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Circadian regulation of the light input pathway in *Neurospora crassa*

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FREQUENCY (FRQ) is a critical element of the circadian system of *Neurospora*. The white collar genes are important both for light reception and circadian function. We show that the responsiveness of the light input pathway is circadianly regulated. This circadian modulation extends to light-inducible components and functions that are not rhythmic themselves in constant conditions. FRQ interacts genetically and physically with WHITE COLLAR-1, and physically with WHITE COLLAR-2. These findings begin to address how components of the circadian system interact with basic cellular functions, in this case with sensory transduction.

Keywords: circadian/frequency/light/Neurospora/white collar

Introduction

Circadian systems coordinate the temporal program of organisms in all phyla to accommodate and anticipate the daily changes of the environment (Pittendrigh, 1993). Experimentally, circadian rhythms are characterized by their self-sustained, ~24 h oscillation in constant conditions. Rhythms are entrained (in nature to 24 h) by appropriate environmental signals (zeitgeber), of which light is the most studied (Roenneberg and Foster, 1997). An intact circadian system includes input pathways, a mechanism that generates rhythmicity (rhythm generator) and outputs. Physiological experiments show that light input pathways (LIPs) to the rhythm generator may themselves be under circadian control. For example, in the marine unicell *Gonyaulax*, one of the circadian LIPs is only active during the subjective night (for definition of subjective day and night, see legend to Figure 1; Roenneberg and Taylor, 1994; Roenneberg and Deng, 1997). There are also indications that light receptors of circadian systems are rhythmic, e.g. cryptochrome in *Drosophila* (Emery et al., 1998) or phytochrome B in plants (Bognar et al., 1999).

Thus, circadian light input is probably not a straightforward transduction of signals to the rhythm generator, but rather an active, circadianly regulated mechanism. In principle, input pathways can influence circadian rhythmicity by responding both to external zeitgebers and to the endogenous circadian system. Modeling shows that mutations in components of rhythmic input pathways change period as determined in constant conditions and contribute to self-sustainment. These theoretical results (Roenneberg and Merrow, 1998, 1999) are in accordance with reports showing that mutations in genes encoding input elements can change period or even result in arhythmicity in constant conditions (Millar et al., 1995; Somers et al., 1998; Iwasaki et al., 2000). Either of these observations would also be consistent with mutations in components that are central to the rhythm generator.

In all molecular/genetic model systems, a negative feedback loop is essential for self-sustained circadian rhythmicity in constant conditions and is generally considered to be central to the rhythm generator. These loops involve expression of genes to proteins (transcription, translation and modification, e.g. phosphorylation) which, in turn, inhibit their own expression. Such a transcription/translation feedback loop is also necessary for self-sustainment of circadian rhythms in *Neurospora*, involving the genes frequency (frq), white collar-1 (wc-1) and white collar-2 (wc-2) as central players (Dunlap, 1999). Initially, wc-1 and wc-2 were identified in screens for lack of light reception (Harding and Turner, 1981; Degli-Innocenti and Russo, 1984). They are both light-inducible, DNA-binding, putative transcription factors (Ballario et al., 1996; Linden and Macino, 1997), and WC-1 shares similarity with a class of proteins from diverse species that actively process electrons or photons (Huala et al., 1997). Also, WC-1 and WC-2 regulate basal levels of frq (Crosthwaite et al., 1997), which was identified in screens for circadian period mutants (Feldman and Hoyle, 1973). FRQ negatively regulates its own transcription in constant conditions (Aronson et al., 1994b), providing a mechanistic basis for continuous oscillations (self-sustained rhythmicity). However, circadian properties remain in the absence of the frq/FRQ transcription/translation feedback loop. FRQ-deficient strains are capable of circadian entrainment (a special, circadian form of synchronization) in temperature cycles, whereas light entrainment fails, indicating a functional role for FRQ in the LIP (Merrow et al., 1999) and opening up the possibility of additional circadian machinery that functions in the absence of FRQ (see also Lakin-Thomas and Brody, 2000).

