript synthesis and turnover of clock components, especially FRQ [23•], and is strongly influenced by levels of certain kinases (A Mehra, M Shi, J Loros and J Dunlap, unpublished).

FRQ is made, acts and is acted upon

By early subjective morning, within a few hours of when its transcript appears, FRQ proteins are synthesized [21], and they dimerize [24] and interact with the RNA helicase FRH [25••] before entering the nucleus [26] where they fulfill their first and major role in the biological clock. FRH is a member of the SKI2 subfamily of RNA helicases, and its closest homolog is the Mtr4/Dob1 helicase found in the *Saccharomyces* exosome, a complex of 3′–5′ exonucleases involved in RNA maturation and quality control [27]. Within the nucleus, specific interactions occur between the FRQ–FRH complex and the WCC (Figure 1, point B) [28–30] that block the activity of the WCC [16], probably by affecting the phosphorylation status of either or both WC-1 and WC-2 (see Update) [31••,32••,33•]. As a result, by mid-subjective day WCC activity declines to its lowest level [16,34], and this dampened expression of *frq* RNA also causes its level to begin to fall. FRQ synthesis continues as long as *frq* mRNA is present, and so the peak in FRQ levels occurs near the end of the day.

Figure 1



Molecular events in the FRQ–WCC based oscillator over the course of a circadian cycle. On the top are shown the daily cycles in levels of *frq* mRNA, FRQ protein and WC-1 protein in constant darkness; subjective day and night are marked by white and black bars. The bottom panel shows the sequential molecular actions and interactions in the oscillator; dotted lines connecting the top with the bottom panel show when these events happen over the course of a day. 'P' indicates phosphorylation of a protein. Straight solid arrows show positive action, dashed arrows show negative effects, and wavy arrows show activation by light. See text for more details. Figure adapted from [12] and from National Academies Keck Futures Initiative Signaling National Conference, http://63.251.167.36/nakfi/progressive/GeneticandMolecularDissectionofE/index.htm.

As soon as FRQ appears, it begins to be phosphorylated by several kinases — CKI, CKII (casein kinase I and II), CAMK1 (calcium/calmodulin-dependent protein kinase 1) and conditionally by PRD-4 (period-4) — that determine its stability [35–39,40••]. Phosphorylation of FRQ serves to facilitate interaction of FRQ with the SCF (Skp1-Cul1-F-box-protein)-type ubiquitin ligase FWD-1 that eventually targets FRQ for turnover in the nucleosome (Figure 1, around point D) [41], and FWD-1 is later recycled through the action of the COP9 signalosome [42•]. FRQ phosphorylation might also influence FRQ–WCC interactions [37]. Overall, *frq* mRNA levels peak in the subjective mid-morning [43,44], about 6 h before the peak of total FRQ that occurs in the late afternoon [21].

The rate of phosphorylation of FRQ is an important determinant of period length, and it's now clear that environmentally induced changes in phosphorylation can affect period and can also reset the clock (e.g. see [40••]). This came to light when a mutant clock gene, *prd-4* was cloned and shown to encode the important cell cycle regulator checkpoint kinase-2 (CHK2). Expression of *prd-4* is clock-regulated, and when activated by DNA damage, PRD-4 phosphorylates FRQ (to reset the clock), as well as cell cycle regulators to stop the cell cycle. The clock thus gates both its own response and the response of the cell cycle to DNA damage, an observation that might help to explain the role of the circadian system in tumorigenesis in mammals (e.g. see [40••]).

Although FRQ enters the nucleus soon after its synthesis in order to fulfill its initial and perhaps dominant clock role in depressing its own synthesis, a third role of FRQ relies on its continued presence in the cytoplasm, as well as on its phosphorylation. Later (Figure 1, around point C), probably triggered by previous phosphorylation events of FRQ at different sites, FRQ becomes phosphorylated at Ser885 and Ser887, and this enables it to promote accumulation of WC-1 [45•]. Because WC-1 is stabilized by its interaction with WC-2, an attractive model is that appropriately phosphorylated FRQ fosters the assembly of the WCC. In this way, a relatively invariant pool of spliced *wc-1* mRNA arising from several promoters [46] yields a low amplitude (and apparently dispensable) rhythm in WC-1 [28,34]. It should be noted, however, that additional mechanisms must contribute to this low-amplitude dispensable rhythm as it is still seen in *frq*-null strains [15••]. If this was not enough for one protein to do, FRQ also promotes the expression of *wc-2* mRNA [28,31••,47], although WC-2 levels are constitutively high in any case [29].

The end results of all these functions of FRQ result in a stable and robust cycle: early action results in negative feedback (Figure 1, point B), late action promotes the appearance of both WC-1 and WC-2 (Figure 1, point C) so that WCC is maintained at an elevated level but, moreover, is held inactive [16,29,34] through the action of FRQ in promoting the phosphorylation of the WCC [31••]. Eventually the precipitous phosphorylation-mediated turnover of FRQ (Figure 1, point D) releases the WCC and it can then be recycled to an active state through the action of protein phosphatases. Once active it is able to reinitiate the transcription of *frq* mRNA in the next cycle (Figure 1, point E).

