6 Photobiology and Circadian Clocks in *Neurospora*

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I. Introduction

Neurospora emerged as a durable model for dissection of the mechanisms underlying circadian regulation in the late 1950s (Pittendrigh et al. 1959). What recommended this system to those researchers, and to subsequent generations, have been the facts that it has a terrific genetic system, it is easy to use for biochemical follow-ups, and it is supported by a relatively large and supportive community. These advantages remain and keep *Neurospora* as a useful and popular model. Although a great deal of work on the photobiology of *Neurospora* emerged independent of circadian research, it is nonetheless true that a large impetus for dissecting the photobiology of *Neurospora* came from the fact that light is a major entraining agent for the circadian system. As a result, much of what we know about the molecular details of photobiology in fungi, nearly all of what we know about circadian rhythms in fungi, and even much of what we know about circadian rhythms in animals (which share a circadian mechanism similar to that of *Neuros-*

pora) can be traced to work on this system. For these historical reasons, and because the light-sensing system and circadian system share some components, it makes sense to cover both of these in a single review.

We begin with the photobiology of fungi in general and of *Neurospora* in particular, introducing the White Collar proteins that make up the principle photoreceptor and other real and potential photoreceptive proteins, and then describe how light sensing works. This nicely sets the stage for a discussion of the circadian system, from input via light using the White Collar photoreceptor, to the oscillator mechanism using the same proteins as well as others, to a discussion of how the circadian oscillator acts to control the metabolism of cells.

II. Photobiology of Fungi

A. Why Do Fungi Sense Light?

Despite the fact that fungi can neither visualize adjacent objects nor utilize electromagnetic energy for photosynthesis, it has been estimated that the majority of fungal species respond to light in some form (Idnurm et al. 2010). An intriguing question then arises: How does the perception of light provide a competitive advantage to a fungus? To understand this question, one may first look to processes that are commonly light regulated in diverse fungal species, principle among which is **sporulation**. One obvious role for light is that it likely serves as a positional marker for optimal spore dispersal into the open air. This is strikingly illustrated by the bending of sporangiophores of various Mucormycotina species (e.g., *Phycomyces* and *Mucor*) toward blue light and the phototropic orientation of protoperithecial beaks of *Neurospora crassa* (Linden et al. 1997; Corrochano and Garre 2010). More generally, light frequently serves as a signal to simply induce or repress sporulation in various zygomycete, ascomycete, and basidiomycete species and may even have differential effects on which form of sporulation takes place (Corrochano 2007; Bayram et al. 2010; Idnurm et al. 2010; Rodriguez-Romero et al. 2010). In *Phycomyces blakesleeanus*, for

example, light promotes macrophorogenesis while inhibiting microphorogenesis, both of which are forms of asexual development (Corrochano and Cerd-Olmedo 1992).

Ultraviolet (UV) radiation represents a major source of genotoxic and oxidative stress for the cell. As such, the first photosensitive proteins to emerge in the prokaryotes were likely the photolyases, which utilize light in the blue/near-UV range to catalyze the repair of DNA lesions (Hitomi et al. 2000). The involvement of the light response in UV defense is evident in numerous fungal species. For example, various ascomycete, basidiomycete, and zygomycete species demonstrate a light-induced production of carotenoid or melanin **pigments**, which can absorb UV photons and convert the energy to heat (Chen and Loros 2009; Chen et al. 2009; Sano et al. 2009; Avalos and Estrada 2010; Corrochano and Garre 2010; Tisch and Schmoll 2010). In addition, light may also induce the expression of numerous DNA repair enzymes, including photolyases and UV endonucleases. Indeed, the loss of certain photoreceptor genes leads to hypersensitivity to UV stress in numerous species (Idnurm and Heitman 2005a; Bluhm and Dunkle 2008; Ruiz-Roldan et al. 2008). Moreover, light serves as a negative regulator of sexual development in numerous species (e.g., *Aspergillus nidulans*, *P. blakesleeanus*, *C. neoformans*) (Yamazaki et al. 1996; Blumenstein et al. 2005; Idnurm and Heitman 2005a); this may serve to prevent UV-mediated damage of energetically costly meiotic progeny, thereby providing an evolutionary basis for the connection between the regulation of development and genomic protection in fungi.

Essentially all organisms experience predictable changes in their environment associated with the **day/night cycles** of the rotating Earth. As such, the perception of light each morning may serve as an anticipatory signal for daily rise in UV (as discussed), increasing temperature and changes in nutrient availability. In at least a few fungal species, the light signal may entrain the **circadian** clock, which allows an organism to anticipate these daily changes, rather than simply respond to them each day (Greene et al. 2003; Lombardi and

Brody 2005; Bluhm et al. 2010; Baker et al. 2012). The molecular basis for circadian rhythmicity is discussed further in this chapter.

Taken together, these examples underscore the generalized importance of light in promoting fungal fitness. This then leads to the next important question: How does a fungus perceive light? As to be expected, the specific influence of light on an organism's physiology (light output) may vary markedly from species to species; however, the biochemical basis for photoperception (light input) is fundamentally similar and is achieved through specialized and conserved light-sensitive proteins.

B. Fungal Photoreceptors: Biochemistry and Distribution

Fungi are capable of responding to light qualities from the UV to far-red ends of the electromagnetic spectrum. This is accomplished through a variety of wavelength-specific photoreceptor proteins, some of which are highly conserved across the Mycota, whereas others are present in only discrete lineages (Table 6.1). The common feature of all photosensory proteins is that they interact with a **light-absorbing chromophore** that undergoes physicochemical and structural changes on photon absorption (Losi and Gärtner 2011). The protein must then respond to conformational changes in the chromophore by altering its own structural or biochemical properties. Although there are proteins whose function is directly modulated by light, such as photolyases and light-sensitive ion channels, the term *photoreceptor* is reserved for those that absorb light and initiate a signaling cascade to downstream targets and pathways.

1. Flavin-Based Blue Light Receptors

a) White Collar Orthologs
Strictly speaking, the first cloned fungal photoreceptor was White Collar-1 (WC-1) of *N. crassa* (Ballario et al. 1996; Losi and Gärtner 2011), although at the time it was cloned its identity as a photoreceptor was unknown. This was later established through proof that WC-1 bound the flavin-adenine dinucleotide (FAD) chromophore, and that the fluence response for the biochemical and molecular changes in the WC-1 protein in vitro matched the in vivo responses (Froehlich et al. 2002). The name *white collar* stems from the fact that the mutants are defective for light-induced carotenoid pigment production in their hyphae; consequently, in contrast to the completely orange appearance of light-exposed wild-type *Neurospora* test-tube slants, *wc-1* mutants display a nonpigmented mycelial border (a white collar) beneath the constitutively pigmented conidia at the top of the slant. The *Neurospora* WC-1, along with its essential partner WC-2 (so named because its mutants yield the same phenotype as those of *wc-1*) (Linden et al. 1997), is by far the most well-characterized fungal photoreceptor in terms of its biochemistry and molecular biology. The description of the WCC that follows stems from work done in *Neurospora* but is presumed to apply to many White Collar orthologs found in other species based on sequence conservation.

The WC-1 proteins are GATA-like Zn-finger transcription factors that contain three **PAS (Per-Ant-Sim) domains**. The N-terminal-most PAS domain is of a special subclass, called the **LOV domain** (for light, oxygen, and voltage), which noncovalently binds a single blue-light-absorbing chromophore, FAD. Through its C-terminal-most PAS domain, WC-1 interacts with the PAS domain of WC-2 to form a heterodimer known as the **White Collar Complex (WCC)** (Linden et al. 1997; Cheng et al. 2002). WC-2 also contains a Zn-finger domain, but lacks a LOV domain for direct light sensing. A simple model for WCC signaling proposes that when the FAD absorbs blue light it forms a transient cysteinyl adduct with the WC-1 LOV domain, thereby inducing a conformational change that promotes the transcriptional activity of the complex (Froehlich et al. 2002; He et al. 2002; Zoltowski et al. 2007). The WCC binds to specific sequences (called light responsive elements, LREs) in the promoters of its target genes, where it is primarily described as a transcriptional activator; however, the downregulation of genes in response

Table 6.1. Conservation of photoreceptors and FRQ across the Mycota

	WC-1	WC-2	VVD	CRY	OPS	OPS-REL	PHY	FRQ
Ascomycetes								
Saccharomyces cerevisiae						3		
Candida albicans						2		
Fusarium oxysporum	1	1	1	2	3	2	1	1
Magnaporthe oryzae	1	1	1	2		1	1	1
Neurospora crassa	1	1	1	1	1	1	2	1
Trichoderma atroviride	1	1	1	1		1	1	1
Trichoderma reesei	1	1	1	1			1	1
Botrytis cinerea	1	1	1	1	2		2	1
Sclerotinia sclerotiorum	1	1	1	1	2		3	1
Stagonospora nodorum	1	1		2	2		2	1
Histoplasma capsulatum	1	1				1		
Coccidioides immitis						1		
Paracoccidioides brasiliensis	1	1				1		
Aspergillus nidulans	1	1		1[a]		1	1	
Schizosaccharomyces pombe						1		
Basidiomycetes								
Phanerochaete chrysosporium	1	1				5	1	
Coprinus cinerea	1	1					1	
Cryptococcus neoformans	1	1			1		1	
Malassezia globosa								
Ustilago maydis	1	1		2	2	1	1	
Sprobolomyces roseus	2	2		1	2		1	
Puccinia graminis	1	1				5	1	
Zygomycetes								
Phycomyces blakesleeanus	3	4		1				
Rhizopus oryzae	3	5		1				
Mucor circinelloides	3	4		1				
Encephalitozoon cuniculi								
Chytrids								
Batrachochytrium dendrobatidis								
Spizelomyces punctatus	2	2					1	

The distribution of photoreceptor orthologs in selected species from various fungal lineages is shown. The numbers in the chart reflect the number of paralogs present within the organism's genome, and the absence of a number reflects the absence of any detectable ortholog. Orthologs of the *Neurospora* clock protein FREQUENCY (FRQ) are also shown, a discussion of which is found in the section on circadian clocks. *CRY* chryptochrome, *PHY* phytochrome, *OPS* rhodopsin, *OPS-REL* opsin-related protein, *VVD* vivid, *WC-1* White Collar 1, *WC-2* White Collar 2 (Information in this chart is integrated from several sources: Idnurm et al. (2010), Dunlap and Loros (2006), and Salichos and Rokas 2010)

[a]For the cryptochrome category, the CPD photolyase class is not included; the only exception is CryA of *A. nidulans*, which has a demonstrated signaling function

to signaling mediated by the WCC has been reported in both *A. nidulans* and *Trichoderma atroviride* (Rosales-Saavedra et al. 2006; Purschwitz et al. 2008; see Chap. 10 in this volume), although whether this is a direct action of the WCC is unclear.

With orthologs present in the genomes of essentially all fungal lineages, including two species of the anciently diverged chytrids (*Spizellomyces punctatus* and *Allomyces macrogynus*), the White Collars are the most well-conserved fungal photoreceptors in terms of evolutionary depth (Idnurm et al. 2010). There are a few organisms that notably lack *wc-1/2* orthologs, including most of the Saccharomycotina yeasts, which is likely a result of gene loss in those lineages (Dunlap and Loros 2006). This also includes numerous pathogenic species, such as the ascomycete yeast *Candida*, the basidiomycete yeasts *Malassezia* and *Microsporidia*, and numerous dermatophytes (*Microsporum* and *Trichophyton* spp.). As

these organisms represent obligate pathogens with a close association with their hosts, it is possible that genomic reductions have led to the loss of superfluous genes involved in environmental light sensing. Conversely, other fungal pathogens, including *Aspergillus fumigatus*, *Cryptococcus*, *Paraccocidioides*, and *Histoplasma* all have an environmental life cycle as well as a *wc-1* ortholog (Idnurm et al. 2010).

In contrast to the loss of *wc-1* in some lineages, sequence analysis of several Mucormycotina has revealed multiple copies of the *wc-1* orthologs, a likely reflection of gene duplication events early in that lineage (Idnurm et al. 2010). Interestingly, the WC paralogs may display specialized roles in the light response, such as in *Mucor circinelloides*, in which one copy, *mcwc-1a*, regulates phototropism, and a second copy, *mcwc-1c*, regulates the photoinduction of carotenoids (Silva et al. 2006). Interestingly, a third paralog, *mcwc-1b*, is an activator of carotenogenesis but a repressor of sporulation (Silva et al. 2008).

Theoretically, WC-1 should function alone as a photoreceptor, as it contains both light-sensing (LOV) and DNA-binding (Zn-finger) domains. In *Neurospora*, however, WC-2 is essential for the light response because its Zn-finger domain is required for binding of the WCC to DNA (Collett et al. 2002; Froehlich et al. 2002; Liu et al. 2003). This essential interaction seems to have occurred early in fungal evolution, as a *wc-2* ortholog has been found in all species that contain *wc-1*. Indeed, many of the WC-1 proteins of the basidiomycetes, including *C. neoformans*, *Ustilago maydis*, *Phanerochaete chrysosporium*, and *Coprinus cinereus*, have lost their Zn-finger domains altogether (Idnurm and Heitman 2005a).

b) Small LOV Domain Proteins: VIVID and
   ENVOY

In addition to WC-1 homologs, several species encode a small protein that is essentially a single LOV domain with an N-terminal cap (Zoltowski et al. 2007). The first identified member of this family was the 186-amino-acid protein in *N. crassa* and was named VIVID due to the bright orange pigmentation phenotype of

vvd mutants grown in light (Heintzen et al. 2001). Like, WC-1, VIVID binds FAD as a chromophore (Schwerdtfeger and Linden 2003).