Here, we characterize how FRQ is involved in light signal transduction. Two distinct physiological responses to light (conidial banding and carotenogenesis) represent two separate destinations of this light transduction pathway. The former has an absolute requirement for FRQ to
respond to light, the latter only requires FRQ for circadian regulation and overall magnitude of light induction. We further show genetic and physical interaction of FRQ and the WC proteins, demonstrating that circadian regulation and light signal transduction are hard wired together.

**Results**

**Regulation of LIP components**

The components that have been characterized most thoroughly for their early involvement in light responses in *Neurospora* are WC-1 and WC-2. Both proteins are critical for normal circadian rhythmicity; however, *wc-1* mRNA is regulated more robustly by light than *wc-2* (Linden and Macino, 1997). Thus, for questions regarding the relationship between light and circadian regulation, we focused our studies on interactions of *wc-1* and *frq*.

One of the important circadian features of *frq* RNA and protein is their rhythmicity in constant darkness (DD; Aronson *et al*., 1994b; Garceau *et al*., 1997). We analyzed *frq* and *wc-1* RNA and protein levels under these conditions. The RNA levels of *wc-1* were variable, but not circadian (Figure 1C; Lee *et al*., 2000). WC-1 protein levels, however, changed with circadian time (Figure 1D; Lee *et al*., 2000). The period of the WC-1 oscillation is specific for different circadian period mutants (Lee *et al*., 2000). The differences for *frq* and *wc-1* RNA and protein profiles in constant conditions show that their regulation is distinct. This is also indicated by the fact that maximum WC-1 protein levels coincide with the FRQ minimum (Figure 1B and D; Lee *et al*., 2000).

**Regulation of FRQ and WC-1 is interdependent**

Basal *frq/FRQ* levels are low in *wc-1* and *wc-2* mutants (Crosthwaite *et al*., 1997). Here, these levels were determined in a Δwc-1 strain (RIPed to a functional knockout; see Materials and methods and Talora *et al*., 1999), confirming that *frq* RNA and FRQ protein (Figure 2A and B) levels are substantially lower in the Δwc-1 than in a *wc-1*+ strain. These observations suggest that regulation of *frq* lies downstream of WC-1.

Recently, the activators of genes in the *Drosophila* circadian transcription/translation feedback loop were shown to depend on ‘downstream’ gene products for their expression (Bae *et al*., 1998; Glossop *et al*., 1999). A comparative analysis would suggest that this might be a common regulatory mechanism in circadian molecular networks. The rhythmicity of WC-1 (Figure 1D; Lee *et al*., 2000) suggests a similar interactive network between FRQ and the white collar gene products. We therefore determined *wc-1* RNA levels in *frq*10 (a *frq* null strain, see Materials and methods for description). Basal RNA levels were lower than those observed in *frq*+ grown in DD (Figure 2C; in contrast to Lee *et al*., 2000), and WC-1 protein levels were reduced similarly (Figure 2D; and as in Lee *et al*., 2000). Thus, at least under some conditions (see Discussion), FRQ apparently is required to maintain basal levels of *wc-1* RNA and protein in DD.

**FRQ deficiency and light responsiveness**

We probed the functional consequences of *frq* deficiency, and the resultant depressed WC-1 levels, on light-induced physiology by investigating several light-inducible outputs: conidial band formation, carotenogenesis and expression of specific RNAs. Figure 3A shows light regulation of conidial banding in a wild-type strain (tubes 1 and 2). As
previously reported, frq null strains do not respond to light:dark (LD) cycles with succinct formation of conidial bands or their synchronization (Figure 3A, tube 3; Chang and Nakashima, 1997; Merrow et al., 1999; Lakin-Thomas and Brody, 2000; Roenneberg and Merrow, 2001). In contrast, temperature cycles entrain conidial band formation in frq9 (Merrow et al., 1999).