Light and temperature changes can reset the clock (reviewed in [11,48–50]). Temperature appears to act within the oscillator by affecting the levels of components [51], whereas light resets the clock by acting on and through the blue light photoreceptor WC-1 [18,52], apparently causing a change in the quaternary state of the WCC at the *frq* promoter [18]. When cells are held in darkness and then exposed to light, there is a sudden burst of *frq* transcription [44] that pushes the clock from any point in the cycle to around point A in Figure 1. Under natural light–dark cycles the VVD (a flavoprotein and auxiliary photoreceptor) protein [53] dampens this response at dawn, thereby enabling the clock to continue keeping time during the day so that the clock is reset at dusk [54•].

Modeling the *Neurospora* clock

The overall kinetics of the circadian cycle fits remarkably well with this simple conceptual model. A time of 3–6 h is required for FRQ synthesis and 14–18 h for recovery from FRQ repression by processing and degradation [55]. This consistency, in addition to the advanced state of understanding of interrelationships among components of the *Neurospora* clock, has fostered development of mathematical models that enable critical analysis of the assumptions which underlie the intuitive models the original molecular experiments were driven by [56–59]. These models have underscored the similarities between fungal and animal circadian systems [60], and are providing insights into some of the more cryptic clock properties, such as temperature compensation [23•,61,62].

Roles for other oscillators?

As noted in the introduction, if cultured in just the right way, many fungi including *Neurospora*, are able to exhibit long period oscillations that, nonetheless, fail several of the essential criteria for being classed as circadian. These rhythms do not require FRQ and are collectively known as FRQ-less oscillators or FLOs [63]. The question of whether these rhythms are the key to understanding circadian clocks or just a manifestation of organism-specific biology that can operate in parallel to the circadian oscillator is difficult, and perhaps irresolvable (e.g. see [64•–66•]). Having been summarized elsewhere [11,67] it won't be further discussed here because of space constraints.

Very recently though, evidence has emerged for a novel oscillator in *Neurospora* that requires the WC proteins but not FRQ. A microarray study [68] identified several genes encoding cycling transcripts with unusual phases, and one of these (now called *ccg-16*) cycles in constant light and in *frq*-null strains, but not in Δ*wc-1* or Δ*wc-2* strains [15••]. Although the evidence for temperature and nutritional compensation are not yet robust — period lengths can only be estimated from time-series samples of RNA assayed by Northern blot analysis — the data suggest the presence of an additional feedback loop (initially called the WC-FLO) involving the WC proteins and possessing circadian properties. The mechanism of such an oscillator is anyone's guess, but as FRQ is not required, we might (tentatively) call this new oscillator the un-FRQ oscillator (yes, the UFO!).

Conservation of the transcription–translation feedback loop circadian mechanism

The basic layout of the transcription–translation feedback loop described above is well conserved between *Neurospora* and the animals (reviewed in [1,69]), and WC-1 is both a functional homolog and a weak sequence homolog of mammalian BMAL1 (brain and muscle Arnt-like protein-1) [34,70]. This mechanism is also consistent with another universal observation from the pre-molecular era: that in all circadian clocks examined there is a time of day when proteins must be made (e.g. see [71]). Both plant and cyanobacterial clocks, however, appear to be distinct from the fungal and animals clocks, both in terms of components and, perhaps more compellingly, in the

