A Novel Cryptochrome-Dependent Oscillator in *Neurospora crassa*

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ABSTRACT Several lines of evidence suggest that the circadian clock is constructed of multiple molecular feedback oscillators that function to generate robust rhythms in organisms. However, while core oscillator mechanisms driving specific behaviors are well described in several model systems, the nature of other potential circadian oscillators is not understood. Using genetic approaches in the fungus *Neurospora crassa*, we uncovered an oscillator mechanism that drives rhythmic spore development in the absence of the well-characterized FRQ/WCC oscillator (FWO) and in constant light, conditions under which the FWO is not functional. While this novel oscillator does not require the FWO for activity, it does require the blue-light photoreceptor CRYPTOCHROME (CRY); thus, we call it the CRY-dependent oscillator (CDO). The CDO was uncovered in a strain carrying a mutation in *cog-1* (*cry-dependent oscillator gate-1*), has a period of ~1 day in constant light, and is temperature-compensated. In addition, *cog-1* cells lacking the circadian blue-light photoreceptor WC-1 respond to blue light, suggesting that alternate light inputs function in *cog-1* mutant cells. We show that the blue-light photoreceptors VIVID and CRY compensate for each other and for WC-1 in CRY-dependent oscillator light responses, but that WC-1 is necessary for circadian light entrainment.

CIRCADIAN clocks, composed of molecular transcription/translation-based feedback loop (TTFL) oscillators, generate daily rhythms in gene expression, physiology, and behavior in all kingdoms of life. The circadian clock provides a mechanism for organisms to anticipate cyclic changes in the environment to carry out specific tasks at advantageous times of the day. During investigations of clock mechanisms, several studies have revealed evidence for the existence of multiple autonomous oscillators in cells and/or tissues. First, there exist free-running rhythms of different periods in the same organism (Morse *et al.* 1994; Sai and Johnson 1999; Cambras *et al.* 2007). Second, residual rhythmicity is found in some strains defective in known oscillator components (Loros and Feldman 1986; Stanewsky *et al.* 1998; Emery *et al.* 2000; Collins *et al.* 2005). Third, some tissue-specific oscillators are constructed differently from core oscillators located in the brains of insects and animals (Stanewsky *et al.* 1998; Emery *et al.* 2000; Ivanchenko *et al.* 2001; Krishnan *et al.* 2001; Collins *et al.* 2005). Thus, multiple oscillators may exist both within, and among, cells in organisms with differentiated tissues. Furthermore, recent data revealed circadian rhythms in the oxidation state of highly conserved peroxiredoxin in the absence of transcription and thus a functional TTFL, adding additional levels of complexity to the circadian clock system (Edgar *et al.* 2012).

The fungus *Neurospora crassa* is a leading model for studying the circadian clock (Bell-Pedersen 2000; Heintzen and Liu 2007; Lakin-Thomas *et al.* 2011; Baker *et al.* 2012) and light signaling (Linden *et al.* 1997; Bell-Pedersen *et al.* 2001; Liu 2003; Merrow *et al.* 2006; Chen and Loros 2009). In *N. crassa*, the core FRQ/WCC oscillator (FWO) contains the negative elements FREQUENCY (FRQ) and FRQ-interacting RNA helicase (FRH), the blue-light photoreceptor WHITE COLLAR 1 (WC-1), and WHITE COLLAR 2 (WC-2). WC-1 and WC-2 form a complex, called the WCC that functions as a positive element in the oscillator loop. WCC binds the *frq* promoter

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and directly activates transcription of the *frq* gene (Froehlich *et al.* 2003). As FRQ protein accumulates, it interacts with itself and FRH (Cheng *et al.* 2001, 2005) and then binds to and promotes the phosphorylation and inactivation of the WCC (Schafmeier *et al.* 2005; He *et al.* 2006). Inhibition of WCC activity results in reduced *frq* transcription and FRQ protein levels. Once FRQ protein levels are sufficiently decreased, FRQ/FRH-directed inhibition of the activity of the WCC is released, and the cycle reactivates the next day. The FWO controls daily rhythms in expression of ~20% of the genome and overt rhythms in asexual spore development (conidiation) (Vitalini *et al.* 2006). The conidiation rhythm, typically measured using the race tube assay, has a period in constant dark (DD) conditions of ~22 h in wild-type strains (Loros and Dunlap 2001).