VIVID homologs seem to be limited to the genomes of a few ascomycetes. An apparent ortholog is the 207-amino-acid protein, ENVOY, in *Hypocrea jecorina*, which is involved in regulation of cellulose in the dark (Schwerdtfeger and Linden 2003). Despite the fact that VIVID and ENVOY contain only a LOV domain, the two genes do not cross complement one another. Moreover, ENVOY seems to play a role in up- and downregulating genes in both the dark and the light (Schuster et al. 2007). Therefore, it is unclear how these small proteins facilitate their downstream function, but as will be discussed for *N. crassa*, likely involves direct protein-protein interactions with WC-1 via the LOV domain.

c) Cryptochromes

Photolyases and cryptochromes are members of a family of blue/UV-A light receptors. Both proteins contain an N-terminal domain (photolyase-related [PHR] region) that binds noncovalently to two chromophores, FAD and 5,10-methenyltetrahydrofolate (MTHF). **Photolyases** are typically characterized by their ability to use light energy to catalyze DNA repair (e.g., pyrimidine dimers), whereas **cryptochromes** have lost DNA repair capability and instead serve as bona fide light-signaling proteins (Losi 2007). Comparative genomics and functional analyses can divide photolyase/cryptochrome proteins into several family members, which are sporadically distributed among species in the ascomycetes, basidiomycetes, and zygomycetes (Mucormycotina). In general, bona fide photolyase orthologs (cyclobutane pyrimidine dimer, CPD, photolyases) are found in essentially all species, where they serve as light-activated DNA repair enzymes (Idnurm et al. 2010). This discussion focuses on those family members with a proposed or demonstrated photosensory function, the cryptochromes; however, as will be seen, some proteins display both repair and signaling capabilities.

The cryptochrome-*Drosophila*, *Arabidopsis*, *Synechocystis*, human (CRY-DASH) family is

believed to represent the earliest divergence of cryptochromes from photolyases as it is the only cryptochrome found in bacteria. Several fungi have CRY-DASH members, including the ascomycetes *N. crassa*, *Sclerotinia sclerotiorum*, and *Stagonospora nodorum* and the basidiomycete *U. maydis*. CRY-DASH proteins display minimal to no photorepair activity for double-stranded DNA (dsDNA) but do display activity against cyclobutane adducts on single-stranded DNA (ssDNA) (Selby and Sancar 2006). Their involvement in fungal light sensing is poorly understood but has been described in *Sclerotinia sclerotium*, in which it plays a small role in both promoting sclerotium accumulation and repressing hyphal pigmentation in response to UV-A light (Veluchamy and Rollins 2008).

The corn gray leaf spot pathogen, *Cercospora zeae-maydis*, encodes a class 1 CPD photolyase, called CPD1, as well as a member of the so-called 6-4 photolyases, called PHL1. Interestingly, PHL1 not only plays a role in photoreactivation following UV treatment but also regulates the light induction of CPD1 and other DNA repair genes. In addition, *phl1* regulates sporulation and cercosporin biosynthesis. Therefore, PHL1 displays true photosensory functions in the fungus (Bluhm and Dunkle 2008).

Interestingly, several species of *Aspergillus*, including *A. nidulans*, *A. fumigatus*, *A. flavus*, *A. terreus*, and *A. oryzae*, each encode only a single member of the photolyase/cryptochrome family (Bayram et al. 2010). Their sequence most closely resembles a class I CPD photolyase; however, the ortholog of *A. nidulans* demonstrates both photolyase activity and developmental regulation in response to light (Bayram et al. 2008a). It is unclear if the dual function of this protein reflects a photolyase that gained signaling function, or if it represents an ancestral form of photolyase/cryptochrome proteins that displayed both functions before their divergence. Orthologs in other species have not been characterized.

2. Red-Light-Sensing Phytochromes

Although blue light responses dominate the fungal kingdom, many species overtly respond to light above 600 nm (red light). Around 40 years ago, in fact, the ability of red light to reverse the inhibitory effect of blue light on conidiation was described in *Alternaria solani* and *Bortytis cinerea* (Lukens 1965; Tan 1974). Since then, the regulation of growth and development by red light has been described in *A. nidulans* (Bayram et al. 2010), *T. atroviride* (Casas-Flores et al. 2004), *Magnaporthe oryzae* (Lee et al. 2006), *Puccinia graminis* (Schneider and Murray 1979), and *A. fumigatus* (K. Fuller unpublished data), among others.

In both plants and bacteria, red light responses are achieved through phytochromes, which bind the linear tetrapyrrole bilin as a chromophore. The phytochrome protein coverts between two **conformational states**, the red light absorbing Pr confirmation and the far-red light absorbing Pfr confirmation, with the ratio of the two states determining the signaling state within the cell (Idnurm and Heitman 2005b). All phytochromes, regardless of type, have a modular architecture consisting of an N-terminal photosensory module (GAF [cGMP-specific phosphodiesterase, adenylyl cyclase, FhlA] and PHY [phytochrome] domains), which binds the bilin chromophore. The C-terminal output domains can vary considerably between species but will always contain a histidine kinase as part of the regulatory output (Blumenstein et al. 2005).

Phytochromes are also present in the genomes of many fungi, and overall sequence analyses indicate they are most similar to bacterial phytochromes, suggesting perhaps an evolutionary origin (Blumenstein et al. 2005; Karniol et al. 2005). Like the cryptochromes, the phytochromes are found sporadically throughout the Mycota. They are widely conserved among the ascomycete and basidiomycete phyla, and a putative ortholog was even identified in the

chytridiomycete *Spizelomyces punctatus* (Idnurm et al. 2010). However, despite the described red light responses of several species and the presence of phytochromes within their genomes, only FphA of *A. nidulans* has known regulatory functions (Idnurm and Heitman 2005b). Deletion of the phytochrome genes in both *C. neoformans* and *N. crassa* does not lead to obvious photobiological defects (Froehlich et al. 2005; Idnurm and Heitman 2005a); however, neither species is overtly responsive to red light. Molecular studies involving additional red-light-sensing fungi will likely demonstrate a conserved role for phytochrome in the fungal red light response.

3. Rhodopsins and Opsin-Related Proteins

Opsins are a family of seven-transmembrane receptors that bind retinal as a chromophore via a Schiff base linkage to a conserved lysine residue. Based on spectral analyses, opsins typically absorb light in the green to blue-green portions of the spectrum and, on absorption, can use the light signal to facilitate ion transport or for downstream light signaling. Generally, rhodopsins are divided into two categories, distinguished by highly divergent sequence homology between the two. The first is the **type II rhodopsins**, which include the G-protein-coupled receptors used primarily for vision in the higher eukaryotes; these proteins bind 11-*cis*-retinal as the chromophore (Brown 2004). A type II opsin has been reported in the chytrid *Allomyces reticulatus*, and it is clearly implicated in zoospore phototaxis toward green light (Sharma and Foster 1997); however, no orthologs of this type have been identified outside chytrid fungi and may represent an independent gene transfer after chytrid split from the other fungi.

The second type is the **type I rhodopsins**, which are found in the Archea, Bacteria, Fungi, and other lower eukaryotes and have either a transport or sensing role; these proteins bind all-*trans*-retinal as a chromophore. Most fungal rhodopsins are type I, and comparative genome sequencing suggested a horizontal gene transfer event from a haloarchea into an ancestor of the

ascomycete and basidiomycete fungi (Sharma et al. 2006). The role for these proteins in light signaling is poorly understood as their disruption in several organisms has not yielded a robust photoresponsive defect, for example, *N. crassa* (Bieszke et al. 1999, 2007), *Gibberella (Fusarium) fujikuroi* (Estrada and Avalos 2009), and *C. neoformans* (Idnurm and Heitman 2005a). Instead, these proteins may have a function in ion transport, such as that described in *Leptosphaeria maculans* (Waschuk et al. 2005).

Understanding the evolutionary basis for fungal opsins is complicated by the fact that most species contain a family of **opsin-related proteins**, which are distinguished based on their absence of the conserved lysine residue required for binding the retinal chromophore (Brown 2004). These proteins display a wide distribution and are even present in multiple copies in the genomes of the Saccharomycotina yeasts, which lack a light response or additional photoreceptors (Idnurm et al. 2010).

4. Conclusions

Although the various photoreceptor families were discussed separately for the sake of exposition, it is important to consider that the light response undoubtedly involves the integration of multiple light inputs via discrete photosensory pathways. For example, the aforementioned positive phototropism of *P. blakesleeanus* sporangiophores is positive for blue light but negative for UV light (Sharma et al. 2006). This illustrates the complexity of the phenotype, considering both wavelengths are likely present at the same time. Moreover, the relative spectral quality and light intensities change over the course of the day, with blue/near-UV peaks near midday and red/far-red peaks at morning and dusk. Regardless, most laboratory experiments are performed under constant conditions. Therefore, photoreceptors that have been reported as having no contribution to an organism's light response (e.g., rhodopsin in *Cryptococcus*) may indeed have subtle, yet important, functions in a natural light environment. Given this, we now discuss what is known about light sensing in two

organisms in which multiple photosensors have been analyzed in some detail.

C. Photobiology of *Neurospora crassa*

1. The Light Response of *Neurospora*

The combination of genetic, physiologic, biochemical, and molecular analyses have made the ascomycete mold *Neurospora crassa* the best-understood fungal system with regard to photosensation. At the phenotypic level, a variety of processes are light regulated in *Neurospora*, including the resetting of the circadian clock, biosynthesis of carotenoid pigments, conidiation, the formation of protoperithecia, and the directionality of ascospore release from perithecia (Lee et al. 2003; Chen and Loros 2009). In recent years, a variety of genome-wide transcriptional analyses have provided novel insight into the influence of light on *Neurospora* beyond those readily observed (Lewis et al. 2002; Chen et al. 2009). Using oligonucleotide arrays, for example, Chen et al. found that approximately 5.6 % of detectable transcripts were influenced by a white light stimulus. Among the light-induced genes were those involved in pigmentation, vitamins and prosthetic groups, secondary metabolism, DNA processing, cell signaling, oxidation of fatty acids, and oxygen detoxification (Chen et al. 2009).

The *Neurospora* genome encodes orthologs to all the photoreceptor types described, including the White Collar genes, a small LOV domain protein, two phytochromes, a cryptochrome, and an archaeal rhodopsin. Interestingly, then, essentially all light responses in *N. crassa*, at both the phenotypic and transcriptional levels, are ablated in either a *wc-1 or wc-2* mutant background. Indeed, screens for mutants that are completely "blind" have yielded over 30 mutants to date, all of which have been alleles of either *wc-1* or *wc-2* (Bieszke et al. 1999; Collett et al. 2002; Froehlich et al. 2005, 2010; Chen and Loros 2009). This suggests the two genes are the only essential regulators of the light response in this fungus,

although other photoreceptors such as VVD (vivid) may modify the response. The genetic data agree with the fact that all aforementioned photoreponses in *N. crassa* occur in response to blue light, with the action spectrum peaking at about 465 nm, the same as the absorption spectrum of flavin chromophores. Since the cloning of *wc-1* and *wc-2* in 1996 and 1997, respectively and the proof of their identity as light receptors in 2002, extensive analyses have made the WCC the paradigm for light regulation in fungi.

2. Molecular Basis for Signaling Through the White Collar Complex

As previously described, WC-1 and WC-2 interact to form a heterodimeric complex (WCC) that binds to the LREs in the promoters of light-regulated genes. However, various studies have revealed that the WCC of *Neurospora* actually exists in two forms (Froehlich et al. 2002; Cheng et al. 2003). The first is a simple **WC-1/2 heterodimer** (small complex) that binds to promoter elements most strongly in the dark. It is this small WCC that drives the circadian clock in the dark by promoting the expression of *frq*, the protein of which in turn inhibits WCC expression to form a free-running negative feedback loop that cycles every 22.5 h. The ability of the small WCC to drive *frq* expression is independent of the LOV domain of WC-1 and is thus independent of the photosensing role of the WCC.