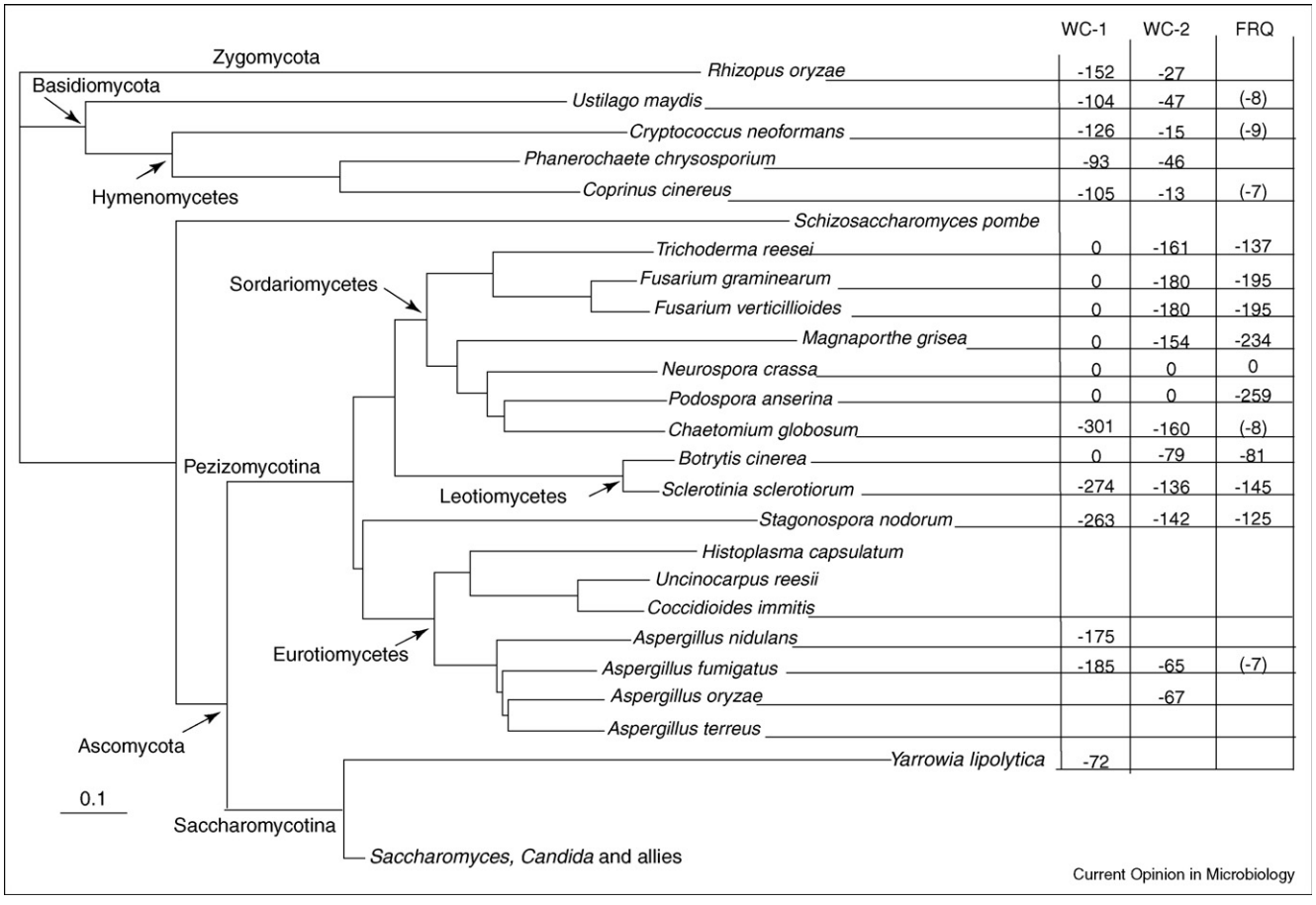
general layout of the loops (reviewed in [1,72]). The existing models cannot of course explain every detail from every circadian system, and this is interpreted by some [73] to mean that the true mechanism has yet to be elucidated. Nonetheless, the success of conceptual models lies in their ability to explain and predict, and by these criteria the existing transcription–translation models are eminently successful.

How conserved are these clocks amongst the fungi?

A large amount of environmental biology exists describing daily and annual cycles of airborne spores for instance [5], but such rhythms could easily be environmentally driven by winds or the light–dark cycle and cannot be taken as evidence of biological rhythms. Although older surveys exist [4,74], the literature describing genuine circadian rhythms amongst the fungi is both limited and largely several years old (predating PubMed), and so it's largely inaccessible to today's computer-tethered armchair scholars. Well-studied bona fide circadian rhythms exist in the Zygomycete *Pilobolus* [75], and growth or developmental rhythms are known in a variety of filamentous Ascomycetes (reviewed in [4]), although most of these fail several of the requisite tests for being classed as a circadian clock (discussed in [11,67]). The only novel true fungal circadian rhythm described recently is an entrainable temperature-compensated rhythm of sclerotia formation in *Aspergillus flavus* [76], and in the same study an enzyme rhythm was also described in *Aspergillus nidulans*.

Given this paucity of literature and the contrasting depth of knowledge describing the *Neurospora* clock, a better approach might be to look for conservation of known

Figure 2



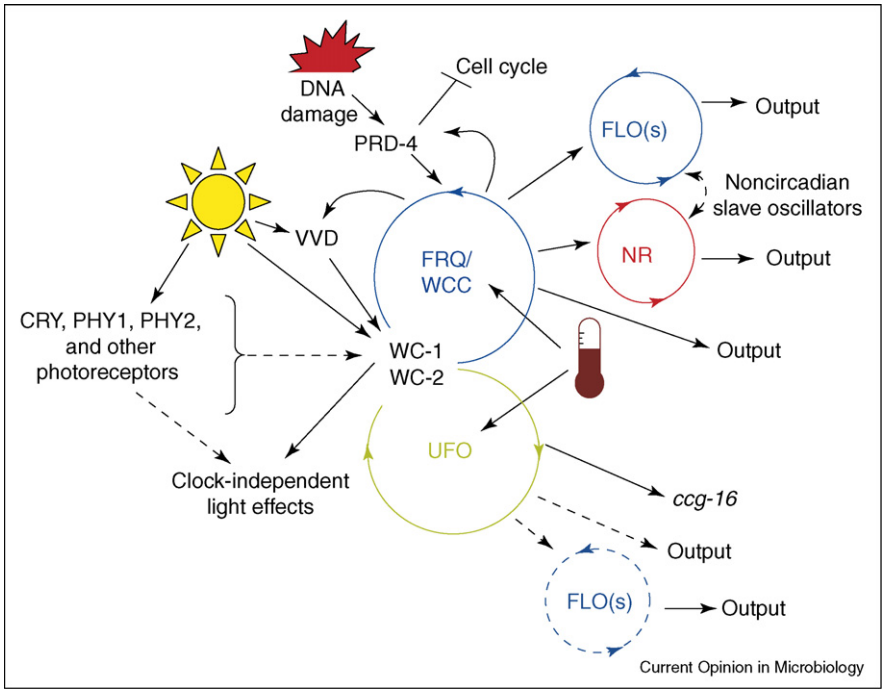
Conservation of FRQ, WC-1 and WC-2 amongst the fungi. Shown is a phylogenetic tree of the fungi with major groups noted and all filamentous fungi with sequenced genomes positioned. BLAST scores — precomputed by SIMAP at MIPS (http://mips.gsf.de/genre/proj/fungi/) or done individually through the Broad Institute (http://www.broad.mit.edu/annotation/genome/neurospora/Blast.html) — reflect homologies of protein sequences in sequenced genomes to *Neurospora* FRQ, WC-1 and WC-2. Only the exponents of scores are marked, thus 0 reflects near identity and a score of −200 reflects greater similarity than a score of −100. When paralogs were found in some species only the highest scoring member is noted. No attempt was made to further examine similarities in domains or to confirm the biological significance of the relatedness. Numbers in parentheses reflect similarities than may not extend over the entire protein and might therefore not be biologically informative.