Several studies revealed that rhythms can persist in the absence of a functional FWO under certain growth conditions and/or in specific genetic backgrounds, providing evidence to suggest the existence of additional oscillators in *N. crassa* cells (Loros and Feldman 1986; Aronson *et al.* 1994; Merrow *et al.* 1999; Ramsdale and Lakin-Thomas 2000; Dragovic *et al.* 2002; Correa *et al.* 2003; Granshaw *et al.* 2003; Christensen *et al.* 2004; He *et al.* 2005; de Paula *et al.* 2006; Brody *et al.* 2010; Hunt *et al.* 2012). The term FLO (*frq*-less-oscillator) was coined to collectively describe these putative circadian and/or noncircadian oscillators (Iwasaki and Dunlap 2000). Indeed, in most cases, rhythms attributed to FLOs were shown to lack one or more of the three canonical clock properties, including the generation of a free-running rhythm of ~24 hr in the absence of environmental cues, entrainment of the free-running rhythm to 24 hr by environmental cues, and temperature compensation of the clock (Lakin-Thomas 2000; Baker *et al.* 2012). In entrainment of the clock, the period of the rhythm becomes equal, on average, to an imposed environmental cycle, and a unique stable phase relationship is established between the imposed environmental cycle and the entrained oscillator (Johnson *et al.* 2003). Synchronization is distinguished from entrainment in that the cycle output occurs in response to the stimulus in a set time frame and does not depend on the length of the imposed environmental cycle. Temperature compensation of the clock means that the rate is relatively independent of temperature within the physiological range, with a temperature coefficient (Q_{10}) near 1. The lack of full circadian properties of the FLOs led to suggestions that the FWO serves as a pacemaker in *N. crassa* cells, driving rhythms in downstream, so-called slave FLOs (Dunlap and Loros 2005). In this model, the FLOs are intrinsically rhythmic but require the FWO for full circadian properties. Furthermore, the number of FLOs in *Neurospora* cells is not known. While studies have been undertaken to identify molecular components of the FLOs (Lombardi *et al.* 2007; Shi *et al.* 2007; Yoshida *et al.* 2008; Schneider *et al.* 2009; Lakin-Thomas *et al.* 2011; Li *et al.* 2011; Hunt *et al.* 2012), little is understood regarding their nature and role in the circadian system.

In an attempt to identify key components of the FLOs, we carried out a genetic screen for mutations that enhance rhythmicity in strains that are deficient in both positive and negative components of the FWO. We identified a mutation called *cry-dependent oscillator gate*-1 (*cog-1*) that displayed robust rhythms in conidiation in constant light (LL), independently of the FWO. The oscillator controlling these rhythms fulfills two of the three criteria for a circadian oscillator: it free-runs under constant conditions with a period close to 1 day and is temperature-compensated. However, while *cog-1* mutant strains were synchronized by light/dark (LD) cycles independently of the photoreceptor and clock component WC-1, circadian entrainment in LD required WC-1. Finally, we show that the blue-light photoreceptor CRYPTOCHROME (CRY), a core component of the mammalian circadian oscillator (Griffin *et al.* 1999), is necessary for the novel oscillator activity in *Neurospora* cells in LL. We therefore call the oscillator the CRY-dependent oscillator (CDO),

Materials and Methods

Strains

The *N. crassa* strains used in this study are listed in Table 1, their periods under different growth conditions in Table 2, and growth rates of representative strains in Table 3. The *vvd* knockout (KO) strain was obtained from Christian Heintzen (Heintzen *et al.* 2001). The *Δwc-1::hph* strain was obtained from Jay Dunlap (Dartmouth Medical School), and *Δwc-1::bar* was generated in our lab (Bennett *et al.* 2013). The *cry* KO [Fungal Genetics Stock Center (FGSC) 12981] was generated by the *N. crassa* KO project (Colot *et al.* 2006) and obtained from the FGSC. All strains used in this study carry the *ras-1^{bd}* mutation, which clarifies the conidiation rhythm on race tubes (Sargent *et al.* 1966; Belden *et al.* 2007). The *ras-1^{bd}* strain serves as the clock wild-type (WT) control strain. To generate the *cog-1* mutation, a strain defective in the positive and negative arm of the FWO (*wc-2^{234W}*, *ras-1^{bd}*, *Δfrq*) was mutagenized by ultraviolet (UV) light according to standard procedures (Davis and De Serres 1970). The construction of double and triple photoreceptor deletion strains is described in Supporting Information, Figure S1, A and B. To complement *Δcry, cog-1* cells, a 4.9-kb PCR fragment containing the *cry* gene was cloned into pCR-BluntBar that contains the *bar* gene for selection and transformed to *Δcry, cog-1* cells using standard *Neurospora* transformation techniques (Pall and Brunelli 1993). PCR was used to verify the genotypes of double and triple photoreceptor mutant strains from genomic DNA isolated from 7-day-old slant cultures as described (Jin *et al.* 2007) (Figure S1C). The PCR primers used to verify the KO strains are listed in Table S1.