On light exposure, the small complex is replaced on the DNA by a larger WCC, which consists of the WC-1/2 heterodimer with the addition of several more WC-1 proteins that interact via their LOV domains. Thus, WC-1 and WC-2 interact to form both a light-independent complex that drives the expression of the circadian clock and a light-dependent complex that drives the photoreresponse. Although WC-2 is the limiting protein in the light-induced WCC, its Zn-finger domain is required for DNA binding; therefore, the light-sensing function of WC-1 and the DNA-binding function of WC-2 makes both proteins
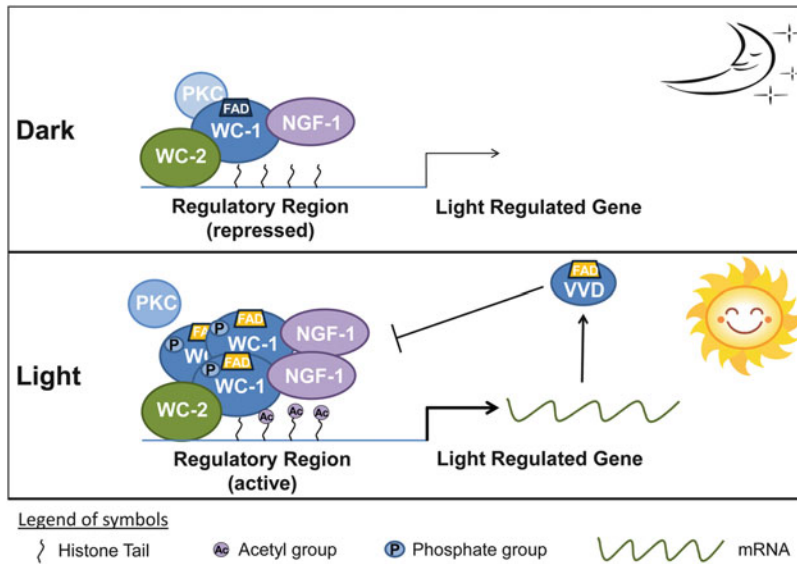
Fig. 6.1. Model for the light-induction of genes by the White Collar Complex (*WCC*). *Top* In the dark, the WC-1/2 heterodimer is preassembled on light-responsive elements (*LREs*) along with NGF-1 and protein kinase C (*PKC*) via their interaction with WC-1. In this state, expression of light-regulated genes is low. *Bottom* In the presence of light, flavin-adenine dinucleotide (*FAD*) absorbs a photon, which leads to conformational changes in the LOV (light, oxygen, and voltage) domain of WC-1. This conformational change promotes LOV domain interactions with additional WC-1 proteins and increases the acetyltrasferase activ-ity of NGF-1. As a result, histone tails are transiently acetylated, leading to chromatin remodeling and an increase in transcriptional activity of the WCC. The activity of the WCC is negatively regulated in two ways: (*1*) WC-1 is rapidly phosphorylated, at first by PKC and then subsequently by others, which leads to its degradation. (*2*) *vvd* expression is induced by the WCC, the protein of which is biochemically activated by light and interrupts the activity of the WCC through a direct LOV domain interaction with WC-1 (This model is modified from a figure in Brenna et al. (2012))

essential in the transmission of the light signal (Linden and Macino 1997; Cheng et al. 2002; Collett et al. 2002).

The induction of light-regulated genes also involves chromatin modifications. For example, histone H3-K14 at light-inducible promoters is transiently acetylated by the **histone acetyltransferase** (HAT) NGF-1 (Grimaldi et al. 2006). Interestingly, acetylation of H3-K14 is transiently decreased at the *frq* promoter (Belden et al. 2007b). The NGF-1-mediated acetylation seems essential for light regulation and is dependent on a direct interaction with WC-1 (Brenna et al. 2012). Interestingly, it was shown that the WCC/NGF-1 complex occurs in the dark. Therefore, the current model for the light induction of target genes is as follows: (a) The small WC-1/2 heterodimer, in complex with NGF-1 via a WC-1 interaction, binds LREs in the dark. (b) Flavin absorbs blue light, which leads to the formation of a cysteine adduct with the WC-1 LOV domain, thereby changing its conformation and allowing for the recruitment of additional WC-1 proteins to the complex (large WCC). (c) The conformational change also enhances the HAT activity of NGF-1 and perhaps changes in other HAT activities, leading to (d) chromatin remodeling and enhanced transcriptional activity of the WC1/2 heterodimer, ultimately leading to the induction of WCC target genes (summarized in Fig. 6.1).

Not all light-induced genes are a direct target of the WCC. Instead, the WCC regulates a subset of genes known as the early-induced genes, which demonstrate transcriptional induction as early as 5 min (average peak at 30 min) post–light transfer (Chen et al. 2009; Smith et al. 2010).

Included in the early-induced genes are additional transcription factors that regulate a second group of late-light-induced genes that show induction as between 45 and 90 min (average peak at 60 min) post–light transfer. For example, Sub-1 is a transcription factor that is directly induced by the WCC, and its deletion results in the loss of most late-light-induced genes (Chen et al. 2009). Thus, the *Neurospora* light response is facilitated by a **hierarchy of transcriptional regulators** but is entirely dependent on the initial perception of light and early transcriptional activity of the WCC.

3. Negative Regulation of the White Collar Complex by Protein Kinases and VVD

Fungi must be able to attenuate the light response to maintain sensitivity to increasing light intensities. To illustrate, carotenoids protect cells from genotoxic and photooxidative stress, but their continued synthesis may represent an unnecessary energy expenditure in low-light conditions; therefore, the amount of carotenoids produced should correlate to the light intensity. This process, termed *photoadaptation*, is obvious in *N. crassa* as the steady-state transcript levels of light-induced genes begin to decrease over time under constant-light conditions (Chen et al. 2009). In *Neurospora*, photoadaptation is achieved primarily through direct inhibition of the WCC, one mechanism of which is through **hyperphosphorylation**, and ultimate turnover, of WC-1. Protein kinase C (PKC), for example, directly interacts with and phosphorylates WC-1 (Franchi et al. 2005). The importance of this in the attenuation of the light response is demonstrated by the fact that PKC inhibition leads to an increase in WC-1 levels and an increase in *albino-3* (involved in carotenogenesis) expression (Arpaia et al. 1999); however, PKC interacts with WC-1 only in the dark, suggesting that PKC can only be responsible for early WC-1 phosphorylation (Fig. 6.1). Therefore, additional kinases are likely involved in the hyperphosphorylation seen after 20 min post–light exposure.

A major advance in understanding photoadaption came with the identification of the small LOV domain protein VIVID (VVD). The name stems from its loss-of-function mutants, which display bright orange conidia when grown in constant light, attributed to the persistence activation of carotenoid pigments (i.e., loss of photoadaptation) (Heintzen et al. 2001). It is a direct target of the WCC, and *vvd* transcript levels are robustly induced following illumination. Various studies have now shown that, on light reception by VVD, its LOV domain interacts with the WC-1 LOV domain, ultimately blocking the transcriptional activity of the WCC (Fig. 6.1) (Chen et al. 2010; Hunt et al. 2010; Malzahn et al. 2010). Ultimately, the amount of WCC activity is dependent on the amount of light-activated VVD present in the system, which itself is a function of the WCC-mediated light response.

4. Additional Photoreceptors in *Neurospora*: All You Need Is LOV?

a) CRY
Neurospora contains a single ortholog to the CRY-DASH family of cryptochromes, called CRY. CRY can bind both FAD and MTHF as chromophores and, interestingly, to both single- and double-stranded DNA and RNA; however, it is not required for photoinduced DNA repair. Similar to *wc-1*, both transcript and protein levels of CRY rapidly accumulate in a WC-1 dependent manner; however, the disruption of the *cry* gene does not lead to any observable photobiology phenotype or seem to interfere with the normal light-regulated transcriptional response. Interestingly, however, CRY may play a role in the circadian clock, as its deletion leads to a small phase delay under light entrainment regimens (Froehlich et al. 2010).

b) PHY-1/PHY-2
Although red light does not regulate the aforementioned growth and developmental programs in *Neurospora*, a half-century ago it was reported that far-red light potentiated X-ray-induced genetic damage in this fungus. Furthermore, this effect was indicative of a

phytochrome response as it could be reversed by exposure to red light following the far-red light treatment (Klein and Klein 1962). *Neurospora* encodes two putative phytochromes for red light sensing, called PHY-1 and PHY-2. The two proteins display similar domain architecture, including an N-terminal domain for chromophore binding and a C-terminal histidine kinase and response regulator motifs, indicating that they may function as two-component sensor kinases. In vitro studies of PHY-2 have indeed shown that the protein can bind bilin or phycocyanobilin, and the holoprotein displays both red and far-red absorption spectra. Unlike *cry-1*, transcripts of neither *phy-1* nor *phy-2* are light induced, and deletion of either does not affect circadian phasing, although the expression of *phy-1* is regulated in a circadian fashion (Froehlich et al. 2005). Similar to CRY, however, the phytochromes have no detectable light-signaling function, as disruption of *phy1/2* does not affect photoinducible phenotypes or gene expression (Froehlich et al. 2005; Chen et al. 2009).

c) Neurospora Opsin (NOP-1)

A single rhodopsin ortholog is present in the *Neurospora* genome, called neurospora opsin (NOP-1). Characteristic of archael rhodopsins, NOP-1 has a seven-transmembrane topology and has been shown to bind all-*trans*-retinal in vitro. The expression of *nop-1* is most prominently induced by conditions that promote conidiation; however, the gene does demonstrate a modest influence by light, with more accumulation of messenger RNA (mRNA) in light-grown cultures (Bieszke et al. 1999). Interestingly, the *nop-1* deletion mutant does display light-dependent morphological differences (with respect to wild type) in the presence of the mitochondrial ATPase inhibitor oligomycin, suggesting it does have a biochemical function as a light-dependent proton pump (Bieszke et al. 1999). Similar to the *cry* and *phy-1/2* mutants, the role of NOP-1 as a light sensor seems to be limited as the mutant displays essentially normal patterns of development (conidiation and catotenogenesis) and gene induction in response to light (Bieszke et al. 1999; Chen et al. 2009).

d) A Role for the Secondary Photoreceptors in Inhibition of the White Collar Complex?

Despite the evidence that all of the *Neurospora* photoreceptors mentioned are expressed and display chromophorebinding capability, their involvement in the photoresponse has remained elusive. However, a study has shown that the gene *con-10* is induced earlier and to a greater extent in a *cry*, *nop-1*, or *phy-2* deletion background, compared to wild-type (WT) (Olmedo et al. 2010). Therefore, while the WCC is essential for driving the expression of light-induced genes, the additional photoreceptors may play a role in inhibiting WCC activity, similar to VVD. A direct interaction of these photoreceptors with the WCC has not been shown, however, so their effect on the WCC presumably requires an intermediary inhibitory protein.

D. Photoperception in *Aspergillus*: Seeing the Rainbow Through Multiple Photoreceptors

1. The *Aspergillus nidulans* Light Response

Aspergillus nidulans has served as a model organism for several decades, but only in recent years has its photobiology been the subject of detailed investigation. *Aspergillus nidulans* distinguishes itself from *Neurospora* by being overtly responsive to both blue and red portions of the visible spectrum and, consequently, having a light response that is mediated by multiple photosensory pathways. Accordingly, *A. nidulans* has quickly emerged as the predominant model for fungal **red-light sensing** and **photosensory cross-talk**. The major recent advances are highlighted.

In contrast to *Neurospora*, in which light induces both the sexual and asexual cycle, the two processes are differentially regulated by light in *A. nidulans*. In the presence of white light, asexual developmental genes are induced, and conidiation represents the dominant form of sporulation (Ruger-Herreros et al. 2011). Either blue or red light alone is capable of inducing conidiation, but only to about one third the level of white light. However, the combination of blue and red light fully restores conidiation,

suggesting the two light qualities are sufficient for the response and interact synergistically. In contrast, cleistothecium formation (sexual development) is repressed by both blue and red light, although red light displays the most dominant role in this response (Purschwitz et al. 2008).

Secondary metabolism, particularly the production of the mycotoxin sterigmatocystin (ST), is tightly linked with sexual development. This may serve to protect sexual structures from predation by competing microorganisms. White light represses ST production, but interestingly, blue and red light play opposing roles; blue light represses ST production, whereas red light induces (Purschwitz et al. 2008).

Microarray analysis has recently demonstrated that light regulates approximately 5 % of the *A. nidulans* genome (Ruger-Herreros et al. 2011), similar to the figure described for *Neurospora*. Beyond development, light was shown to induce genes involved in carbon metabolism and transport, redox reactions, and stress response (Ruger-Herreros et al. 2011).

2. Genetic and Physical Interactions Between the WCC and Phytochrome

With the exception of an archael rhodopsin, the genome of *A. nidulans* contains essentially the same complement of photoreceptors as *Neurospora*. The orthologs to *Neurospora* WC-1/2 in *A. nidulans* are called LreA and LreB (for *L*ight *re*sponse), respectively; both have the predicted domain architecture of their respective *Neurospora* proteins, and they have been shown to interact physically and genetically, suggesting LreA/B also form a codependent heterodimer (WCC) (Purschwitz et al. 2008). For red light sensing, *A. nidulans* encodes for a single phytochrome ortholog, called FphA. The protein shows strong covalent interaction with the tetrapyrrole biliverdin and displays red and far-red absorption peaks typical of bacterial and plant phytochromes. Moreover, the protein displays weak autophosphorylation, thus demonstrating a functional kinase domain (Blumenstein et al. 2005).

Analysis of *lreA*, *lreB*, and *fphA* single-deletion mutants has revealed that the WCC and phytochrome have complimentary roles in regulating development. For example, based on levels of conidiation seen in the mutants with respect to wild type, the WCC and FphA serve as negative and positive regulators of asexual development, respectively (Purschwitz et al. 2008). However, only when *fphA* is deleted along with *lreA* or *lreB* is a complete loss in light induction observed, suggesting that the activation of FphA and derepression of WCC must be coordinated to achieve the proper response. Interestingly, the respective roles for the photoreceptors are reversed with regard to regulating both the sexual cycle and ST production. Notably, cleistothecium formation is severely reduced in the dark in the ΔlreA and ΔlreB mutants; therefore, the WCC seems to serve as a transcriptional activator and repressor (Purschwitz et al. 2008). Further work will be required to determine if the WCC of *A. nidulans* acts through intermediary transcription factors, as is seen in *Neurospora*.

Strikingly, in addition to the genetic interactions between the WCC and FphA, the two photoreceptor systems also interact physically. Through a combination of bimolecular fluorescence complementation and coimmunoprecipitation assays, it was shown that FphA binds the WCC in the nucleus through a direct interaction with LreB. Within this complex, FphA also interacts directly with a second protein called **VeA (Velvet A)** (Purschwitz et al. 2008, 2009). VeA is a central regulator of development in *A. nidulans* whose loss of function abolishes the requirement of light for conidiation (Mooney and Yager 1990). However, VeA seems not to sense light directly but instead is light regulated through its interaction with FphA. In addition, it is a central regulator of secondary metabolism and is required for ST production (Bayram et al. 2008b). In this way, the complex consisting of VeA/FphA/LreA/LreB likely serves to balance both developmental programming and secondary metabolism through the integration of multiple light inputs.