To clarify whether FRQ, as such, is required for light-induced conidial band formation or whether this function depends on the intact negative feedback regulation of the frq/FRQ loop, we constitutively expressed FRQ in frq10 (using a qa-2p-frq fusion construct similar to that of Aronson et al., 1994b). Although under these conditions this response is not entirely normal, a light-regulated conidiation response was rescued (Figure 3A, tube 4); this strain remains arhythmic in DD (data not shown; and
Aronson et al., 1994b). The timing of conidiation following the light signal (its phase angle) is similar to that for the frq+ strain. The reconstitution of light responsiveness could be mediated by light-induced rhythmicity of FRQ levels, even in the absence of transcriptional regulation. However, when FRQ was induced in the frq10 strain, there was no difference in phosphorylation state (judged by mobility in SDS–PAGE; Figure 3B, left panel) or protein accumulation (right panel) in light versus darkness (Figure 3B). Thus, expression of FRQ is required for light regulation of conidiation, even in the absence of negative feedback of FRQ on frq. Given that there is not an obvious qualitative or quantitative difference in FRQ protein induced in light or dark (Figure 3B), it is possible that rhythmicity of the protein is not essential for this response.

Because it was reported previously that the frq null strains do show light-induced gene expression (Arpaia et al., 1993, 1995), we investigated light-induced mycelial carotenogenesis (De Fabo et al., 1976). Figure 3C shows that this light response remained qualitatively intact in frq10: the absorption spectrum of hexane-extracted, light-induced tissue was the same in frq10 and frq+. However, final carotenoid concentrations were approximately half in frq10 compared with frq+ (the two superimposed spectra represent extracts of different dilutions; see Figure 3C legend). Note that light-dependent carotenoid synthesis is entirely absent in wc-1 mutants (Harding and Turner, 1981; Linden et al., 1999).

When fluence response curves for light induction of carotenogenesis in frq+ and frq10 were compared, the amplitude of the saturation response in frq10 was about half that of frq+ (Figure 3D). The sensitivity of both strains (fluence rate at half-maximal response, black arrow) was, however, identical. Comparison of the fluence threshold for carotenogenesis with that for light-driven synchronization of conidiation (Figure 3D, gray arrow; Merrow et al., 1999; Roenneberg and Merrow, 2001) suggests two, distinct, light-regulated pathways. Without FRQ, light-regulated conidial band formation is absent even in high light intensity LD cycles, but carotenoids are induced normally based on fluence threshold, although overall accumulation is about half. So, while both branches of this pathway are light blind without WC-1, they are each modulated differently by FRQ (see Figure 7A).

Carotenoid production is the result of a complex, multistep process. In contrast, one of the earliest detectable events after light exposure of Neurospora is induction of wc-1 RNA, which occurs in <2 min at high light intensities (P. Ballario, unpublished data). Induction of wc-1 RNA was, therefore, used to monitor early events in light signal reception and transduction, and contrasts the endpoint that carotenogenesis represents. The amplitudes (maximum response relative to baseline) of light-induced RNA in frq+ and frq10 appeared to be similar (Figure 4); however, the peak light-induced wc-1 levels were low in frq10, reaching, at most, the basal DD levels of wc-1 in frq+ (compare with Figure 2C). al-1 RNA was also induced weakly by light in frq10 (data not shown). al-1 encodes a downstream enzyme on the carotenogenesis pathway (phytoene dehydrogenase) (Schmidhauser et al., 1990). Given that RNA induction is a relatively rapid and discrete response compared with carotenogenesis, the disparity in strength of carotenoid and RNA induction is not directly comparable.

Circadian regulation of light responses

Light responses depend on FRQ and WC-1 (Figures 3A, C and D, and 4), and both proteins show a circadian rhythm in abundance (Figure 1B and D). We therefore investigated the physiological light responses described above at different times of the circadian cycle. Time courses measured over 2 h in the middle of the subjective day and night showed large differences in light-induced gene expression. While basal levels of wc-1 were indistinguishable (see also Figure 1C), the amplitude of the response in the subjective day was less than half when compared with the subjective night (Figure 5A).

frq RNA levels depend on WC-1 and WC-2, for both basal expression and rapid and robust light induction (Crosthwaite et al., 1997). In contrast to wc-1, frq RNA is induced to approximately the same maximal levels at the opposite circadian times (Figure 5B; Crosthwaite et al., 1995). Because frq levels are circadian (Figure 1A; Aronson et al., 1994b; Garceau et al., 1997), frq is already at different levels at the beginning of light incubation.