To determine if the *cog-1* mutation is dominant or recessive, a heterokaryon of strain *matA*, *arg-5*, *ras-1^{bd}* and *matA*, *trp-3*, *ras-1^{bd}*, *cog-1* was generated. Heterokaryons were selected by growth on minimal media as described (Davis and De Serres 1970). Heterokayotic strains with nuclear ratios of 1:1, as determined by plating on selective media, were examined for rhythmicity on race tubes.

Table 1 Strains used in this study

Strain	Genotype	Strain no.	Source/reference
WT	*matA*, *ras-1^{bd}*	DBP369	FGSC 1858
WT	*mat***a**, *ras-1^{bd}*	DBP368	FGSC 1859
cog-1	*matA*, *ras-1^{bd}*, *cog-1*	DBP694	This study
cog-1	*mat***a**, *ras-1^{bd}*, *cog-1*	DBP695	This study
Δ*frq*	*mat***a**, *ras-1^{bd}*, *frq^{10}*	DBP287	Aronson *et al.* (1994)
Δ*frq*	*matA*, *ras-1^{bd}*, *frq^{10}*	DBP776	FGSC 7490
Δ*frq*, *cog-1*	*matA*, *ras-1^{bd}*, *frq^{10}*, *cog-1*	DBP831	This study
Δ*wc-1*	*matA*, *ras-1^{bd}*, Δ*wc-1^{hyg}*	DBP580	Lee *et al.* (2003)
Δ*wc-1*, *cog-1*	*matA*, *ras-1^{bd}*, Δ*wc-1^{hyg}*, *cog-1*	DBP696	This study
Δ*wc-1^{bar}*	*matA*, *ras-1^{bd}*; Δ*wc-1^{bar}*	DBP1223	Bennett *et al.* (2013)
Δ*wc-1^{bar}*, *cog-1*	*mat***a**, *ras-1^{bd}*, Δ*wc-1^{bar}*, *cog-1*	DBP1369	This study (DBP1223 × DBP695)[a]
Δ*vvd*	*mat***a**, *ras-1^{bd}*, Δ*vvd^{hyg}*	DBP693	Heintzen *et al.* (2001)
Δ*vvd*	*matA*, *ras-1^{bd}*, Δ*vvd^{hyg}*	DBP1634	This study (DBP369 × DBP693)
Δ*vvd*, *cog-1*	*matA*, *ras-1^{bd}*, Δ*vvd^{hyg}*, *cog-1*	DBP1335	This study (DBP693 × DBP694)
Δ*vvd*, *cog-1*	*mat***a**, *ras-1^{bd}*, Δ*vvd^{hyg}*, *cog-1*	DBP1638	This study (DBP695 × DBP1335)
Δ*cry*	Δ*cry^{hyg}*, *mat***a**, *ras-1^{bd}*	DBP963	Laboratory stock
Δ*cry*, *cog-1*	Δ*cry^{hyg}*, *matA*, *ras-1^{bd}*, *cog-1*	DBP1022	This study (DBP963 × DBP694)
Δ*cry*, Δ*wc-1*	Δ*cry^{hyg}*, *matA*, *ras-1^{bd}*, Δ*wc-1^{bar}*	DBP1598	This study (DBP963 × DBP1223)
Δ*cry*, Δ*wc-1*	Δ*cry^{hyg}*, *mat***a**, *ras-1^{bd}*, Δ*wc-1^{bar}*	DBP1599	This study (DBP963 × DBP1223)
Δ*cry*, Δ*wc-1*, *cog-1*	Δ*cry^{hyg}*, *matA*, *ras-1^{bd}*, Δ*wc-1^{bar}*, *cog-1*	DBP1645	This study (DBP1022 × DBP1369)
Δ*cry*, Δ*vvd*	Δ*cry^{hyg}*, *mat***a**, *ras-1^{bd}*, Δ*vvd^{hyg}*	DBP1640	This study (DBP693 × DBP963)
Δ*cry*, Δ*vvd*	Δ*cry^{hyg}*, *matA*, *ras-1^{bd}*, Δ*vvd^{hyg}*	DBP1600	This study (DBP693 × DBP963)
Δ*cry*, Δ*vvd*, *cog-1*	Δ*cry^{hyg}*, *matA*, *ras-1^{bd}*, Δ*vvd^{hyg}*, *cog-1*	DBP1601	This study (DBP1022 × DBP1638)
Δ*vvd*, Δ*wc-1*	*matA*, *ras-1^{bd}*, Δ*vvd ^{hyg}*, Δ*wc-1^{bar}*	DBP1639	This study (DBP693 × DBP1223)
Δ*vvd*. Δ*wc-1*	*mat***a**, *ras-1^{bd}*, Δ*vvd^{hyg}*, Δ*wc-1^{bar}*	DBP1637	This study (DBP693 × DBP1223)
Δ*vvd*, Δ*wc-1*, *cog-1*	*matA*, *ras-1^{bd}*, Δ*vvd^{hyg}*, Δ*wc-1^{bar}*, *cog-1*	DBP1635	This study (DBP1335 × DBP1369)
Δ*vvd*, Δ*wc-1*, *cog-1*	*mat***a**, *ras-1^{bd}*, Δ*vvd^{hyg}*, Δ*wc-1^{bar}*, *cog-1*	DBP1636	This study (DBP1335 × DBP1369)
Δ*cry*, Δ*vvd*, Δ*wc-1*	*mat***a**, Δ*cry^{hyg}*, *ras-1^{bd}*, Δ*vvd^{hyg}*, Δ*wc-1^{bar}*	DBP1593	This study (DBP1599 × DBP1634)
Δ*cry*, Δ*vvd*, Δ*wc-1*	*matA*, Δ*cry^{hyg}*, *ras-1^{bd}*, Δ*vvd^{hyg}*, Δ*wc-1^{bar}*	DBP1592	This study (DBP1599 × DBP1634)
Δ*cry*, Δ*vvd*, Δ*wc-1*, *cog-1*	*matA*, Δ*cry^{hyg}*, *ras-1^{bd}*, Δ*vvd^{hyg}*, Δ*wc-1^{bar}*, *cog-1*	DBP1597	This study (DBP1022 × DBP1636)
Δ*cry*, Δ*vvd*, Δ*wc-1*, *cog-1*	*mat***a**, Δ*cry^{hyg}*, *ras-1^{bd}*, Δ*vvd^{hyg}*, Δ*wc-1^{bar}*, *cog-1*	DBP1596	This study (DBP1022 × DBP1636)