3. CryA of *Aspergillus nidulans* Functions as Both a CPD Photolyase and a Light-Sensing Cryptochrome

CryA is the only photolyase/cryptochrome family member encoded by *A. nidulans*. This protein is most closely related to the class I CPD photolyases based on sequence comparisons, and it does indeed demonstrate photoactivated DNA repair in vivo and when expressed in *Escherichia coli*. Interestingly, CryA also seems to function as a bona fide cryptochrome in *A. nidulans*, as it represses sexual development in response to blue light and UV-A irradiation. This repressive action of CryA is, in part, achieved by negatively regulating the expression of *veA* and other positive regulators of the sexual cycle (Bayram et al. 2008a). From a physiological standpoint, the combined action of the WCC (blue) and CryA (UV-A) likely imparts a broad action spectrum to which *A. nidulans* can regulate development. Whether the CryA protein interacts with the FphA/WCC/VeA complex has yet to be established.

4. Summary

In summary, the photoresponse of *A. nidulans* displays important parallels and differences with the WCC-dominated system established in *Neurospora*. While *A. nidulans* does indeed respond to blue light through its WCC (LreA and LreB), the fungus is equally responsive to red light via a phytochrome and UV-A light via a cryptochrome. Because these different light qualities may differentially regulate certain processes (e.g., mycotoxin production) and because different light signals are likely detected concurrently, the photosensory pathways must somehow communicate to integrate the light signals in a meaningful way. If recent breakthroughs are any indication, future studies will undoubtedly reveal a highly complex system that is regulated at the transcriptional, posttranscriptional, posttranslational, and epigenetic levels.

E. General Conclusions

Further investigations into how fungi perceive light will undoubtedly have important implications in a variety of different fields. For example, the use of photoreceptors (Christie et al. 2012) and light-inducible promoters (Hurley et al. 2012) as tools to study cell biology in heterologous systems has been an emerging and exciting area of research. Intriguingly, in two divergent fungal pathogens, *C. neoformans* (Idnurm and Heitman 2005a) and *Fusarium oxysporum* (Ruiz-Roldan et al. 2008), deletion of the *wc-1* ortholog leads to an attenuation of virulence in their respective animal infection models. It is, therefore, exciting to speculate that the WCC, and potentially other photosensory pathways, could be conservatively linked to fungal pathogenesis and represent novel targets for antifungal therapy. Recent work has shown that the predominant mold pathogen of immunocompromised patients, *A. fumigatus*, also regulates many virulence-associated pathways in response to light (K. Fuller unpublished data), and research into how this affects its pathogenic potential is under way. Only time will tell to what extent understanding fungal visual systems will help researchers see the world in new and exciting ways.

III. Circadian Rhythms and Fungi

Originally made famous by Beadle and Tatum's one enzyme/one gene work, *Neurospora* was a premier organism in genetics until the use of *E. coli* became common (Davis and Perkins 2002). Circadian rhythms were first documented in *Neurospora* in 1959 by Pittendrigh et al. (1959). These rhythms fulfilled the criteria to be defined as circadian: (a) They maintained a sustained period (approximately 22.5 h under constant conditions); (b) the phase was set by a single transition from light to dark; (c) the rhythms were entrainable; and (d) the period

was not dependent on temperature. Since then, *Neurospora* has become a highly tractable genetic organism, with a full genome sequence, transformation protocols with an extremely high success rate, and near-complete knockout library (Collopy et al. 2010; Colot et al. 2006; Galagan et al. 2003).

A. What Is a Circadian Rhythm?

The origin of circadian studies started with Jean Jacques Ortous de Mairan, a French scientist who described daily leaf movements, noting that the movements persisted when the plant he was studying was in the dark as well as in the light. More important, these movements persisted with a regular period, leading him to the conclusion that they were not a simple response to the sun but in fact a more complex system regulated by an internal mechanism. The study of circadian rhythms in *Neurospora* started with Colin Pittendrigh's work in the 1950s looking at the rhythmic expression of asexual conidia.

To distinguish a reactive response from a clear circadian rhythm, there are four basic tenants. First, the rhythm must persist in the absence of exogenous cues, such as light/dark cycles. This is a clear sign that there is an **internal timekeeping mechanism** that is creating this cycle, and that the cycle is not just a response to external cues.

Second, the duration of a true circadian rhythm must have a cycle length, also called a **period**, of approximately 24 h. This ensures that the organism is at the same state at the same time every day and builds flexibility into the system so that it may be reset by external cues. This allows for better control of the timing of circadian responses.

A third characteristic of a true circadian rhythm is the ability of the rhythm to be reset (or entrained) to external time cues. This **entrainment** allows the organism to adapt to its local environment, as in resetting the clock when crossing time zones. Without this adaptability, a person could not thrive in an environment that did not have conditions that matched their endogenous rhythm.

Finally, as is well established in chemistry, different temperatures affect the kinetics of a reaction. If a clock were to speed up in higher temperatures and slow down in lower ones, it would simply be a thermometer, not a clock. So, a true circadian rhythm must maintain its periodicity over a range of physiological temperatures, a capability referred to as **temperature compensation**.

Beyond these basic rules of circadian rhythms, it is generally accepted that circadian rhythms are nearly ubiquitous in nature, being found in many organisms and biological processes. They are also generated at the cellular level, whether the organism is a unicellular prokaryote or a multicellular, complex eukaryote.

B. Tracking Circadian Rhythms in *Neurospora crassa*

Neurospora propagates both asexually and sexually. Although both are circadianly regulated, it is the asexual cycle that allowed for the first clear insight into the circadian cycle (Bobrowicz et al. 2002; Dunlap and Loros 2004). In the case of asexual spores, a developmental switch leads to the production of conidia in the subjective night. A common method to assay this rhythmic development of conidia is using a race tube, a long glass tube, bent at both ends, holding agar medium onto which mycelia or conidia are inoculated at one end (Fig. 6.2a). The fungus grows toward the other end, and the growth front is marked each day. Cellular clocks are synchronized by germinating the cultures in constant light for a day and then transferring them to constant darkness.

Expression of the overt rhythm in sporulation is not robust in wild-type cultures unless there is gentle airflow over the culture. However, identification of the **band (bd)** strain in the late 1960s alleviated this (Sargent et al. 1966). This strain exhibited rhythmic and robust formation of asexual conidia in a manner that fit the conditions of a true circadian rhythm without the need to flow air through the system (Belden et al. 2007a). The mutation in the strain was identified as a mutation in *ras-1*,
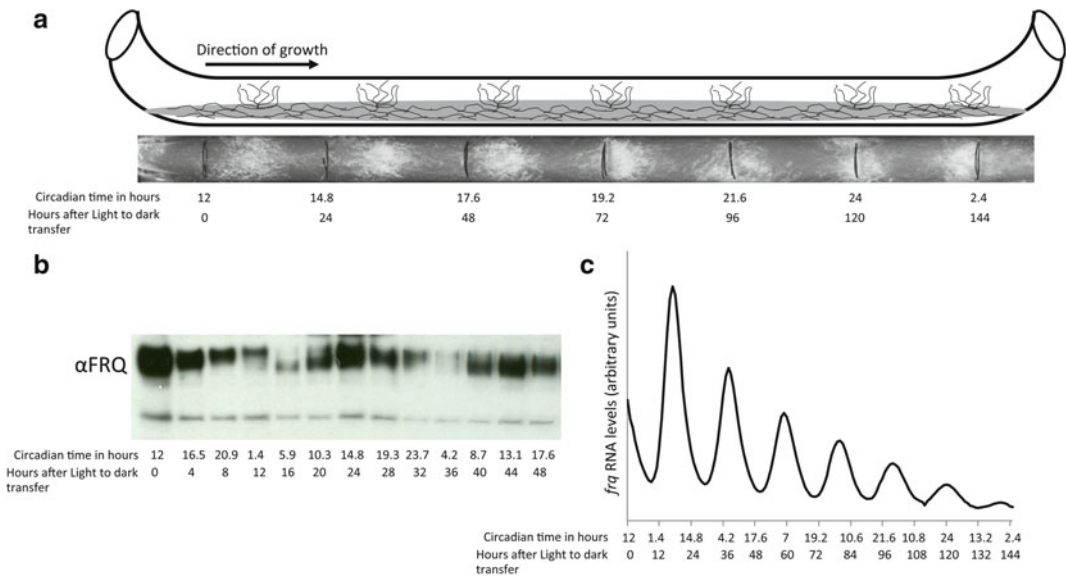
Fig. 6.2. Methods of circadian analysis in *Neurospora*. (**a**) *Neurospora* growing along a race tube with an image of an actual race tube beneath it. Marks represent the growth front (demarcated in sidereal time and in circadian time for the figure) (Unpublished data from J. Hurley) (**b**) Western blot of FRQ (FREQUENCY) protein tracked over 48 sidereal hours (time points taken every 4 sidereal hours) and labeled in circadian hours, highlighting the changes of phosphorylation state of FRQ protein over time (Unpublished data from J. Emerson) (**c**) Luciferase trace of *frq* mRNA expression tracked over 144 sidereal hours and labeled in circadian hours, highlighting the changes of expression levels of *frq* mRNA over time (Unpublished data from J. Emerson)

which increases the levels of reactive oxygen species (ROS) in *Neurospora*. This in turn leads to an increased expression of a subgroup of genes known as the clock-controlled genes (ccgs), in particular the conidial regulation protein, *fluffy*, which accounts for the *bd* phenotype (Belden et al. 2007a).

The *bd* strain has been used in almost all circadian experiments in *Neurospora* since its isolation. However, *bd* turns a subtle circadian regulation into a robust phenotype by increasing transcripts whose gene products control asexual development, suggesting a link between RAS signaling and ROS levels in promotion of sporulation. This means that a subset of the genes and pathways identified as ccgs may be RAS responsive instead of circadianly regulated and be absent in analysis of ccgs from a wild-type strain (Belden et al. 2007a).

In addition to this, rhythms have been tracked when cultures are grown in liquid medium as well, leading to the understanding of the clock on a molecular level as opposed to the overt rhythms followed in the *bd* strain

(Aronson et al. 1994; Loros et al. 1989). More recently, in an effort to correct for this potential problem, rhythms have been monitored on a more direct level. An alternative method of monitoring the output of the circadian clock is by following the expression of the luciferase gene from the firefly beetle *Photinus pyralis*, codon optimized for *Neurospora* and driven by the *frq* or *ccg-2* promoter (Gooch et al. 2008; Morgan et al. 2003). Originally designed as a method to follow the endogenous, core rhythms of the *Neurospora* clock on the transcriptional level, this method has been used to understand temperature compensation as well as translational activity in the core *Neurospora* clock (Fig. 6.2b, c) (Gooch et al. 2008; Larrondo et al. 2012).

C. Clock Conservation in Other Fungi

Beyond the well-studied pacemaker in *Neurospora*, there is evidence of conservation of a

circadian circuit in other fungi (Table 6.1). At the phenotypic level, rhythms have been reported in conidiospore formation in the Zygomycete *Pilobolus* (Bruce et al. 1960). Some less-definite growth and developmental rhythms exist in a variety of Ascomycetes (reviewed in Dunlap and Loros 2006), although sclerotia formation of *A. flavus* and enzyme rhythms in *A. nidulans* have been demonstrated to be truly circadianly regulated, with validation of entrainment as well as temperature compensation (Greene et al. 2003).

On the genotypic level, the identification of conserved core clock components has been well investigated. According to a review of 42 sequenced fungal genomes, while light-sensing mechanisms (which typically work as circadian positive-feedback elements; see Molecular Mechanism of a Circadian Oscillator) are widely conserved in the fungi, the clock-exclusive protein FREQUENCY (FRQ) (the negative-feedback element) is less conserved. However, complete circadian feedback loops are seen universally in the Sordariacea, suggesting that many plant and animal pathogens have a functional clock. As described previously, WC-1 is conserved into Basidiomycetes as well as Zygomycetes. Given that rhythms have been reported in *Aspergillus*, which has no FRQ, it may be that rhythms in these strains have a different negative arm protein but still use the positive arm WCC (Dunlap and Loros 2006).

In a more recent study using 64 fungal genomes, it was determined that FRQ was only seen in Sordariomycetes, Leotiomycetes, and Dothideomycetes. Homologs of other clock components such as the WCC, FRH (frequency-interacting helicase), and FWD-1 (F-box and WD40 repeat-containing protein 1) (see FRQ Degradation) have been found in a wider array of organisms, including Zygomycetes, Basidiomycetes, and Ascomycetes. The Saccharomycetes seem to have lost both the WCC and FRQ in a genome size reduction and have never been demonstrated to possess circadian rhythms (Dunlap and Loros 2006; Salichos and Rokas 2010).

D. Molecular Mechanism of a Circadian Oscillator

The mechanism that drives circadian rhythms is a transcriptional/translational feedback loop that is strictly regulated by a series of proteins that interact with a core clock complex. The core complex is made up of two protein complexes, the positive arm (known in *Neurospora* as the WCC), which drives the expression of a second component, the negative arm (known in *Neurospora* as the FRQ/FRH complex or FFC). The negative arm in turn acts to autoregulate its expression on a time delay, which sets that length of the period (Dunlap 1999).