clock components. This has been pursued at various levels from full functional complementation, using homologs from different genera [77], to simple sequence comparisons [78–80]; but, given the precedent from the former, the sequence comparisons might be a reasonably valid basis for comparison — and are much easier to perform. An update of this for the 42 sequenced fungal genomes (Figure 2) revealed some solid and interesting evolutionary trends.

Elements of the entire FRQ–WCC feedback loop are found sporadically, but not universally among the Pezizomycetes (formerly the Euascomycetes), and as far afield as *Stagonospora* and *Leptosphaeria* in the Loculoascomycetes. At first glance it appears that all the components for complete FRQ–WCC-based feedback loops are universally seen among the Sordariaceae, with the possible exception of *Chaetomium* (although given the otherwise strong conservation there is cause to suspect a sequencing error in this case). Thus, we might expect clocks like that seen in *Neurospora* in a variety of important plant pathogens (e.g. *Fusarium*, *Sclerotinia*, *Cochliobolus* and *Magnaporthe*), and a few opportunistic animal pathogens such as *Fusarium oxysporum*). Genes involved in biological clocks, as well those involved in light perception appear to have been lost in the wholesale reduction in genome size seen in the yeasts.

Light-sensing mechanisms appear to be widely conserved amongst the fungi. Although *Neurospora* [81] and *Aspergillus* [82•] contain phytochromes sensitive to red light, only blue light is known to reset the clock, and this is detected by the flavin-based circadian and blue-light photoreceptor WC-1 [18,52]. WC-1 is strongly conserved at the level of sequence, not just in the Pezizomycetes but throughout the fungi as far afield as the Basidiomycetes (mushrooms) and Zygomycetes (e.g. *Mucor*, *Phycomyces* and the black bread mold *Rhizopus*) and even in the Hemiascomycete yeast *Yarrowia*. And the conservation isn't just in the sequences: recently, a cottage industry has grown up around proof of functional conservation, with genuine blue-light photoreception seen to be the function of WC-1 homologs in the Ascomycetes *Magnaporthe* [83•] and *Trichoderma* [84], the Basidiomycetes *Cryptococcus* [85•,86••] and *Coprinus* [87•], and the Zygomycetes *Phycomyces* [88••] and *Mucor* [89•], demonstrating that indeed, in the fungal kingdom, light is sensed through a conserved mechanism [86••]. Lastly, given wide phylogenetic conservation of the WC proteins and the recent identification of circadian rhythms in strains lacking FRQ (*Aspergillus* [76] or *Neurospora* mutants [15••]), it might be that rhythms in the fungi can also be based in part on an oscillator, in some ways distinct from the well understood FRQ–WCC oscillator, for instance the UFO.

Figure 3



An overview of the Neurospora circadian system showing the oscillators generating time information and the networks integrating this with environmental cues in order to coordinate the life of the organism. Solid lines reflect known feedback loops or regulatory relationships and dotted lines those that are predicted, or that might reasonably exist. Adapted from National Academies Keck Futures Initiative Signaling National Conference (http://63.251.167.36/nakfi/progressive/GeneticandMolecularDissectionofE/index.htm).

Conclusions: the circadian system

The circadian oscillator that has been discussed here receives inputs of ambient light and temperature, which enable it to adjust its phase so that the internal day matches the external day, and then uses the time information it generates to regulate the life of the cell and of the organism as a whole. This circadian system generates the circadian rhythm.

Much is known about output and is reviewed elsewhere [6,7], so its full description is beyond the scope of this review. An important point, however, is that when the FRQ–WCC loop is functional, outputs such as conidiation or enzyme activities tend to be circadianly regulated, whereas when FRQ is lost, the outputs lose their circadian rhythmicity (e.g. compensation, light entrainment and persistence) although in some cases retaining some rhythmicity. A good example is a rhythm in nitrate reductase activity that is circadian in wild type cells but persists in Δ*frq* and in Δ*wc* strains, albeit having lost some circadian characteristics [90]. A satisfying and possibly correct way to view such rhythmic output in the context of the circadian the system is shown in Figure 3. Here the well known FRQ–WCC oscillator and the plausible newly described UFO are at the core, and serve both to directly drive some gene expression and enzyme rhythms as well as to coordinate the activities of various FLOs involved in output, rendering their activities truly circadian. Light input is mediated through FRQ and temperature input through both the FRQ–WCC and UFO. Collectively, these drive rhythms then govern the biology of the whole organism in a coordinated way.