[a] Derived from the indicated cross (×) between lab strains.

Growth conditions

All vegetative cultures were maintained on 1× Vogel's, 2% glucose, minimal medium with the appropriate supplements as required (Vogel 1956; Davis and De Serres 1970). Sexual crosses were performed on Westergaard's crossing agar plates (Westergaard and Mitchell 1947). KO strains containing the *hph* marker were maintained on Vogel's minimal medium supplemented with 200 μg/ml hygromycin B (Sigma Aldrich, St. Louis). KO strains containing the *bar* gene were maintained on nitrate-free Vogel's medium supplemented with 250 μg/ml glufosinate (Sigma Aldrich). The composition of race tube media was 11.5 ml of 1× Vogel's, 0.1% glucose, 0.17% arginine, and 1.5% agar. After autoclaving, race tubes were allowed to dry for 7 days. The dried race tubes were inoculated with mycelia or conidia from 7-day-old slants and grown for 1 day at 25° and then transferred to the indicated conditions in Percival growth chambers (Perry, IA). The growth front was marked at the time of transfer. Light was from cool white fluorescent bulbs, unless otherwise indicated. Light intensity was measured with a VWR Scientific Dual Range Light Meter (Radnor, PA) and maintained at 1200 lx in all light experiments, unless otherwise indicated. To examine the effects of different wavelengths of light on the CDO, four different light sources (red: 60 lx; blue: 80 lx; green: 64 lx; white: 72 lx) were used in Percival incubators. Temperature recording in the incubators was accomplished using an EasyLog EL-USB thermometer (Lascar Electronics, Erie, PA). The growth fronts of the race tubes were marked at 24-hr intervals using a red light for cultures in DD and at lights on for cultures in LD cycles. Race tubes were scanned with an EPSON scanner (Long Beach, CA), and growth rates and periods were calculated. *n* is the number of conidial bands measured on replicate race tubes. The percentage rhythmic was calculated as the number of individual race tubes displaying at least five clear conidial bands for which we could measure period out of the total number of race tubes examined for each strain under a given growth condition.