Finally, al-1 light induction resembles the pattern of wc-1 RNA induction, but the subjective night/day ratio is ~10-fold (Figure 5C). Interestingly, wc-1 and al-1 RNA profiles, and to a lesser extent frq, show a transiency in prolonged light exposure (Schmidhauser et al., 1990; Arpaia et al., 1995; Linden et al., 1999), resembling classical adaptation responses. RNA levels are down-regulated within 1–2 h and, at least for wc-1, remain constant for 10 h of illumination (data not shown). The adaptation profile is apparent at both circadian times that were evaluated.

Lastly, we determined light-induced mycelial carotenogenesis at different circadian times. Light-dependent accumulation of carotenoids was also circadianly regulated, peaking before subjective dawn (notably, the
FRQ in light responses could stem from a physical association with either of these proteins. We probed anti-WC-2 immunoprecipitates of cell extracts and found that FRQ binds to WC-2 (Figure 6A). The amount of FRQ in the complex correlated with its circadian accumulation in crude cell extracts (Figure 1B). For reference, FRQ in total cell extracts from frq+ is shown at two circadian times: one harvested at subjective dawn with low levels of highly phosphorylated FRQ, the other at late subjective day with large amounts of less phosphorylated FRQ (Figure 6A, left panel, right lanes). In this experiment, highly phosphorylated FRQ apparently does not participate in complex formation, although in some others it did. Using anti-WC-1, we were also able to immunoprecipitate FRQ from cell extracts of mycelia grown in constant light (LL; Figure 6B). Thus, FRQ is in complexes containing WC-2 and/or WC-1 in vivo and WC-2 is found in association with WC-1 (Talora et al., 1999).

Discussion

We previously observed circadian entrainment by temperature cycles in Neurospora strains deficient in FRQ (Merrow et al., 1999). The same strains, however, fail to synchronize to light cycles (Figure 3A; Chang and Nakashima, 1997; Merrow et al., 1999; Lakin-Thomas and Brody, 2000), suggesting that FRQ is required in processing light signals, in addition to controlling essential circadian properties, such as self-sustained rhythmicity in DD. Here, we address several questions about the role of frq/FRQ in the LIP and the circadian system of Neurospora crassa. What is the nature of the interaction between the WC proteins and their complex (WCC) and FRQ? What is the role of FRQ in light transduction and how does it contribute to entrainment? Figure 7 is a diagrammatic view of the Neurospora LIP, specifically with respect to WC-1, WC-2 and FRQ at the protein level in Figure 7A and at the level of gene regulation of wc-1 and frq in Figure 7B.

Interdependent regulation of frq and wc-1 by their proteins

The circadian and light input pathways in Neurospora interact genetically. Robust frq RNA and protein expression depends on WC-1 and WC-2 proteins, which are essential for light responses in Neurospora (Figure 2A and B; Crosthwaite et al., 1997). However, frq expression is not simply downstream of the WC gene products; we find that basal, DD levels of wc-1 (Figure 2C) and WC-1 depend on FRQ (Figure 2D; Lee et al., 2000). Since FRQ levels are robustly circadian (Figure 1B; Garceau et al., 1997), one would expect rhythmic wc-1 RNA in DD, but this is not the case (Figure 1C). A recent report (Lee et al., 2000) also finds that wc-1 RNA is constitutive but further describes that basal wc-1 levels are similar in frq+ and frq−. This is in contrast to what we observe. The most obvious difference in the experimental protocols is the time at which the samples are harvested: after 28 h in darkness in the protocol described here, versus only 6 h in that presented by Lee et al. (2000). There could be residual light-induced wc-1 RNA in frq− (Figure 4) that persists for at least 6 h in DD; sustainment of those RNA levels may depend on FRQ. WC-1 protein oscillates with a circadian period, out of phase with FRQ (Figure 1D; and as shown in
Lee et al., 2000), and its regulation includes a post-transcriptional control that is initiated by FRQ expression (Lee et al., 2000). Thus, WC-1 regulates FRQ, and FRQ regulates WC-1, indicating the backbone of the circadian transcription/translation feedback loop in Neurospora (see Figure 7B).