The cycle begins late in the subjective night (Fig. 6.3a–c), as the WCC binds to the promoter of FRQ and induces expression of *frq* mRNA, which reaches its maximum around early subjective morning. About 4 h after the start of *frq* expression, FRQ protein begins to appear; it binds directly to FRH, enters the nucleus, and begins to form homodimers (Dunlap and Loros 2004; Merrow et al. 1997). New FRQ is rapidly phosphorylated in the PEST-1 (a protein domain rich in proline, glutamic acid, serine and threonine) and FFD domains (sites of FRQ/FRQ and FRQ/FRH interactions), with phosphorylation events occurring via interaction with several kinases in the C-terminal region shortly thereafter. These C-terminal phosphorylations stabilize the protein (reviewed in Baker et al. 2012; Heintzen and Liu 2007).

On nuclear entry, FRQ represses *frq* transcription by inhibiting the activity of the WCC while simultaneously increasing the levels of WC-1 (Dunlap and Loros 2004). This inhibition is caused by a direct interaction between FRQ and the WCC, which leads to the phosphorylation of the WCC, inactivating the WCC as well as clearing it from the *frq* promoter (reviewed in Brunner and Kaldi 2008; Liu and Bell-Pedersen 2006). The WCC begins to exit the nucleus at this point in the cycle, perhaps reflecting its phosphorylation status, further decreasing activation of the *frq* promoter
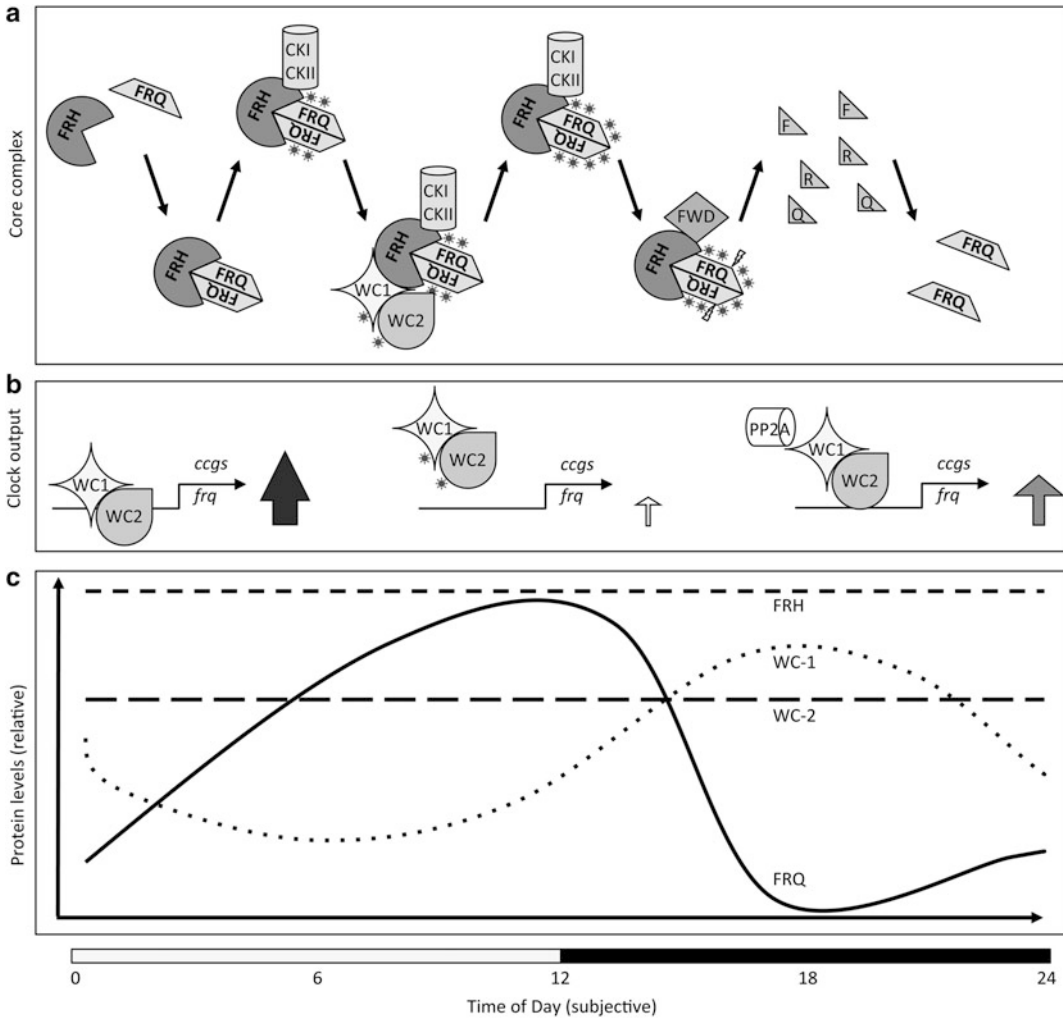
Fig. 6.3. Molecular underpinning of *Neurospora* circadian rhythm. (**a**) In the late subjective night, the WCC induces expression of *frq* mRNA, leading to high levels of FRQ (FREQUENCY) translation. FRQ forms homodimers and binds to FRH. FRQ is phosphorylated via the interaction with several kinases. FRQ is autoregulatory, inhibiting the activity of the WCC by promoting the phosphorylation of the WCC. Lack of WCC leads to a decrease in FRQ synthesis, while old FRQ is increasingly phosphorylated, which leads to ubiquitination facilitated by FWD-1, leading to FRQ degradation. (**b**) The output of the circadian clock. When FRQ levels are low, WCC activity is high, and it subsequently binds to the *frq* promoter as well as other *ccg* promoter and increases expression at those loci. When FRQ is high, it binds to the WCC, promoting its phosphorylation and causing it to become inactive. As FRQ ages and is degraded, phosphatases bind the WCC, dephosphorylating it and allowing it to become active again, leading the WCC to bind to target promoters again. (**c**) Protein levels of the core clock components. While FRH and WC-2 remain constant, FRQ and WC-1 oscillate in opposite phases to one another. Stars represent phosphorylation and lightning bolts represent ubiquitination

(Hong et al. 2008). In the late afternoon, due to the lack of WCC activation, *frq* expression begins to decline, and in accordance, so does FRQ synthesis (Merrow et al. 1997). FRQ is then increasingly phosphorylated at the PEST domain and the N-terminal domain, which leads to FRQ being ubiquitinated by an SCF-ubiquitin ligase complex containing the F-box protein FWD-1 and targeted to the proteasome for degradation (reviewed in Baker et al. 2012;

Heintzen and Liu 2007). The mass of WC-1 that was created and held in the inactive state by FRQ is now released, and the cycle restarts, with the WCC again binding to the *frq* promoter (Dunlap and Loros 2004). The delays between *frq* expression and FRQ synthesis (3–6 h) and FRQ phosphorylation-directed degradation (14–18 h) leads to the nearly 24-h rhythm in *Neurospora* and is how the circuit keeps its specific time (Merrow et al. 1997).

E. Core Clock components

1. The Negative Arm: FRQ and FRH

The use of *Neurospora* as a model for circadian rhythms at the molecular level began with the discovery of several mutants, each of which directly affect the period of banding in *Neurospora*. Eventually, these mutants were all mapped to the *frequency* (*frq*) locus and displayed long, short, or arrhythmic periods; some alleles also altered or disrupted temperature compensation (Gardner and Feldman 1980; Loros and Feldman 1986). *frq* was cloned, and this began the molecular dissection of the core clock of *Neurospora* and a greater understanding of clocks in general (McClung et al. 1989). In *Neurospora*, FRQ expression periodicity matches the conidiation rhythm seen in the *bd* mutant. Altering or inhibiting the FRQ rhythm had a direct and equivalent effect on the clock, demonstrating that FRQ is the driver of *Neurospora* period (Aronson et al. 1994; Belden et al. 2007a; Garceau et al. 1997).

FRQ constitutes the one of two proteins that make up the negative arm of the clock. Full-length FRQ contains 989 amino acids and self-associates via the coil-coil region near the N′ terminus (Aronson et al. 1994; Cheng et al. 2001). *frq* message as well as FRQ protein are rhythmically expressed in a 22.5-h cycle under constant conditions with a phase difference of approximately 4 h (Aronson et al. 1994; Garceau et al. 1997), and it is this rhythmic expression that sets the **period of the clock**. FRQ is a highly transcriptionally, posttranscriptionally, translationally, and posttranslationally controlled protein (see FRQ Transcriptional and Posttranscriptional Regulation) (Baker et al. 2012). FRQ is both nuclear and cytoplasmic; however, the most understood activity of FRQ is nuclear, where it binds to the WCC and blocks the transcriptional activity of the WCC (Liu et al. 2003). FRQ is able to increase WC-1 levels; this probably is the result of inhibiting the activity of WC-1, a protein believed to be unstable when it is active (Shi et al. 2010; reviewed in Baker et al. 2012). FRQ also increases the abundance of *wc-2* through an unknown mechanism (Liu et al. 2003).

a) FRQ Transcriptional and Posttranscriptional Regulation

First, the rhythmic binding of the pertinent transacting factors must occur to maintain a functional circadian rhythm. Transcriptional regulation at the *frq* promoter occurs through binding of the WCC proteins to a pair of *cis*-acting sequences termed the **Clock box** (C box) and the **proximal light-regulated element** (PLRE) (Froehlich et al. 2003). The C box is required for rhythmic expression of *frq* and overall clock function in continual darkness, whereas the PLRE is necessary to establish the proper phase when entrained by light. Both elements are necessary for high levels of light-induced *frq* expression via WCC influence on the *frq* promoter, but each element acts differentially as chromatin is remodeled during the transcriptional activation and deactivation of *frq* (Belden et al. 2007b).

The gene *clockswitch* (*csw-1*) is required for normal *frq* expression and acts directly or indirectly to negatively regulate WCC activity at *frq* by altering chromatin structure, creating a more compact chromatin structure at the C box (Belden et al. 2007b). Chromodomain helicase DNA–binding (CHD-1) contributes to changes in chromatin structure at *frq* and is also needed for normal *frq* expression. DNA methylation at *frq*, which is promoted by loss of CHD-1, is transient and reversible and catalyzed by the DNA methyltransferase DIM-2 (Defective in Methylation-2), which limits the onset of circadian-regulated transcription via regulation of methylation at the *frq* promoter (Belden et al. 2007b, 2011).

The *frq* mRNA regulation extends beyond simple transcriptional regulation. Indeed, there is alternative initiation of translation that results in the production of two distinct FRQ polypeptides, FRQ$^{1-989}$ (L-FRQ) and FRQ$^{100-989}$ (S-FRQ) (Garceau et al. 1997). While both forms of FRQ are able to maintain rhythmicity at 25 °C, the amplitude and robustness of the rhythm are affected across a range of physiological temperatures. Ratios of the two proteins are known to vary with temperature, with more L-FRQ being produced at higher temperatures versus even amounts of long and short FRQ protein at lower temperatures (Liu et al. 1997). Phosphorylation sites on the 100 amino acids on L-FRQ that are not present in S-FRQ have been shown to decrease period length even in the presence of S-FRQ. The **ratio of FRQ polypeptides** is believed to allow the clock to fine-tune the period in response to environmental cues while remaining a robust timekeeping mechanism in their absence (Baker et al. 2009; Diernfellner et al. 2007; Liu et al. 1997).

The selective transcription of either S- or L-FRQ is determined by the temperature-dependent, alternative splicing event of a small intron encompassing the AUG of L-FRQ. The retention of this intron results in the exclusive use of the AUG from L-FRQ, whereas its removal makes the AUG from S-FRQ the first bona fide start codon. Strains unable to splice this intron fail to produce S-FRQ (Colot et al. 2005; Diernfellner et al. 2005).

In addition to this splice site, there are alternative splicing events farther upstream in the 5′ UTR that remove five upstream AUGs with four uORFs from all major *frq* transcripts. Two AUGs remain, and these uORFs may be differentially regulating S- and L-FRQ at the translational level by targeting transcripts for nonsense-mediated decay (NMD), either as a way to remove improperly spliced transcripts or as a mechanism for quantitative control of gene expression (Colot et al. 2005; Diernfellner et al. 2005).

Another regulation of the fine-tuning of the *frq* mechanism is an **antisense transcript,** which comprises the entire length of the FRQ open reading frame (ORF). Elimination of this transcript causes a slight increase in period as well as a loss of rhythmicity at low physiological temperatures and an earlier phase setting on light-to-dark transfer. It is postulated that this antisense RNA may be an additional mechanism to regulate *frq* posttranscriptionally or posttranslationally to further insolate the clock from environmental stresses (Kramer et al. 2003).

b) FRQ Associations and Subcellular Localization

FRQ is only half of the negative arm of the clock. Frequency-interacting RNA helicase (FRH) is a homolog of Mtr4p, a well-studied cofactor of the *Saccharomyces cerevisiae* exosome (Cheng et al. 2005). Mtr4p is a known member of the TRAMP complex and has been suggested to play many roles in a variety of cellular processes (LaCava et al. 2005). FRH is so important to the clock complex that all FRQ is bound to FRH. When FRH is removed from the system via small interfering RNA (siRNA) knockdown (*frh* is an essential gene in *Neurospora*), the clock loses rhythmicity completely, and the FRQ level decreases dramatically while the inverse happens to the mRNA (Cheng et al. 2005).

It has been suggested that the role of FRH in the clock is to regulate the levels of *frq* posttranscriptionally, as when FRH is knocked down, *frq* mRNA is stabilized (Guo et al. 2009). When taken in combination with the demonstrated interaction between FRH and components of the exosome complex in *Neurospora*, the general theory is that FRH directs *frq* mRNA to the TRAMP complex for degradation (Guo et al. 2009).