Update

After submission of this review an additional paper has appeared documenting the importance of phosphorylation to the operation of the negative feedback loop [91•].

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References and recommended reading

Papers of particular interest, published within the annual period of review, have been highlighted as:

- • of special interest
- •• of outstanding interest

1. Dunlap JC: **Molecular biology of circadian pacemaker systems**. In *Chronobiology: Biological Timekeeping.* Edited by Dunlap JC, Loros JJ, Decoursey P: Sinauer Assoc; 2004:210-251.

2. Tu BP, Kudlicki A, Rowicka M, McKnight SL: **Logic of the yeast metabolic cycle: temporal compartmentalization of cellular processes**. *Science* 2005, **310**:1152-1158.

3. Jerebzoff S: **Metabolic steps involved in periodicity**. In *Dahlem Workshop on the Molecular Basis of Circadian Rhythms.* Edited by Hastings JW, Schweiger H-G: Dahlem Conferenzen; 1976:193-213.

4. Bünning E: *The Physiological Clock*. edn 3. Springer-Verlag; 1973.

5. Ingold CT: *Fungal Spores*. Oxford: Clarendon Press; 1971.

6. 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.

7. Bell-Pedersen D, de Paula RM, Vitalini MV: **The rhythms of life: circadian output pathways in *Neurospora*.** *J Biol Rhythms* 2006, **21**: in press.

8. 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.

9. Loros JJ, Denome SA, Dunlap JC: **Molecular cloning of genes under the control of the circadian clock in *Neurospora***. *Science* 1989, **243**:385-388.

10. Heintzen C, Liu Y: **The *Neurospora* circadian clock**. *Adv Genet* 2006, in press.

11. Dunlap JC, Loros JJ: **The *Neurospora* circadian system**. *J Biol Rhythms* 2004, **19**:414-424.

12. Dunlap JC: **Proteins in the *Neurospora* circadian clockwork**. *J Biol Chem* 2006, **281**:28489-28493.

13. Brunner M, Schafmeier T: **Transcriptional and post-transcriptional regulation of the circadian clock of cyanobacteria and *Neurospora***. *Genes Dev* 2006, **20**:1061-1074.

14. Tan Y, Merrow M, Roenneberg T: **Photoperiodism in *Neurospora crassa***. *J Biol Rhythms* 2004, **19**:135-143.

15. •• dePaula RM, Lewis ZA, Greene AV, Seo KS, Morgan LW, Vitalini MW, Bennett L, Gomer RH, Bell-Pedersen D: **Two circadian timing circuits in *Neurospora crassa* share components and regulate distinct rhythmic processes**. *J Biol Rhythms* 2006, **21**:159-168.
Physiological and molecular evidence are presented for the existence of a novel timing circuit that might contribute to the circadian system. Although WC-1 and WC-2 are required for the oscillator, FRQ is not, and so this new circuit might be called the un-FRQ oscillator or UFO.

16. Froehlich AC, Loros JJ, Dunlap JC: **Rhythmic Binding of a WHITE COLLAR Containing Complex to the *frequency* Promoter is Inhibited by FREQUENCY**. *Proc Natl Acad Sci USA* 2003, **100**:5914-5919.

17. 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.

18. Froehlich AC, Loros JJ, Dunlap JC: **WHITE COLLAR-1, a circadian blue light photoreceptor, binding to the *frequency* promoter**. *Science* 2002, **297**:815-819.

19. • Colot HV, Loros JJ, Dunlap JC: **Temperature-modulated alternative splicing and promoter use in the Circadian clock gene frequency**. *Mol Biol Cell* 2005, **16**:5563-5571.
See annotation for [20•].

20. • Diernfellner AC, Schafmeier T, Merrow MW, Brunner M: **Molecular mechanism of temperature sensing by the circadian clock of Neurospora crassa**. *Genes Dev* 2005, **19**:1968-1973.
Along with [19•], the authors describe the complex environmentally influenced use of multiple promoters, alternative splicing and translational control that regulated FRQ expression.

21. Garceau N, Liu Y, Loros JJ, Dunlap JC: **Alternative initiation of translation and time-specific phosphorylation yield multiple forms of the essential clock protein FREQUENCY**. *Cell* 1997, **89**:469-476.

22. 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.

23. • Ruoff P, Loros JJ, Dunlap JC: **The relationship between FRQ-protein stability and temperature compensation in the *Neurospora* circadian clock**. *Proc Natl Acad Sci USA* 2005, **102**:17681-17686.

A combined mathematical simulation and experimental study that convincingly models the temperature compensation process as the dynamic balance between the integrals of two opposing sets of processes.

24. 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.