Western blots

Protein was extracted from ground tissue (Garceau *et al.* 1997), and 100 μg of total protein was run on 10% SDS PAGE gels and transferred to polyvinylidene difluoride membranes (IPVH00010; Millipore, Billarica, MA). CRY protein was detected by immunoblotting with primary anti-CRY antibody (polyclonal, 1:1000 v/v, a gift from Jay Dunlap) and secondary goat anti-rabbit horseradish peroxide 2-conjugated antibody (diluted 1:20,000 v/v, 170-6515, Bio-Rad, Hercules, CA). Protein bands were visualized using a chemiluminescence

Table 2 Period of strains

	DD				LL			
	cog-1$^+$		*cog-1*$^-$		*cog-1*$^+$		*cog-1*$^-$	
Genotype	Period (hr)	% Rhythmic	Period (hr)	% Rhythmic	Period (hr)	% Rhythmic	Period (hr)	% Rhythmic
WT	22.3 ± 0.8[a] *n* = 60[b]	100	22.7 ± 0.5, *n* = 77	100	AR	0	17.1 ± 1.3, *n* = 194	100
Δ*wc-1*	AR	0	23.9 ± 1.3, *n* = 77	40	AR	0	25.1 ± 0.8, *n* = 191	100
Δ*frq*	AR	0	21.7 ± 2.7, *n* = 34	50	AR	0	23.0 ± 2.0, *n* = 80	75
Δ*cry*	22.2 ± 0.5, *n* = 60	100	22.4 ± 0.5, *n* = 60	100	AR	0	AR	0
Δ*vvd*	21.7 ± 0.4, *n* = 60	100	23.2 ± 0.9, *n* = 77	100	AR	0	12.9 ± 1.7, *n* = 47	100
Δ*vvd*, Δ*wc-1*	AR	0	21.3 ± 1.8, n = 44	60	AR	0	23.0 ± 1.3, *n* = 62	100
Δ*cry*, Δ*wc-1*	AR	0	AR	0	AR	0	AR	0
Δ*cry*, Δ*vvd*	22.2 ± 0.5, *n* = 60	100	22.2 ± 0.5, *n* = 60	100	AR	0	AR	0
Δ*cry*, Δ*vvd*, Δ*wc-1*	AR	0	AR	0	AR	0	AR	0