**A bifurcated light input pathway**

The effect of FRQ on light transduction was investigated by comparing various light-induced responses. Carotenogenesis and conidiation each represent the result of extensive coordination of gene expression and metabolism. Both are regulated by light but, unlike conidiation, carotenogenesis is not circadianly rhythmic in DD. The WCs are essential for both of these light-regulated physiological processes (Russo, 1988; Linden et al., 1999), while FRQ is only essential for light-regulated conidiation (Figure 3), indicating a bifurcation in the LIP (Figure 7A). The effect of FRQ on conidiation is specific for light transduction and not for development, per se, as conidiation proceeds normally in FRQ-less strains, though without photic or circadian regulation (Figure 3A, tube 3). FRQ has a different function in the branch leading to carotenogenesis: without FRQ, the quantities of light-induced RNAs and carotenoids are lower (Figures 3C and D, and 4).

Additional evidence for a bifurcation in the LIP lies in the fluence responses of the respective branches. While light synchronizes conidiation above a threshold of 2 nE/m²/s (gray arrow in Figure 3D; see Merrow et al., 1999; Roenneberg and Merrow, 2001), carotenogenesis is half-maximal at an ~50-fold higher fluence rate (black arrow in Figure 3D).

**Circadian response regulation**

FRQ plays a critical role in the Neurospora circadian system, as well as in potentiating light-induced carotenogenesis. In addition, we have shown that light responses are stronger at specific circadian times compared with others (Figure 5D). The circadian response regulation is apparent within minutes (e.g. in RNA induction, Figure 5A and C). This represents a general phenomenon whereby evoked sensory processes, which are not rhythmic in constant conditions, are modulated by the circadian
system, e.g. olfaction in Drosophila (Krishnan et al., 1999) or chlorophyll production and flowering in plants (Claus and Rau, 1956). In Neurospora, the strength of the light-induced response (in the non-circadianly expressed, evoked branch: wc-1, al-1 RNA and carotenogenesis) approximately correlates with the amount of WC-1 protein: no WC-1, no response; low levels of WC-1, weak response; and higher levels of WC-1, stronger response. Thus, FRQ may transduce the circadian modulation of light responses by dictating WC-1 levels (Figure 7A and B).

This correlation, however, is not entirely straightforward. There are some conditions where Neurospora is not stimulated by light despite abundant WC-1 protein. During prolonged light exposure, some RNA levels adapt (are down-regulated) although WC-1 levels remain the same (see Figures 4, and 5A and C; Talora et al., 1999). Furthermore, Neurospora is refractory to additional light increments for some hours after a dark to light transition (Schmidhauser et al., 1990; Arpaia et al., 1999).

How do FRQ and the WCs functionally participate in the various regulatory processes described above? The WC proteins are thought to possess both photoreceptor and transcription factor activity. FRQ function within the clock has been described with respect to transcriptional control. Indeed, all of these attributes could derive from transcription factor function as modulated by complex formation (Figure 6). Signal transduction by a photoreceptor has been shown to occur via transcriptional control. The plant photoreceptor phytochrome B forms a complex with and modulates the function of a DNA-bound transcription factor (Martinez-Garcia et al., 2000). Interestingly, this particular photoreceptor feeds into the Arabidopsis circadian system (Somers et al., 1998).