25. •• 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.
The authors purified native FRQ, identified the FRH helicase as an obligate stoichiometric interacting partner, and developed novel technology to probe the role of this essential protein in the clock.

26. Luo C, Loros JJ, Dunlap JC: **Nuclear localization is required for function of the essential clock protein FREQUENCY**. *EMBO J* 1998, **17**:1228-1235.

27. LaCava J, Houseley J, Saveanu C, Petfalski E, Thompson E, Jacquier A, Tollervey D: **RNA degradation by the exosome is promoted by a nuclear polyadenylation complex**. *Cell* 2005, **121**:713-724.

28. Cheng P, Yang Y, Liu Y: **Interlocked feedback loops contribute to the robustness of the *Neurospora* circadian clock**. *Proc Natl Acad Sci USA* 2001, **98**:7408-7413.

29. Denault DL, Loros JJ, Dunlap JC: **WC-2 mediates WC-1-FRQ interaction within the PAS protein-linked circadian feedback loop of *Neurospora crassa***. *EMBO J* 2001, **20**:109-117.

30. Merrow M, Franchi L, Dragovic Z, Gorl M, Johnson J, Brunner M, Macino G, Roenneberg T: **Circadian regulation of the light input pathway in *Neurospora crassa***. *EMBO J* 2001, **20**:307-315.

31. •• Schafmeier T, Haase A, Kaldi K, Scholz J, Fuchs M, Brunner M: **Transcriptional feedback of *Neurospora* circadian clock gene by phosphorylation-dependent inactivation of its transcription factor**. *Cell* 2005, **122**:235-246.
With data suggesting that FRQ does not form a stoichiometric partnership with the WCC, the authors show that FRQ influences the phosphorylation of WC-2, thereby decreasing its ability to co-activate transcription. This casts details of the FRQ–WCC negative feedback loop in a new light.

32. •• He Q, Liu Y: **Molecular mechanism of light responses in *Neurospora*: from light-induced transcription to photoadaptation**. *Genes Dev* 2005, **19**:2888-2899.
Light-induced hyperphosphorylation of WC proteins is shown to inhibit their binding to DNA, consistent with WCC hyperphosphorylation being crucial for photoadaptation and, as in [31••], playing an important role in the circadian negative feedback loop.

33. • He Q, Shu H, Cheng P, Chen S, Wang L, Liu Y: **Light-independent phosphorylation of WHITE COLLAR-1 regulates its function in the Neurospora circadian negative feedback loop**. *J Biol Chem* 2005, **280**:17526-17532.
The authors provide the first evidence of regulation of WCC function by phosphorylation and identify several of the residues in WC-1 that mediate this effect.

34. Lee K, Loros JJ, Dunlap JC: **Interconnected feedback loops in the *Neurospora* circadian system**. *Science* 2000, **289**:107-110.

35. Gorl M, Merrow M, Huttner B, Johnson J, Roenneberg T, Brunner M: **A PEST-like element in FREQUENCY determines the length of the circadian period in *Neurospora crassa***. *EMBO J* 2001, **20**:7074-7084.

36. Liu Y, Loros J, Dunlap JC: **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 USA* 2000, **97**:234-239.

37. Yang Y, Cheng P, Liu Y: **Regulation of the *Neurospora* circadian clock by casein kinase II**. *Genes Dev* 2002, **16**:994-1006.

38. Yang Y, Cheng P, Qiyang Q, He Q, Wang L, Liu Y: **Phosphorylation of FREQUENCY protein by casein kinase II is necessary for the function of the *Neurospora* circadian clock**. *Mol Cell Biol* 2003, **23**:6221-6228.

39. 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.

40. •• 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.
Cloning of a novel clock gene, *prd-4*, showed it to encode the universal cell cycle regulator CHK2. Showing that DNA damage resets the clock in a PRD-4-dependent manner, and that the clock regulates PRD-4, revealed a new feedback loop conditionally connecting clock output to input and to the cell cycle. This helps to explain the role of clock genes regulation in mammalian tumorigenesis.

41. 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.

42. • He Q, Cheng P, He Q, Liu Y: **The COP9 signalosome regulates the *Neurospora* circadian clock by controlling the stability of the SCFFWD-1 complex**. *Genes Dev* 2005, **19**:1518-1531.
A nice study showing how small alterations in the cellular protein degradation machinery can impact the circadian machinery and even generate a non-circadian oscillation. Independently, a potentially important paper in explaining the COP9 signalosome paradox.

43. 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.

44. 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.

45. • Schafmeier T, Kaldi K, Diernfellner A, Mohr C, Brunner M: **Phosphorylation-dependent maturation of *Neurospora* circadian clock protein from a nuclear repressor toward a cytoplasmic activator**. *Genes Dev* 2006, **20**:297-306.
The authors show that phosphorylation of FRQ late in the circadian day underlies its cytoplasmic role in promoting accumulation of WC-1. This provides a molecular explanation for the daily cycle in WC-1 levels although, on the basis of [15••], perhaps not the only explanation.

46. Kaldi K, Gonzalez BH, Brunner M: **Transcriptional regulation of the *Neurospora* circadian clock gene *wc-1* affects the phase of circadian output**. *EMBO Rep* 2006, **7**:199-204.