	LD 6:6		LD 9:9		LD 12:12		LD 14:14	
	cog-1$^+$ Period (h)	*cog-1*$^-$ Period (h)	*cog-1*$^+$ Period (h)	*cog-1*$^-$ Period (h)	*cog-1*$^+$ Period (h)	*cog-1*$^-$ Period (h)	*cog-1*$^+$ Period (h)	*cog-1*$^-$ Period (h)
WT	12.1 ± 0.1, *n* = 172	11.9 ± 0.1, *n* = 179	18.1 ± 0.1, *n* = 90	17.8 ± 0.3, *n* = 96	23.9 ± 0.2, *n* = 84	23.9 ± 0.2, *n* = 84	27.3 ± 0.5, *n* = 68	27.6 ± 0.4, *n* = 74
Δ*wc-1*	AR	11.9 ± 0.1, *n* = 126	AR	17.9 ± 0.2, *n* = 74	AR	23.8 ± 0.2, *n* = 61	AR	27.8 ± 0.2, *n* = 61
Δ*frq*	ND	ND	ND	ND	ND	ND	ND	ND
Δ*cry*	12.0 ± 0.1, *n* = 162	12.0 ± 0.2, *n* = 150	17.8 ± 0.3, *n* = 78	17.9 ± 0.1, *n* = 83	24.2 ± 0.2, *n* = 69	23.8 ± 0.3, *n* = 70	27.9 ± 0.4, *n* = 53	27.8 ± 0.3, *n* = 70
Δ*vvd*	12.0 ± 0.1, *n* = 168	11.9 ± 0.2, *n* = 190	17.8 ± 0.2, *n* = 84	17.8 ± 0.2, *n* = 108	23.6 ± 0.2, *n* = 72	23.5 ± 0.4, *n* = 99	27.8 ± 0.2, *n* = 62	27.8 ± 0.2, *n* = 72
Δ*vvd*, Δ*wc-1*	AR	12.0 ± 0.5, *n* = 74	AR	17.8 ± 0.3, *n* = 91	AR	23.9 ± 0.4, *n* = 60	AR	27.7 ± 0.5, *n* = 60
Δ*cry*, Δ*wc-1*	AR	12.0 ± 0.5, *n* = 68	AR	18.1 ± 0.5, *n* = 88	AR	24.0 ± 0.2, *n* = 63	AR	27.4 ± 1.7, *n* = 63
Δ*cry*, Δ*vvd*	12.0 ± 0.5, *n* = 155	12.1 ± 0.1, *n* = 132	17.8 ± 0.2, *n* = 101	17.8 ± 0.2, *n* = 88	23.6 ± 0.2, n = 60	23.5 ± 0.2, *n* = 60	27.9 ± 0.2, *n* = 60	28.0 ± 0.2, *n* = 60
Δ*cry*, Δ*vvd*, Δ*wc-1*	AR	AR	AR	AR	AR	AR	AR	AR

AR, arrhythmic; ND, not determined.
[a] Values represent mean ± SD.
[b] *n*, number of bands per strain used to calculate period.

Table 3 Growth rates of strains with various oscillator functions

	DD		LL	
Genotype	Growth rate (cm/day)	Oscillator function	Growth rate (cm/day)	Oscillator function
WT	4.0 ± 0.2[a]	FWO	4.0 ± 0.2	None
cog-1	3.3 ± 0.3	FWO, CDO	3.5 ± 0.2	CDO
Δ*wc-1*	4.3 ± 0.4	None	4.3 ± 0.3	None
Δ*wc-1*, *cog-1*	3.7 ± 0.2	CDO	3.2 ± 0.2	CDO
Δ*cry*	3.8 ± 0.4	FWO	4.5 ± 0.4	None
Δ*cry*, *cog-1*	3.8 ± 0.4	FWO	4.8 ± 0.4	None

[a] Values are mean ± SD; $n \geq 12$.

kit (SuperSignal West Femto, ThermoFischer Scientific, Waltham, MA) according to the manufacturer's instructions. The membranes were subsequently stained with amido black [0.1% amido black (Sigma-Aldrich), 10% acetic acid, 25% isopropanol] for protein-level normalization.

Results

CDO functions independently of the FWO

To identify components of *N. crassa* FLOs, we carried out a screen for mutations that would enhance developmental rhythms on race tubes in strains that were defective in both the positive and negative arms of the FWO. This approach ruled out any possible residual activity from the FWO. The *wc-2*^{234W}, Δ*frq* strain was mutagenized by UV light to 50% survival, and a mutant strain, originally identified as Light Mutant 1 and renamed *cog-1*, that had robust conidiation rhythms in LL was identified (Figure 1 and Table 2). The *cog-1* mutant strain was outcrossed multiple times to the clock WT strain *ras-1*^{bd} to isolate the *cog-1* mutation from all other mutations. The *cog-1* mutant phenotype segregated with a 1:1 ratio in crosses, suggesting that the rhythmic phenotype is due to a single gene mutation. The resulting *ras-1*^{bd}, *cog-1* strain (called *cog-1*) was used for all subsequent analyses.