Beyond this, FRH is known to play a role in the complex interaction between FRQ and the WCC; FRH is integral to the interaction between the FFC and the WCC and is also able to interact with the WCC in the absence of FRQ (Cheng et al. 2005; Guo et al. 2010; Shi et al. 2010). FRH is also implicated in the proper methylation of *frq* (Belden et al. 2011), as well as an essential interactor of VVD in suppression of FRQ expression via interaction with the WCC (Hunt et al. 2010).

FRQ association with FRH is essential for proper phosphorylation (discussed in FRQ Phosphorylations and the Role of Kinases and

Phosphatases in the Clock) as well as FRQ stability, and FRH plays a role in the proper localization of FRQ protein (Cha et al. 2011; Guo et al. 2010). FRQ nuclear localization is essential for its function in the circadian clock, and the nuclear localization signal located downstream of the coiled-coil domain is sufficient to direct the localization of FRQ protein (Luo et al. 1998). However, a large portion of FRQ is cytoplasmic, and the nuclear-cytoplasmic shuttling has been suggested to be dependent on the phosphorylation state of FRQ (Diernfellner et al. 2009). This model stands in contrast to the idea that FRH is the key link to proper subcellular localization of FRQ (Cha et al. 2011).

Independently of the pull-down assay used to identify FRH originally, FRH was identified through a mutagenesis screen for negative-feedback loop mutants. This screen identified a point mutation that was outside the highly conserved helicase region of FRH. This mutation eliminated the interaction of FRH with the WCC but not with FRQ (Shi et al. 2010). This mutation hints that the role of FRH that is specific to the clock may be different from its role in the TRAMP/exosome complex function that FRH plays for overall cell fitness.

c) FRQ Phosphorylations and the Role
  of Kinases and Phosphatases in the Clock
FRQ undergoes dual molecular rhythms, in both abundance (described previously) and phosphorylation, both of which influence its turnover kinetics (Garceau et al. 1997; Liu et al. 2000). FRQ is phosphorylated immediately after synthesis and further phosphorylated in a highly regulated manner throughout the circadian day (Baker et al. 2009). FRQ is also phosphorylated in constant light, although in a less-specific and regulated manner (Baker et al. 2009; Tang et al. 2009). Mutations that reduce the level of phosphorylated FRQ tend to increase its stability, which in turn leads to increased period lengths (Liu et al. 2000; Ruoff et al. 2005).

No specific phosphorylation event has a role that is essential to protein turnover. Instead, most phosphorylations occur in clusters, added at different locations and times. FRQ is unmodified after synthesis, and phosphorylation events occur in a strict sequence throughout the day. At first, FRQ is rapidly phosphorylated in the middle part of the protein between the PEST-1 and the FFD domain. There has been no function assigned to these events, and mutations at these sites did not alter circadian rhythms. Phosphorylation at the C-terminus is next in the sequence, and mutations at specific sites in the C-terminal region result in FRQ protein that is less stable and in a short-period rhythm. Midday, the PEST-1 domain shows a dramatic increase in phosphorylation. Mutations of sites in this region showed an increase in period and more stable FRQ, suggesting that phosphorylation of these residues is needed to promote turnover of FRQ. Further phosphorylation of FRQ, predominantly in residues specific to the long FRQ isoform, occurs late in the cycle, and mutations in this region result in a longer period, suggesting a role in promoting turnover (Baker et al. 2009).

Beyond stability, **FRQ structure** is predicted to be affected directly by the phosphorylation of FRQ protein (Querfurth et al. 2011). Hyperphosphorylation of FRQ at many sites causes a conformational change caused by charge-charge repulsion. In the hypophosphorylated state, FRQ is in a closed conformation, which opens on increasing phosphorylation, to reveal a degradation signal in the middle portion of FRQ. The key regulation site to this opening is in the N-terminal domain of FRQ; new FRQ adopts preferentially the closed conformation with the positively charged N-terminal domain interacting with the negatively charged remainder portion of the protein. The N-terminal domain of FRQ is progressively phosphorylated, which lowers the pI of the domain, increasing negative surface charge of the N-terminal domain and weakening the interaction with the negatively charged middle and C-terminal domains (Querfurth et al. 2011). While in some ways appealing, this model suffers from the fact that N-terminal phosphorylations were previously shown to be among the last modifications during the circadian cycle rather than among the first (Baker et al. 2009).

Phosphorylation of a protein to this extent, around 100 distinct modifications, needs a complex network of **kinases and phosphatases** for proper modification. Kinases known to play

a role in the clock include casein kinases 1 and 2 (CK1a and CK2), a *Neurospora* homolog of checkpoint kinase-2 (PRD-4), as well as CAMK-1 and basophilic protein kinase A (Klengel et al. 2005); FRQ has been shown to have direct physical interaction with CK1a, CK2, and PRD-4 (Baker et al. 2012; Diernfellner and Schafmeier 2011). CK1a interacts with FRQ via two FRQ/CK1a interacting domains (FCDs), and this interaction not only catalyzes the phosphorylation of FRQ (as many as 41 times), leading to FRQ degradation, but also may catalyze the phosphorylation of the WCC (He et al. 2006; Querfurth et al. 2011), leading to the hypothesis that FRQ acts as a scaffold for major components of the clock. The interaction between CK2 and FRQ is significantly weaker than that of CK1a but plays a unique role in the clock in that the phosphorylations that are attributed to CK2 are involved in maintaining the temperature compensation function of the clock (Mehra et al. 2009).

Several phosphatases are known to be involved in the clock as well, including protein phosphatase-1 (PP1), PP2a, and PP4. Phosphatases play a role in everything from regulating FRQ stability to influencing *frq* transcription, to phosphorylation of the WCC and affecting WCC subcellular localization (Baker et al. 2012).

d) FRQ Degradation

As described previously, proper phosphorylation of FRQ has been shown to regulate the degradation of FRQ as well as affect the function of the clock. But, there are other posttranslational modifications that affect the degradation of FRQ, including **ubiquitination** (He and Liu 2005). One gene that plays a role in ubiquitination is the F-box/WD40 repeat-containing protein FWD-1. FWD-1 has been shown to directly interact with FRQ, particularly the phosphorylated form, and is required for the proper degradation of FRQ. It is predicted that phosphorylated FRQ is a substrate for an FWD-1-containing SCF-type ubiquitin ligase complex, and moreover, that the SCF complex can recognize different phosphorylated motifs within FRQ. Increase in FRQ phosphorylation increases the number of potential

FWD-1-binding sites and its overall affinity toward FWD-1 (He et al. 2003). Progressive phosphorylation of FRQ may be a dynamic process that fine-tunes the stability of FRQ through its role in the ubiquitination of FRQ, and this determines the period of the clock. Beyond the FWD-1 role in ubiquitination, it is believed that there may be other FRQ mechanisms of degradation (He et al. 2003).

2. The Positive Arm: The White Collar Complex

Although the WCC was extensively described previously, it is also known to play a major role in addition to its photoreceptor function in *Neurospora*; the WCC is the positive arm of the clock. Knockouts of either of the White Collar genes eliminate clock function (Crosthwaite et al. 1997). The WCC complex binds directly to the *frq* promoter and induces expression of *frq* mRNA (Froehlich et al. 2002). WCC activity is then inhibited when a direct interaction with the FFC causes the complex constituents to be phosphorylated. Individually, WC-1 is necessary for the interaction between the WCC and FRQ. The amount of WC-1 protein also cycles, although this rhythm is not necessary for the clock, and WC-1 is stabilized by WC-2. WC-2 is also necessary for interaction between the WCC and FRQ, shows neither mRNA nor protein rhythms, and is both positively regulated by FRQ and negatively regulated by WC-1 (Liu et al. 2003). Interestingly, WC-1 can be found at the FRQ promoter throughout the day, even when it is inactivated, whereas WC-2 binds cyclically (Belden et al. 2007b).

a) Rhythmic Phosphorylation of the White Collar Complex

Like FRQ, both WC-1 and WC-2 are phosphorylated in a circadian manner, and this phosphorylation regulates the activity of the complex. Direct phosphorylation of WC-2 is rhythmic through the circadian day, and this phosphorylation regulates the binding of the WCC to DNA. Only one site has been identified on WC-2, but it has been suggested that there are many more that could affect both the period

and the stability of the protein (Sancar et al. 2009). WC-1 is also circadianly phosphorylated; phosphorylation of some sites near the Zn-finger DNA binding domain is believed to regulate the ability of WC-1 to activate transcription (He et al. 2005), but otherwise the role of WC-1 phosphorylation is not well understood. What is known is that phosphorylation in the C-terminus of WC-1 is sequential and FRQ dependent and is required for negative feedback (Baker et al. 2012).

F. Clock Inputs and Outputs

1. Inputs

The *Neurospora* clock is designed to be sensitive to many environmental inputs. **Light induction** by the WCC (described previously) drives an increase in *frq* levels as well as entrainment and phase resetting. This effect on *frq* expression by light impacts the clock differently depending on the time in the cycle that the light is seen. At the time of low *frq* expression in the morning, *frq* levels that increase sharply in accordance with exposure to light will advance the clock to the time corresponding to the highest *frq* expression (mid-to late morning), while increasing *frq* levels during the time of declining *frq* (late subjective afternoon and into evening) will lead to phase delays as *frq* levels are forced to return to their maximum midday levels after light exposure (Crosthwaite et al. 1997).

Light input to the clock can be modified by VVD (described previously), which itself is clock regulated and is able to gate the light response on the clock (Heintzen et al. 2001). VVD inhibition of the WCC directs the clock to take its principal cues from the dusk transition as well as contributing to temperature compensation (Elvin et al. 2005; Hunt et al. 2007). Furthermore, VVD levels in the dark are able to inactivate any WCC induced by moonlight and keep the clock on pace in the brightest of nights (Malzahn et al. 2010).

Temperature has a great effect on the clock, both on entrainment and on period, and can be a stronger resetting cue than light. Higher temperatures induce higher levels of L-FRQ as well as higher levels of FRQ overall (Garceau et al. 1997; Liu et al. 1997). When shifting to higher temperatures, the FRQ levels at the shift are lower than the lowest FRQ levels at the higher temperature, so the clock will reset to subjective morning, the time of day corresponding to low FRQ (Liu et al. 1998). Finally, it has been hypothesized that **metabolism** can play a role in clock input via a feedback loop on the positive arm by CSP-1 (Sancar et al. 2012).

2. Outputs

The internal mechanism of the clock has to have a method to provide rhythmic information to regulate the cell. The primary output of the clock is through a subset of rhythmically expressed genes termed the **clock-controlled genes**, or ccgs. Depending on the method of experimentation, anywhere from 1 % to 15 % of the genome is considered to be circadianly regulated in *Neurospora* (Dong et al. 2008; Dunlap and Loros 2004). These genes are not all expressed at the same time but are actually staggered in their expression around the clock, although late night to morning genes are the most common. Not surprisingly, most ccgs are WCC driven, and although most WCC-driven/light-induced genes are also ccgs, there are some distinct subsets of WCC-driven genes in both the light and dark conditions, showing that distinct from its role in light response (described previously), the WCC can have a different set of target genes in the dark (Dunlap and Loros 2004).

Rhythmic gene regulation must occur via either transcription or mRNA turnover, and transcription seems to be the most common mechanism of regulation. The promoter of the well-studied CCG, *ccg-2*, has several regulatory regions that individually confer light, developmental, and circadian regulation. One such element, termed the activating circadian element (ACE), is sufficient to confer clock regulation on these promoters and has been seen in other clock promoters (Bell-Pedersen et al. 1996). The core ACE sequence is distinct from the core LRE sequence (discussed previously), and

both of these elements mediate clock control as they have been identified in some of the promoters of ccgs identified using microarrays. Some genes have neither element, suggesting additional clock control elements and the presence of hierarchical control, in which the oscillator directly regulates oscillator proximal controllers, which in turn regulate more downstream genes, as has been noted for light-responsive genes in *Neurospora* (Chen et al. 2009; Dunlap and Loros 2004).

In addition to transcriptional regulation of ccgs, it has recently been hypothesized that another level of regulation can occur when the FRQ-FRH complex interacts with mRNA and the exosome via an association with the TRAMP complex. This mechanism would allow for control of message stability rather than production (Guo et al. 2009). Beyond this, it has recently been shown that the mitogen-activated protein kinase (MAPK) pathways are regulated by the clock at the transcriptional level by the WCC (Bennett et al. 2013; Lamb et al. 2011, 2012). In addition, MAK-1 has been shown to be phosphorylated in a circadian manner, and its targets are ccgs, demonstrating the circadian clock signal can be propagated outside WCC regulation (Bennett et al. 2013).

G. Molecular Advancements in *Neurospora*

Although historically genetic manipulation of filamentous fungi was difficult, within the past decade methods have been developed to perfect gene replacements and knockouts in *Neurospora*. Wild-type *Neurospora* strains exhibit a low frequency of homologous recombination after transformation. However, *Neurospora* recipient strains in which ectopic insertions are virtually eliminated have been developed. Mutations of either of two *Neurospora* genes required for nonhomologous end-joining DNA repair (*mus-51* and *mus-52*) result in an increase in homologous recombination (Ninomiya et al. 2004). Homologous recombination rates are around 90 % in strains that are mutant at these loci. In addition to this advancement, recombinational cloning in

yeast is used to anneal polymerase chain reaction (PCR) fragments, which are synthesized individually with short overlapping ends by PCR and cotransformed into yeast for assembly (Colot et al. 2006). These generated fragments can then be transformed into *Neurospora* strains that are mutant at either of the *mus* loci, creating a streamlined and highly efficient system for gene replacements and knockouts.