47. Cheng P, Yang Y, Wang L, He Q, Liu Y: **WHITE COLLAR-1, a multifunctional *Neurospora* protein involved in the circadian feedback loops, light sensing, and transcription repression of *wc-2***. *J Biol Chem* 2003, **278**:3801-3808.

48. Liu Y: **Molecular mechanisms of entrainment in the *Neurospora* circadian clock**. *J Biol Rhythms* 2003, **18**:195-205.

49. Merrow M, Boesl C, Ricken J, Messerschmitt M, Goedel M, Roenneberg T: **Entrainment of the *Neurospora* circadian clock**. *Chronobiol Int* 2006, **23**:71-80.

50. Price-Lloyd N, Elvin M, Heintzen C: **Synchronizing the *Neurospora crassa* circadian clock with the rhythmic environment**. *Biochem Soc Trans* 2005, **33**:949-952.

51. Liu Y, Merrow M, Loros JJ, Dunlap JC: **How temperature changes reset a circadian oscillator**. *Science* 1998, **281**:825-829.

52. 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.

53. Heintzen C, Loros JJ, Dunlap JC: **VIVID, gating and the circadian clock: the PAS protein VVD defines a feedback loop that represses light input pathways and regulates clock resetting**. *Cell* 2001, **104**:453-464.

54. • Elvin M, Loros JJ, Dunlap JC, Heintzen C: **The PAS/LOV protein VIVID supports a rapidly dampened daytime oscillator that facilitates entrainment of the *Neurospora* circadian clock**. *Genes Dev* 2005, **19**:2593-2605.
A carefully executed and data-rich paper describing how a novel daytime oscillator influenced by the VVD protein acts to keep the clock running during the day and to enable the clock to reset at dusk.

55. Merrow M, Garceau N, Dunlap JC: **Dissection of a circadian oscillation into discrete domains**. *Proc Natl Acad Sci USA* 1997, **94**:3877-3882.

56. Gonze D, Leloup JC, Goldbeter A: **Theoretical models for circadian rhythms in *Neurospora* and *Drosophila***. *C R Acad Sci III* 2000, **323**:57-67.

57. Gonze D, Halloy J, Goldbeter A: **Robustness of circadian rhythms with respect to molecular noise**. *Proc Natl Acad Sci USA* 2002, **99**:673-678.

58. Francois P: **A model for the *Neurospora* circadian clock**. *Biophys J* 2005, **88**:2369-2383.

59. Kurosawa G, Goldbeter A: **Amplitude of circadian oscillations entrained by 24-h light-dark cycles**. *J Theor Biol* 2006, **242**:478-488.

60. Smolen P, Baxter DA, Byrne JH: **Reduced models of the circadian oscillators in *Neurospora crassa* and *Drosophila melanogaster* illustrate mechanistic similarities**. *OMICS* 2003, **7**:337-354.

61. Ruoff P, Vinsjevik M, Mohsenzadeh S, Rensing L: **The Goodwin Model: simulating the effect of cycloheximide and heat shock on the sporulation rhythm of *Neurospora crassa***. *J Theor Biol* 1999, **196**:483-494.

62. Ruoff P, Vinsjevik M, Mohsenzadeh S, Rensing L: **The Goodwin Oscillator: on the importance of degradation reaction in the circadian clock**. *J Biol Rhythms* 1999, **14**:469-479.

63. Iwasaki H, Dunlap JC: **Microbial circadian oscillatory systems in *Neurospora* and *Synechococcus*: models for cellular clocks**. *Curr Opin Microbiol* 2000, **3**:189-196.

64. • Roenneberg T, Dragovic Z, Merrow M: **Demasking biological oscillators: properties and principles of entrainment exemplified by the *Neurospora* circadian clock**. *Proc Natl Acad Sci USA* 2005, **102**:7742-7747.
See annotation for [66•].

65. • Pregueiro AM, Price-Lloyd N, Bell-Pedersen D, Heintzen C, Loros JJ, Dunlap JC: **Assignment of an essential role for the Neurospora frequency gene in circadian entrainment to temperature cycles**. *Proc Natl Acad Sci USA* 2005, **102**:2210-2215.
See annotation for [66•].

66. • Lakin-Thomas PL: **Circadian clock genes frequency and white collar-1 are not essential for entrainment to temperature cycles in *Neurospora crassa***. *Proc Natl Acad Sci USA* 2006, **103**:4469-4474.
This paper, along with [64•,65•], are three studies aimed at understanding how temperature cycles influence FLOs, and what, if any, role FLOs might have in the circadian system.

67. Loros JJ, Dunlap JC: **Genetic and molecular analysis of circadian rhythms in *Neurospora***. *Annu Rev Physiol* 2001, **63**:757-794.

68. 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 USA* 2003, **100**:13597-13602.

69. Dunlap JC: **Molecular bases for circadian clocks**. *Cell* 1999, **96**:271-290.

70. Tauber E, Last KS, Olive PJ: **Kyriacou CP-: clock gene evolution and functional divergence**. *J Biol Rhythms* 2004, **19**:445-458.