**FRQ and light entrainment**

The response of circadian systems to a given zeitgeber signal (e.g. a light pulse) is different at different circadian times. The quantification of these differential responses results in a phase response curve (PRC), a signature for a circadian system and the respective zeitgeber. This quality is the basis for circadian synchronization (entrainment). FRQ is required for the Neurospora circadian system to respond to light. Induction of *frq* RNA by short light pulses has been invoked to explain the light PRC of Neurospora (Crostwaite et al., 1995). When *frq* is increased to or above its DD maximum, then the circadian system is reset to the phase of FRQ max in DD (Figure 1B). Maximum phase shifts are thus achieved when a light pulse is given at the FRQ minimum (Crostwaite et al., 1995).

Entrainment is distinct from a driven synchronization whereby a system is turned on or off directly by an exogenous signal. We have shown that light drives conidiation rather than entraining it in a PRC-dependent fashion (Merrow et al., 1999). In DD cycles, conidiation occurs with a constant lag after lights-off, regardless of zeitgeber period (i.e. in full photoperiods the response is the same for all tested circadian times). This feature is apparent even at the lowest light fluences that synchronize conidiation (2 nE/m2/s, comparable with moonlight, see Figure 3D). In spite of this driven synchronization, the lag between lights-off and conidiation onset is strain specific. It correlates with the period of the specific *frq* allele in DD, indicating that it depends on the activity of FRQ in the dark phase of the LD cycle. Although light pulse-induced *frq* levels correlate well with phase shifting (Crostwaite et al., 1995), synchronization of conidiation in full LD cycles appears to function via FRQ protein, independently of its transcription. In the experiments shown in Figure 3A and B (constitutive *frq* expression from the qa-2 promoter), the lag between lights-off and conidiation is similar to that for *frq* (note that the qa promoter *per se* is non-responsive to light; Crosthwaite et al., 1995). One would predict that constitutive expression of different *frq* alleles (similar to the experiments in Figure 3A and B) would also give a strain-specific lag. With regard to mechanism, our experiments show no qualitative or quantitative effects of light on constitutively expressed FRQ protein after 6 or 12 h incubation (Figure 3B); however, acute light effects on FRQ and/or on the formation of the WCC–FRQ complex are possible, and might contribute to the rhythmicity.

Whether circadian systems are entrained primarily by the prolonged presence of light in LD cycles (parametric entrainment) or by acute changes in light (non-parametric entrainment) has long been a point of discussion (Beersma et al., 1999). In Drosophila, cryptochrome has been implicated in mediating non-parametric, but not parametric, entrainment (Stanewsky et al., 1998). Our results indicate that synchronization by full photoperiods does not require *frq* transcription, although phase shifting by short light pulses correlates well with *frq* induction (Crostwaite et al., 1995). Thus, *frq* transcription may be involved in non-parametric light effects, and FRQ protein, together with the WCC complex, may mediate parametric light effects.

So, where does FRQ act within the circadian system: upstream of, central to or downstream of the rhythm generator? Our results show that the participation of FRQ is more complex. Previous results suggest that FRQ is not necessarily central to the rhythm generator (Merrow et al., 1999; Lakin-Thomas and Brody, 2000). Given the fact that conidiation is not regulated by light in its absence, FRQ appears to function as a gateway through which the light signal must pass, placing it upstream of the rhythm generator. Yet without FRQ, light responsiveness (e.g. of carotenogenesis) is not circadianly regulated, placing FRQ downstream of the rhythm generator.

The close association between light input and molecular components of circadian systems is one of the functional parallels characterized from cyanobacteria to mice (Crostwaite et al., 1997; Shigeyoshi et al., 1997; Emery et al., 1998; Stanewsky et al., 1998; Bognat et al., 1999; Ceriani et al., 1999; Iwasaki et al., 2000; Roenneberg and Merrow, 2000). In addition, the Neurospora transcription/translation feedback loop, as described here (and in Lee et al., 2000) shares many features with those of Drosophila (Lee et al., 1998; Glossop et al., 1999) and mice (Shearman et al., 2000). In Neurospora, it also serves as the LIP.