In addition to the targeted gene placement system, the ability to insert single-copy DNA sequences into a known locus is useful. In *Neurospora*, integration into the *his-3* gene (replacing a nonfunctional mutant copy of *his-3* with the wild-type allele as well as the DNA sequence of interest) is the most frequently used method. A drawback, however, is that the strain must be backcrossed to obtain a homokaryon, thereby exposing duplicated sequences to RIP. In addition, the *his-3* mutant is a potentially limiting genetic background. To compensate for these limitations, a system has been developed using *csr-1* as a targetable locus. *csr-1* encodes cyclophilin, a gene that, when mutated, confers resistance to cyclosporin A, an antifungal agent (Bardiya and Shiu 2007; Hurley et al. 2012). The advantages of targeting to this locus are that there is a scorable phenotype (resistance to cyclosporin A) and that all transformants are homokaryotic (untransformed *csr-1*[+] nuclei are selected against based on their abilities to produce cyclosporin A–binding protein).

References

Aronson BD, Johnson KA, Loros JJ, Dunlap JC (1994) Negative feedback defining a circadian clock: autoregulation of the clock gene *frequency*. Science 263:1578–1584

Arpaia G, Cerri F, Baima S, Macino G (1999) Involvement of protein kinase C in the response of *Neurospora crassa* to blue light. Mol Gen Genet 262:314–322

Avalos J, Estrada AF (2010) Regulation by light in *Fusarium*. Fungal Genet Biol 47:930–938

Baker CL, Kettenbach AN, Loros JJ, Gerber SA, Dunlap JC (2009) Quantitative proteomics reveals a dynamic interactome and phase-specific phosphorylation in the *Neurospora* circadian clock. Mol Cell 34:354–363

Baker CL, Loros JJ, Dunlap JC (2012) The circadian clock of *Neurospora crassa*. FEMS Microbiol Rev 36:95–110

Ballario P, Vittorioso P, Magrelli A, Talora C, Cabibbo A, Macino G (1996) White collar-1, a central regulator of blue light responses in *Neurospora*, is a zinc finger protein. EMBO J 15:1650–1657

Bardiya N, Shiu PK (2007) Cyclosporin A-resistance based gene placement system for *Neurospora crassa*. Fungal Genet Biol 44:307–314

Bayram Ö, Biesemann C, Krappmann S, Galland P, Braus GH (2008a) More than a repair enzyme: *Aspergillus nidulans* photolyase-like CryA is a regulator of sexual development. Mol Biol Cell 19:3254–3262

Bayram O, Krappmann S, Ni M, Bok JW, Helmstaedt K, Valerius O, Braus-Stromeyer S, Kwon NJ, Keller NP, Yu JH, Braus GH (2008b) VelB/VeA/LaeA complex coordinates light signal with fungal development and secondary metabolism. Science 320:1504–1506

Bayram Ö, Braus GH, Fischer R, Rodriguez-Romero J (2010) Spotlight on *Aspergillus nidulans* photosensory systems. Fungal Genet Biol 47:900–908

Belden WJ, Larrondo LF, Froehlich AC, Shi M, Chen CH, Loros JJ, Dunlap JC (2007a) The band mutation in *Neurospora crassa* is a dominant allele of ras-1 implicating RAS signaling in circadian output. Genes Dev 21:1494–1505

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

Belden WJ, Lewis ZA, Selker EU, Loros JJ, Dunlap JC (2011) CHD1 remodels chromatin and influences transient DNA methylation at the clock gene frequency. PLoS Genet 7:e1002166

Bell-Pedersen D, Dunlap JC, Loros JJ (1996) Distinct *cis*-acting elements mediate clock, light, and developmental regulation of the *Neurospora crassa* eas (ccg-2) gene. Mol Cell Biol 16:513–521

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

Bieszke JA, Braun EL, Bean LE, Kang S, Natvig DO, Borkovich KA (1999) The nop-1 gene of *Neurospora crassa* encodes a seven transmembrane helix retinal-binding protein homologous to archaeal rhodopsins. Proc Natl Acad Sci U S A 96:8034–8039

Bieszke JA, Li L, Borkovich KA (2007) The fungal opsin gene nop-1 is negatively-regulated by a component of the blue light sensing pathway and influences conidiation-specific gene expression in *Neurospora crassa*. Curr Genet 52:149–157

Bluhm BH, Dunkle LD (2008) PHL1 of *Cercospora zeae-maydis* encodes a member of the photolyase/cryptochrome family involved in UV protection and fungal development. Fungal Genet Biol 45:1364–1372

Bluhm BH, Burnham AM, Dunkle LD (2010) A circadian rhythm regulating hyphal melanization in *Cercospora kikuchii*. Mycologia 102:1221–1228

Blumenstein A, Vienken K, Tasler R, Purschwitz J, Veith D, Frankenberg-Dinkel N, Fischer R (2005) The *Aspergillus nidulans* phytochrome FphA represses sexual development in red light. Curr Biol 15:1833–1838

Bobrowicz P, Pawlak R, Correa A, Bell-Pedersen D, Ebbole DJ (2002) The *Neurospora crassa* pheromone precursor genes are regulated by the mating type locus and the circadian clock. Mol Microbiol 45:795–804

Brenna A, Grimaldi B, Filetici P, Ballario P (2012) Physical association of the WC-1 photoreceptor and the histone acetyltransferase NGF-1 is required for blue light signal transduction in *Neurospora crassa*. Mol Biol Cell 23:3863–3872

Brown L (2004) Fungal rhodopsins and opsin-related proteins: eukaryotic homologues of bacteriorhodopsin with unknown functions. Photochem Photobiol Sci 3:555–565

Bruce VG, Weight F, Pittendrigh CS (1960) Resetting the sporulation rhythm in *Pilobolus* with short light flashes of high intensity. Science 131:728–730

Brunner M, Kaldi K (2008) Interlocked feedback loops of the circadian clock of *Neurospora crassa*. Mol Microbiol 68:255–262

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

Cha J, Yuan H, Liu Y (2011) Regulation of the activity and cellular localization of the circadian clock protein FRQ. J Biol Chem 286:11469–11478

Chen CH, Loros J (2009) *Neurospora* sees the light: light signaling components in a model system. Commun Integr Biol 2:448–451

Chen C, Ringelberg CS, Gross RH, Dunlap JC, Loros JJ (2009) Genome-wide analysis of light-inducible responses reveals hierarchical light signalling in *Neurospora*. EMBO J 28:1029–1042

Chen C, DeMay BS, Gladfelter AS, Dunlap JC, Loros JJ (2010) Physical interaction between VIVID and white collar complex regulates photoadaptation in *Neurospora*. Proc Natl Acad Sci U S A 107:16715–16720

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

Cheng P, Yang Y, Gardner KH, Liu Y (2002) PAS domain-mediated WC-1/WC-2 interaction is essential for maintaining the steady-state level of WC-1 and the function of both proteins in circa-

dian clock and light responses of *Neurospora*. Mol Cell Biol 22:517–524

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

Cheng P, He Q, Wang L, Liu Y (2005) Regulation of the *Neurospora* circadian clock by an RNA helicase. Genes Dev 19:234–241

Christie JM, Gawthorne J, Young G, Fraser NJ, Roe AJ (2012) LOV to BLUF: flavoprotein contributions to the optogenetic toolkit. Mol Plant 5:533–544

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

Collopy PD, Colot HV, Park G, Ringelberg C, Crew CM, Borkovich KA, Dunlap JC (2010) High-throughput construction of gene deletion cassettes for generation of *Neurospora crassa* knockout strains. Methods Mol Biol 638:33–40

Colot HV, Loros JJ, Dunlap JC (2005) Temperature-modulated alternative splicing and promoter use in the circadian clock gene *frequency*. Mol Biol Cell 16:5563–5571

Colot HV, Park G, Turner GE, Ringelberg C, Crew CM, Litvinkova L, Weiss RL, Borkovich KA, Dunlap JC (2006) A high-throughput gene knockout procedure for *Neurospora* reveals functions for multiple transcription factors. Proc Natl Acad Sci U S A 103:10352–10357

Corrochano LM (2007) Fungal photoreceptors: sensory molecules for fungal development and behavior. Photochem Photobiol Sci 6:725–736

Corrochano LM, Cerd-Olmedo E (1992) Sex, light and carotenes: the development of *Phycomyces*. Trends Genet 8:268–274

Corrochano LM, Garre V (2010) Photobiology in the zygomycota: multiple photoreceptor genes for complex responses to light. Fungal Genet Biol 47:893–899

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

Davis RH, Perkins DD (2002) Timeline: *Neurospora*: a model of model microbes. Nat Rev Genet 3:397–403

Diernfellner AC, Schafmeier T (2011) Phosphorylations: making the *Neurospora crassa* circadian clock tick. FEBS Lett 585:1461–1466

Diernfellner AC, Schafmeier T, Merrow MW, Brunner M (2005) Molecular mechanism of temperature sensing by the circadian clock of *Neurospora crassa*. Genes Dev 19:1968–1973

Diernfellner A, Colot HV, Dintsis O, Loros JJ, Dunlap JC, Brunner M (2007) Long and short isoforms of *Neurospora* clock protein FRQ support

temperature-compensated circadian rhythms. FEBS Lett 581:5759–5764

Diernfellner AC, Querfurth C, Salazar C, Hofer T, Brunner M (2009) Phosphorylation modulates rapid nucleocytoplasmic shuttling and cytoplasmic accumulation of *Neurospora* clock protein FRQ on a circadian time scale. Genes Dev 23:2192–2200

Dong W, Tang X, Yu Y, Nilsen R, Kim R, Griffith J, Arnold J, Schuttler HB (2008) Systems biology of the clock in *Neurospora crassa*. PLoS One 3:e3105

Dunlap JC (1999) Molecular bases for circadian clocks. Cell 96:271–290

Dunlap JC, Loros JJ (2004) The *Neurospora* circadian system. J Biol Rhythms 19:414–424

Dunlap JC, Loros JJ (2006) How fungi keep time: circadian system in *Neurospora* and other fungi. Curr Opin Microbiol 9:579–587

Elvin M, Loros JJ, Dunlap JC, Heintzen C (2005) The PAS/LOV protein VIVID supports a rapidly damp-ened daytime oscillator that facilitates entrainment of the *Neurospora* circadian clock. Genes Dev 19:2593–2605

Estrada AF, Avalos J (2009) Regulation and targeted mutation of *opsA*, coding for the NOP-1 opsin orthologue in *Fusarium fujikuroi*. J Mol Biol 387:59–73

Franchi L, Fulci V, Macino G (2005) Protein kinase C modulates light responses in *Neurospora* by regulating the blue light photoreceptor WC-1. Mol Microbiol 56:334–345

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

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

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

Froehlich AC, Chen CH, Belden WJ, Madeti C, Roenneberg T, Merrow M, Loros JJ, Dunlap JC (2010) Genetic and molecular characterization of a cryptochrome from the filamentous fungus *Neurospora crassa*. Eukaryot Cell 9:738–750

Galagan JE, Calvo SE, Borkovich KA, Selker EU, Read ND, Jaffe D, FitzHugh W, Ma LJ, Smirnov S, Purcell S, Rehman B, Elkins T, Engels R, Wang S, Nielsen CB, Butler J, Endrizzi M, Qui D, Ianakiev P, Bell-Pedersen D, Nelson MA, Werner-Washburne M, Selitrennikoff CP, Kinsey JA, Braun EL, Zelter A, Schulte U, Kothe GO, Jedd G, Mewes W, Staben C, Marcotte E, Greenberg D, Roy A, Foley K, Naylor J, Stange-Thomann N, Barrett R, Gnerre S, Kamal M, Kamvysselis M, Mauceli E, Bielke C, Rudd S, Frishman D, Krystofova S, Rasmussen C, Metzenberg RL, Perkins DD, Kroken S, Cogoni C, Macino G,

Catcheside D, Li W, Pratt RJ, Osmani SA, DeSouza CP, Glass L, Orbach MJ, Berglund JA, Voelker R, Yarden O, Plamann M, Seiler S, Dunlap J, Radford A, Aramayo R, Natvig DO, Alex LA, Mannhaupt G, Ebbole DJ, Freitag M, Paulsen I, Sachs MS, Lander ES, Nusbaum C, Birren B (2003) The genome sequence of the filamentous fungus *Neurospora crassa*. Nature 422:859–868

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

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

Gooch VD, Mehra A, Larrondo LF, Fox J, Touroutou-toudis M, Loros JJ, Dunlap JC (2008) Fully codon-optimized luciferase uncovers novel temperature characteristics of the *Neurospora* clock. Eukaryot Cell 7:28–37

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

Grimaldi B, Coiro P, Filetici P, Berge E, Dobosy JR, Freitag M, Selker EU, Ballario P (2006) The *Neurospora crassa* White Collar-1 dependent blue light response requires acetylation of histone H3 lysine 14 by NGF-1. Mol Biol Cell 17:4576–4583

Guo J, Cheng P, Yuan H, Liu Y (2009) The exosome regulates circadian gene expression in a posttranscriptional negative feedback loop. Cell 138:1236–1246

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

He Q, Liu Y (2005) Degradation of the *Neurospora* circadian clock protein FREQUENCY through the ubiquitin-proteasome pathway. Biochem Soc Trans 33:953–956

He Q, Cheng P, Yang Y, Wang L, Gardner KH, Liu Y (2002) White collar-1, a DNA binding transcription factor and a light sensor. Science 297:840–843

He Q, Cheng P, Yang Y, Yu H, Liu Y (2003) FWD1-mediated degradation of FREQUENCY in *Neurospora* establishes a conserved mechanism for circadian clock regulation. EMBO J 22:4421–4430