71. Dunlap JC, Feldman JF: **On the role of protein synthesis in the circadian clock of *Neurospora crassa***. *Proc Natl Acad Sci USA* 1988, **85**:1096-1100.

72. Bell-Pedersen D, Cassone VM, Earnest DJ, Golden SS, Hardin PE, Thomas TL, Zoran MJ: **Circadian rhythms from multiple oscillators: lessons from diverse organisms**. *Nat Rev Genet* 2005, **6**:544-556.

73. Lakin-Thomas PL: **New models for circadian systems in microorganisms**. *FEMS Microbiol Lett* 2006, **259**:1-6.

74. Edmunds LN Jr: *Cellular and Molecular Bases of Biological Clocks*. Springer-Verlag; 1988.

75. Uebelmesser ER: **The endogenous daily rhythm of conidiospore formation of *Pilobolus***. *Arch Mikrobiol* 1954, **20**:1-33.

76. 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**:231-237.

77. Merrow M, Dunlap JC: **Intergeneric complementation of a circadian rhythmicity defect: phylogenetic conservation of the 989 amino acid open reading frame in the clock gene *frequency***. *EMBO J* 1994, **13**:2257-2266.

78. Lombardi LM, Brody S: **Circadian rhythms in *Neurospora crassa*: clock gene homologues in fungi**. *Fungal Genet Biol* 2005, **42**:887-892.

79. Lewis M, Morgan L, Feldman JF: **Cloning of *(frq)* clock gene homologs from the *Neurospora sitophila* and *Neurospora tetrasperma, Chromocrea spinulosa* and *Leptosphaeria australiensis***. *Mol Gen Genet* 1997, **253**:401-414.

80. Lewis M, Feldman JF: **Evolution of the *frequency* clock locus in ascomycete fungi**. *Mol Biol Evol* 1997, **13**:1233-1241.

81. Froehlich AC, Noh B, Vierstra RD, Loros J, Dunlap JC: **Genetic and molecular analysis of phytochromes from the filamentous fungus *Neurospora crassa***. *Eukaryot Cell* 2005, **4**:2140-2152.

82. • Blumenstein A, Vienken K, Tasler R, Purschwitz J, Veith D, Frankenberg-Dinkel N, Fischer R: **The Aspergillus nidulans phytochrome FphA represses sexual development in red light**. *Curr Biol* 2005, **15**:1833-1838.
This is the first description of an activity for a fungal phytochrome; a nicely crafted and satisfying study.

83. • Lee K, Singh P, Chung WC, Ash J, Kim TS, Hang L, Park S: **Light regulation of asexual development in the rice blast fungus, *Magnaporthe oryzae***. *Fungal Genet Biol* 2006, **43**:694-706.
See annotation for [89•].

84. Casas-Flores S, Rios-Momberg M, Bibbins M, Ponce-Noyola P, Herrera-Estrella A: **BLR-1 and BLR-2, key regulatory elements of photoconidiation and mycelial growth in Trichoderma atroviride**. *Microbiol* 2004, **150**:3561-3569.

85. • Lu YK, Sun KH, Shen WC: **Blue light negatively regulates the sexual filamentation via the Cwc1 and Cwc2 proteins in *Cryptococcus neoformans***. *Mol Microbiol* 2005, **56**:480-491.
See annotation for [89•].

86. •• Idnurm A, Heitman J: **Light controls growth and development via a conserved pathway in the fungal kingdom**. *PLoS Biol* 2005, **3**:e95.
See annotation for [89•].

87. • Terashima K, Yuki K, Muraguchi H, Akiyama M, Kamada T: **The *dst1* gene involved in mushroom photomorphogenesis of *Coprinus cinereus* encodes a putative photoreceptor for blue light**. *Genetics* 2005, **171**:101-108.
See annotation for [89•].

88. •• Idnurm A, Rodriguez-Romero J, Corrochano LM, Sanz C, Iturriaga EA, Eslava AP, Heitman J: **The Phycomyces *madA* gene encodes a blue-light photoreceptor for phototropism and other light responses**. *Proc Natl Acad Sci USA* 2006, **103**:4546-4551.
See annotation for [89•].

89. • Silva F, Torres-Martínez S, Garre V: **Distinct white collar-1 genes control specific light responses in Mucor circinelloides**. *Mol Microbiol* 2006, **61**:1023-1037.
This group of seven papers [83•,84,85•,86••,87•,88••,89•] comprise a sudden wave of research describing the conserved role of WC-1-like proteins in photoreception amongst the fungi. Because WC-1 also has a non-light-associated function in the *Neurospora* circadian system in both the FRQ—WCC oscillator, and potentially in the UFO, this conservation raises the possibility of a similarly conserved role in fungal circadian systems.

90. Christensen M, Falkeid G, Hauge I, Loros JJ, Dunlap JC, Lillo C, Ruoff P: **A *frq*-independent nitrate reductase rhythm in *Neurospora crassa***. *J Biol Rhythms* 2004, **19**:280-286.

91. • 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 Dev* 2006, **20**:2552-2565.
Casein kinase 1 and casein kinase 2 are identified as kinases that phosphorylate WC-1 and WC-2 in a FRQ-dependent manner to reduce the activity of the WCC.