**Materials and methods**

**Strains and media**

*frq* and *frq* alleles are standard laboratory strains with the *bd* mutation in their background (Loros et al., 1986; Merrow et al., 1999). *frq* is a knockout of the *frq* locus (Aronson et al., 1994a) and is also on the *bd* background. The Δwc-1 strain is a functional knockout, generated by repeat-induced
point (RIP) mutation (Talora et al., 1993), which was crossed with bd (bd Δwc-1 4–7). RIP is a method for gene inactivation in Neurospora, whereby the presence of a duplicate copy of DNA signals methylation and inactivation of both copies when the strain is put through a sexual cross (Selker and Garrett, 1988). This Δwc-1 strain makes no RNA or protein.

wc-2 234W is a loss-of-function mutant, which produces a truncated protein (Linden and Macino, 1997), similarly to the frq- strain (data not shown). frqρ0 qa-2p-frq describes a strain with the genotype bd frq10 his3::his3qα2p-frqhis3. To generate this, the bd frq10 HIS3 strain was transformed at the his3 locus with a his-tagged copy of the frq open reading frame fused with the Neurospora qa-2 promoter (for a similar construction see Aronson et al., 1994).

Race tube experiments and circadian time courses
Race tubes were inoculated with 1X Vogel’s medium (Vogel, 1964) with 0.5% t-arginine, 10 ng/ml biotin and 2% agar (i.e. no glucose). In some race tubes and liquid cultures (~quinate acid adjusted to pH 6.0 was added to 10−3 M. For light induction experiments (Figures 4 and 5A–C), as well as for the experiments shown in Figure 2, stationary, liquid cultures were grown in 1X Vogel’s medium with 2% glucose, 0.5% t-arginine and 10 ng/ml biotin. For the circadian time course (Figure 1), the same medium was used but with shaking cultures. For induction of carotenoids (Figure 3C–D and 5D), arginine was omitted from this medium.

Light induction experiments
In the experiments shown in Figures 4 and 5, 25 ml of medium in 250 ml flasks was inoculated with 3.5 × 105 conidia. These cultures were held in constant light at 30°C for ~1–2 days prior to transfer to constant darkness (DD) at 25°C. Following the indicated time in darkness, age-matched cultures were exposed to continuous light (fluences indicated in the figures legend) until they were harvested by filtration and frozen in liquid nitrogen.

RNA analyses
RNA was prepared essentially as previously described (Crosthwaite et al., 1995), except that a single phenol extraction was performed. RNA was run on 1% agarose–formaldehyde gels (otherwise as in strain Merrow et al., 1997), and blotted onto nylon (HybondN, Amersham Corp.). Riboprobes were used to probe the blots for frq, wc-1 and rRNA. The frq riboprobe was described elsewhere (Crosthwaite et al., 1995). The wc-1 riboprobe was generated from pGEM4wc-1 using T7 RNA polymerase with the linearized plasmid. The riboprobe for rRNA was generated from PV325 with T3 polymerase, without linearizing. The riboprobe for rRNA was then on western blots with recombinant deletion proteins and cell extracts from frqρ0. The isotype of mFRQ3G11 is IgG1. The antibodies for WC-1 and WC-2 used in Figures 1, 2 and 6A have been described previously (Talora et al., 1999). Co-immunoprecipitations were performed as described (Talora et al., 1999). The specificity of the rabbit anti-WC-1 used in co-immunoprecipitations (Figure 6B) was confirmed with the Δwc-1 strain.

Data analysis
All rhythmic time courses were fitted with a cosine function with one exception: due to the non-sinusoidal shape of the time series, WC-1 (Figure 1D) was fitted by eye. The S-curves in Figure 3D were fitted with the following equation:

\[ y = G + S + D; \]

with \( G = a \cdot \frac{x}{\sqrt{1 + (\frac{x}{b})^2}}; \) \( S = d \cdot \frac{x}{\sqrt{1 + (\frac{x}{b})^2}}; \) and \( D = \frac{f}{(x + 1)} \).

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