To confirm that the *cog-1* mutation rescued developmental rhythms in strains that lack a functional FWO, the *cog-1* strain was crossed to *frq*-null (Δ*frq*) and *wc-1*-null (Δ*wc-1*) strains. Progeny that were Δ*frq*, *cog-1* and Δ*wc-1*, *cog-1* were isolated and examined, along with control sibling strains, for rhythmicity in LL (Figure 2 and Table 2). Consistent with previous observations, the WT strain was arrhythmic in LL (Crosthwaite *et al.* 1995; Elvin *et al.* 2005). Strains that lacked FWO components were also arrhythmic in LL. However, rhythms in conidial development within the circadian range were observed in all strains that contained the *cog-1* mutation. In the absence of the FWO components, the period of the *cog-1* rhythm was almost 24 hr, whereas *cog-1* strains with a functional FWO had a shorter period. These data suggested that the FWO and the CDO genetically interact in LL. While all *cog-1* and Δ*wc-1*, *cog-1* replicate race-tube cultures displayed rhythms in LL (100%), the number of rhythmic race tubes of Δ*frq*, *cog-1* was reduced to 75% (Table 2).

CDO free-runs under constant conditions and is temperature-compensated

Using heterokaryon analyses, we determined that the *cog-1* mutation was recessive (Figure S2), suggesting *cog-1* is a loss-of-function mutation that uncovers an autonomous CDO. To test if the CDO has properties of a circadian oscillator, *cog-1* strains were further examined for free-running rhythms in DD, light entrainment, and temperature compensation. Consistent with the LL data, and independent of the FWO, strains containing the *cog-1* mutation are rhythmic in DD (Figure 3A). However, the robustness of the rhythms (% rhythmic) in *cog-1* strains that also lacked components of the FWO was decreased in DD, as compared to LL (Table 2), and robustness of the rhythms in *cog-1* strains was independent of whether the clock was initially synchronized in these cells by a light and/or a temperature transition (data not shown). Together, these data suggest that in DD the FWO overrides and/or enhances the CDO rhythms. Consistent with the FWO overriding the CDO in DD, the *cog-1* period in DD was similar to the WT strain (22 hr), whereas when only the CDO is expressed (*cog-1* in LL), the period is ~17 hr (Figure 2).

To determine if the CDO rhythm is temperature compensated, we assayed the developmental rhythm in *cog-1* and Δ*wc-1*, *cog-1* strains in LL, conditions under which the FWO is not functional but the CDO rhythm on race tubes is pronounced. While most biochemical reactions are temperature-dependent, with Q_{10} temperature coefficient values of 2–3, temperature-compensated clocks have Q_{10} values of between 0.8 and 1.4 (Sweeney and Hastings 1960). The period of the rhythm differed between the *cog-1* and Δ*wc-1*, *cog-1* strains as expected; however, the Q_{10} values measured for both strains between 17° and 27° was 1.2, confirming that the CDO is temperature-compensated (Figure 3B).

The third defining property of a circadian clock is entrainment. The best way to demonstrate entrainment is to examine environmental cycles with periods that are not equal to 24 hr (Johnson *et al.* 2003). If the rhythm entrains to environmental cycles it will display a period that equals the length of the environmental cycle and have stable phase angles that differ in different environmental cycle lengths. If instead, the rhythms display periods equal to the cycle length, but have similar phase angles in different cycles, then the rhythms are synchronized (driven), not entrained, by the imposing cycle.

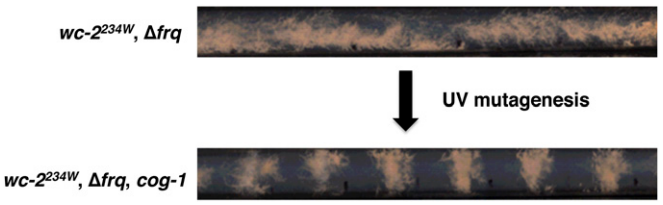

Figure 1 Identification of the *cog-1* mutant strain that uncovers the CDO. A FWO mutant strain (*wc-2*^{234W}, Δ*frq*) was mutagenized by UV light, and the resulting strains were assayed in LL (1200 lx) at 25° for rescue of developmental rhythms on race tubes. The *cog-1* mutation displayed rhythmic development under these conditions, whereas the parental strain was arrhythmic. The direction of growth is from left to right, and the black marks correspond to 24 hr of growth.