He Q, Shu H, Cheng P, Chen S, Wang L, Liu Y (2005) Light-independent phosphorylation of WHITE COLLAR-1 regulates its function in the Neurospora circadian negative feedback loop. J Biol Chem 280:17526–17532

He Q, Cha J, Lee HC, Yang Y, Liu Y (2006) CKI and CKII mediate the FREQUENCY-dependent phosphorylation of the WHITE COLLAR complex to close the *Neurospora* circadian negative feedback loop. Genes Dev 20:2552–2565

Heintzen C, Liu Y (2007) The *Neurospora crassa* circadian clock. Adv Genet 58:25–66

Heintzen C, Loros JJ, Dunlap JC (2001) The PAS protein VIVID defines a clock-associated feedback loop that represses light input, modulates gating, and regulates clock resetting. Cell 104:453–464

Hitomi K, Okamoto K, Daiyasu H, Miyashita H, Iwai S, Toh H, Ishiura M, Todo T (2000) Bacterial cryptochrome and photolyase: characterization of two photolyase-like genes of *Synechocystis* sp PCC6803. Nucleic Acids Res 28:2353–2362

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

Hunt SM, Elvin M, Crosthwaite SK, Heintzen C (2007) The PAS/LOV protein VIVID controls temperature compensation of circadian clock phase and development in *Neurospora crassa*. Genes Dev 21:1964–1974

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

Hurley JM, Chen CH, Loros JJ, Dunlap JC (2012) Light-inducible system for tunable protein expression in *Neurospora crassa*. G3 2:1207–1212

Idnurm A, Heitman J (2005a) Light controls growth and development via a conserved pathway in the fungal kingdom. PLoS Biol 3:e95

Idnurm A, Heitman J (2005b) Photosensing fungi: phytochrome in the spotlight. Curr Biol 15:R829–R832

Idnurm A, Verma S, Corrochano LM (2010) A glimpse into the basis of vision in the kingdom Mycota. Fungal Genet Biol 47:881–892

Karniol B, Wagner JR, Walker JM, Vierstra RD (2005) Phylogenetic analysis of the phytochrome superfamily reveals distinct microbial subfamilies of photoreceptors. Biochem J 392:103–116

Klein RM, Klein DT (1962) Interaction of ionizing and visible radiation in mutation induction in *Neurospora crassa*. Am J Bot 49:870–874

Klengel T, Liang WJ, Chaloupka J, Ruoff C, Schröppel K, Naglik JR, Eckert SE, Mogensen EG, Haynes K, Tuite MF, Levin LR, Buck J, Mühlschlegel FA (2005) Fungal adenylyl cyclase integrates CO_2 sensing with cAMP signaling and virulence. Curr Biol 15:2021–2026

Kramer C, Loros JJ, Dunlap JC, Crosthwaite SK (2003) Role for antisense RNA in regulating circadian clock function in *Neurospora crassa*. Nature 421:948–952

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

Lamb TM, Goldsmith CS, Bennett L, Finch KE, Bell-Pedersen D (2011) Direct transcriptional control

of a p38 MAPK pathway by the circadian clock in *Neurospora crassa*. PLoS One 6:e27149

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

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

Lee K, Dunlap JC, Loros JJ (2003) Roles for WHITE COLLAR-1 in circadian and general photoperception in *Neurospora crassa*. Genetics 163:103–114

Lee K, Singh P, Chung W, Ash J, Kim TS, Hang L, Park S (2006) Light regulation of asexual development in the rice blast fungus, *Magnaporthe oryzae*. Fungal Genet Biol 43:694–706

Lewis ZA, Correa A, Schwerdtfeger C, Link KL, Xie X, Gomer RH, Thomas T, Ebbole DJ, Bell-Pedersen D (2002) Overexpression of White Collar-1 (WC-1) activates circadian clock-associated genes, but is not sufficient to induce most light-regulated gene expression in *Neurospora crassa*. Mol Microbiol 45:917–931

Linden H, Macino G (1997) White Collar 2, a partner in blue-light signal transduction, controlling expression of light-regulated genes in *Neurospora crassa*. EMBO J 16:98–109

Linden H, Ballario P, Macino G (1997) Blue light regulation in *Neurospora crassa*. Fungal Genet Biol 22:141–150

Liu Y, Bell-Pedersen D (2006) Circadian rhythms in *Neurospora crassa* and other filamentous fungi. Eukaryot Cell 5:1184–1193

Liu Y, Garceau NY, Loros JJ, Dunlap JC (1997) Thermally regulated translational control of FRQ mediates aspects of temperature responses in the *Neurospora* circadian clock. Cell 89:477–486

Liu Y, Merrow M, Loros JJ, Dunlap JC (1998) How temperature changes reset a circadian oscillator. Science 281:825–829

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

Liu Y, He Q, Cheng P (2003) Photoreception in *Neurospora*: a tale of two White Collar proteins. Cell Mol Life Sci 60:2131–2138

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

Loros JJ, Feldman JF (1986) Loss of temperature compensation of circadian period length in the *frq-9* mutant of *Neurospora crassa*. J Biol Rhythms 1:187–198

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

Losi A (2007) Flavin-based blue-light photosensors: a photobiophysics update. Photochem Photobiol 83:1283–1300

Losi A, Gärtner W (2011) Old chromophores, new photoactivation paradigms, trendy applications: flavins in blue light-sensing photoreceptors. Photochem Photobiol 87:491–510

Lukens RJ (1965) Reversal by red light of blue light inhibition of sporulation in *Alternaria solani*. Phytopathology 55:1032

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

Malzahn E, Ciprianidis S, Kldi K, Schafmeier T, Brunner M (2010) Photoadaptation in *Neurospora* by competitive interaction of activating and inhibitory LOV domains. Cell 142:762–772

McClung CR, Fox BA, Dunlap JC (1989) The *Neurospora* clock gene frequency shares a sequence element with the Drosophila clock gene period. Nature 339:558–562

Mehra A, Shi M, Baker CL, Colot HV, Loros JJ, Dunlap JC (2009) A role for casein kinase 2 in the mechanism underlying circadian temperature compensation. Cell 137:749–760

Merrow MW, Garceau NY, Dunlap JC (1997) Dissection of a circadian oscillation into discrete domains. Proc Natl Acad Sci U S A 94:3877–3882

Mooney JL, Yager LN (1990) Light is required for conidiation in *Aspergillus nidulans*. Genes Dev 4:1473–1482

Morgan LW, Greene AV, Bell-Pedersen D (2003) Circadian and light-induced expression of luciferase in *Neurospora crassa*. Fungal Genet Biol 38:327–332

Ninomiya Y, Suzuki K, Ishii C, Inoue H (2004) Highly efficient gene replacements in *Neurospora* strains deficient for nonhomologous end-joining. Proc Natl Acad Sci U S A 101:12248–12253

Olmedo M, Ruger-Herreros C, Luque EM, Corrochano LM (2010) A complex photoreceptor system mediates the regulation by light of the conidiation genes *con-10* and *con-6* in *Neurospora crassa*. Fungal Genet Biol 47:352–363

Pittendrigh CS, Bruce VG, Rosensweig NS, Rubin ML (1959) Growth patterns in *Neurospora*: a biological clock in *Neurospora*. Nature 184:169–170

Purschwitz J, Müller S, Kastner C, Schöser M, Haas H, Espeso EA, Atoui A, Calvo AM, Fischer R (2008) Functional and physical interaction of blue- and red-light sensors in *Aspergillus nidulans*. Curr Biol 18:255–259

Purschwitz J, Müller S, Fischer R (2009) Mapping the interaction sites of *Aspergillus nidulans* phytochrome FphA with the global regulator VeA and the white collar protein LreB. Mol Genet Genomics 281:35–42

Querfurth C, Diernfellner AC, Gin E, Malzahn E, Hofer T, Brunner M (2011) Circadian conformational change of the *Neurospora* clock protein FREQUENCY triggered by clustered hyperphosphorylation of a basic domain. Mol Cell 43:713–722

Rodriguez-Romero J, Hedtke M, Kastner C, Müller S, Fischer R (2010) Fungi, hidden in soil or up in the air: light makes a difference. Annu Rev Microbiol 64:585–610

Rosales-Saavedra T, Esquivel-Naranjo EU, Casas-Flores S, Martínez-Hernndez P, Ibarra-Laclette E, Cortes-Penagos C, Herrera-Estrella A (2006) Novel light-regulated genes in *Trichoderma atroviride*: a dissection by cDNA microarrays. Microbiology 152:3305–3317

Ruger-Herreros C, Rodríguez-Romero J, Fernndez-Barranco R, Olmedo M, Fischer R, Corrochano LM, Canovas D (2011) Regulation of conidiation by light in *Aspergillus nidulans*. Genetics 188:809–822

Ruiz-Roldan MC, Garre V, Guarro J, Marine M, Roncero MIG (2008) Role of the White Collar 1 photoreceptor in carotenogenesis, UV resistance, hydrophobicity, and virulence of *Fusarium oxysporum*. Eukaryot Cell 7:1227–1230

Ruoff P, Loros JJ, Dunlap JC (2005) The relationship between FRQ-protein stability and temperature compensation in the *Neurospora* circadian clock. Proc Natl Acad Sci U S A 102:17681–17686

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

Sancar G, Sancar C, Brunner M, Schafmeier T (2009) Activity of the circadian transcription factor White Collar Complex is modulated by phosphorylation of SP-motifs. FEBS Lett 583:1833–1840

Sancar G, Sancar C, Brunner M (2012) Metabolic compensation of the *Neurospora* clock by a glucose-dependent feedback of the circadian repressor CSP1 on the core oscillator. Genes Dev 26:2435–2442

Sano H, Kaneko S, Sakamoto Y, Sato T, Shishido K (2009) The basidiomycetous mushroom *Lentinula edodes white collar-2* homolog PHRB, a partner of putative blue-light photoreceptor PHRA, binds to a specific site in the promoter region of the *L edodes* tyrosinase gene. Fungal Genet Biol 46:333–341

Sargent ML, Briggs WR, Woodward DO (1966) Circadian nature of a rhythm expressed by an invertaseless strain of *Neurospora crassa*. Plant Physiol 41:1343–1349

Schneider MJ, Murray BJ (1979) Phytochrome mediation of uredospore germination in the fungus *Puccinia graminis*. Photochem Photobiol 29:1051–1052

Schuster A, Kubicek CP, Friedl MA, Druzhinina IS, Schmoll M (2007) Impact of light on *Hypocrea jecorina* and the multiple cellular roles of ENVOY in this process. BMC Genomics 8:449

Schwerdtfeger C, Linden H (2003) VIVID is a flavoprotein and serves as a fungal blue light photoreceptor for photoadaptation. EMBO J 22:4846–4855

Selby CP, Sancar A (2006) A cryptochrome/photolyase class of enzymes with single-stranded DNA-specific photolyase activity. Proc Natl Acad Sci U S A 103:17696–17700

Sharma AK, Foster KW (1997) Rhodopsin guides fungal phototaxis. Nature 387:465–466

Sharma AK, Spudich JL, Doolittle WF (2006) Microbial rhodopsins: functional versatility and genetic mobility. Trends Microbiol 14:463–469

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

Silva F, Torres-Martínez S, Garre V (2006) Distinct *white collar-1* genes control specific light responses in *Mucor circinelloides*. Mol Microbiol 61:1023–1037

Silva F, Navarro E, Peñaranda A, Murcia-Flores L, Torres-Martínez S, Garre V (2008) A RING-finger protein regulates carotenogenesis via proteolysis-independent ubiquitylation of a White collar-1-like activator. Mol Microbiol 70:1026–1036

Smith KM, Sancar G, Dekhang R, Sullivan CM, Li S, Tag AG, Sancar C, Bredeweg EL, Priest HD, McCormick RF, Thomas TL, Carrington JC, Stajich JE, Bell-Pedersen D, Brunner M, Freitag M (2010) Transcription factors in light and circadian clock signaling networks revealed by genomewide mapping of direct targets for *Neurospora* white collar complex. Eukaryot Cell 9:1549–1556

Tan KK (1974) Red-far-red reversible photoreaction in the recovery from blue-light inhibition of sporulation in *Botrytis cinerea*. J Gen Microbiol 82:201–202

Tang CT, Li S, Long C, Cha J, Huang G, Li L, Chen S, Liu Y (2009) Setting the pace of the *Neurospora* circadian clock by multiple independent FRQ phosphorylation events. Proc Natl Acad Sci U S A 106:10722–10727

Tisch D, Schmoll M (2010) Light regulation of metabolic pathways in fungi. Appl Microbiol Biotechnol 85:1259–1277

Veluchamy S, Rollins JA (2008) A CRY-DASH-type photolyase/cryptochrome from *Sclerotinia sclerotiorum* mediates minor UV-A-specific effects on development. Fungal Genet Biol 45:1265–1276

Waschuk SA, Bezerra AG, Shi L, Brown LS (2005) *Leptosphaeria* rhodopsin: bacteriorhodopsin-like proton pump from a eukaryote. Proc Natl Acad Sci U S A 102:6879–6883

Yamazaki Y, Kataoka H, Miyazakl A, Watanabe M, Ootakl T (1996) Action spectra for photoinhibition of sexual development in *Phycomyces blakesleeanus*. Photochem Photobiol 64:387–392

Zoltowski BD, Schwerdtfeger C, Widom J, Loros JJ, Bilwes AM, Dunlap JC, Crane BR (2007) Conformational switching in the fungal light sensor Vivid. Science 316:1054–1057