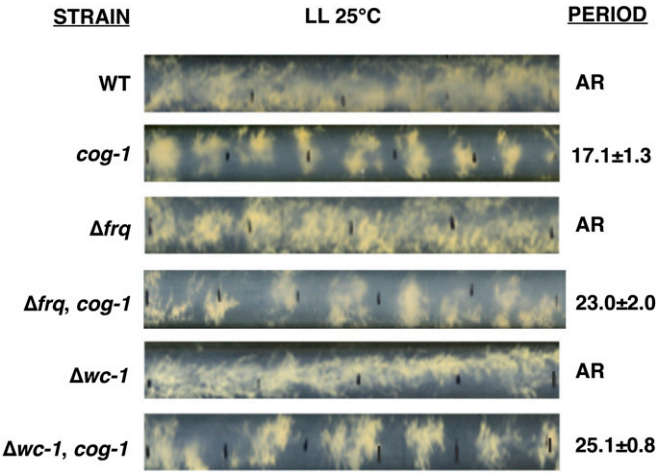

Figure 2 The *cog-1* mutation restores rhythms in strains that lack a functional FWO in LL. Representative photographs of race tubes of the indicated strains in LL (1200 lx) at 25°. Black lines on the tubes represent 24-hr growth fronts. The period of the rhythmic strains is shown on the right (±SD, $n \geq 60$). "AR" indicates that the strain was arrhythmic.

Thus, to investigate entrainment of the CDO, we examined strains that display the CDO in different LD cycle lengths (Figure 4A). For WT, *cog-1*, and Δ*wc-1*, *cog-1* strains, the period of the rhythm matched the imposed LD cycle length. Strains with functional WC-1 displayed conidiation in different phases in LD cycles of different duration, consistent with entrainment. However, the phase angles in Δ*wc-1*, *cog-1* strains stayed relatively constant, with conidiation occurring just after lights on in the different LD cycles, indicative of an LD-driven rhythm (Figure 4B).

To rule out the possibility that the synchronization of Δ*wc-1*, *cog-1* strains in LD cycles was due to changes in temperature, rather than to changes in light, we carefully monitored the temperature of the incubators with a temperature-recording device. The maximum temperature variance recorded was an increase in temperature in the light of 0.5° in each cycle. A 1° temperature change was not sufficient to drive the developmental rhythm in Δ*wc-1* cells (Figure S3). Therefore, we can rule out the possibility that the rhythms observed in Δ*wc-1*, *cog-1* strains in LD cycles was due to 0.5° cycles in the incubators. Together, these data demonstrate that WC-1 is required for stable entrainment of the circadian clock to light in *N. crassa* and that photoreceptors other than WC-1 are capable of responding to light in Δ*wc-1*, *cog-1* cells.

CRY is essential for CDO rhythms

The *N. crassa* clock is known to be responsive to only blue light (Sargent and Briggs 1967). Consistent with this, the response of the CDO to light is restricted to the blue-light range (Figure S4). Therefore, to identify the photoreceptors responsible for light responses in the Δ*wc-1*, *cog-1* strain, we crossed the *cog-1* mutation to strains carrying deletions of the other known blue-light photoreceptors in *N. crassa*, VVD (Zoltowski *et al.* 2007) and CRY (Froehlich *et al.* 2010) to generate Δ*vvd*, *cog-1*, and Δ*cry*, *cog-1* strains. These strains, and the control siblings, were assayed for rhythms in LL and for entrainment in LD cycles at 25° (Figure 5). In the Δ*vvd*, *cog-1* strain in LL, rhythms in development were observed (Figure 5A); however, the period of the rhythm was reduced, and more variable, as compared to *cog-1* (compare Figure 5A to Figure 2). The Δ*vvd*, *cog-1* strains also entrained to LD cycles (Figure 5, B and C). Surprisingly, in Δ*cry*, *cog-1* strains grown in LL, the developmental rhythms were abolished. The rhythm was rescued by ectopic transformation of the *cry* gene into the Δ*cry*, *cog-1* strain (Δ*cry*::*cry*, *cog-1*) (Figure 5A). In addition, the Δ*cry*, *cog-1* strain entrained normally to LD cycles (Figure 5, B and C). Entrainment of Δ*vvd*, *cog-1* and Δ*cry*, *cog-1* strains to LD cycles is consistent with the presence of a functional FWO in these cells. Together, these data demonstrated that CRY is necessary for function of the CDO in LL and that both CRY and VVD are dispensable for light responses in *cog-1* cells. However, the WC-1 photoreceptor likely compensated for the loss of individual VVD or CRY photoreceptors in these strains.

To determine if light responses in the Δ*vvd*, *cog-1* and Δ*cry*, *cog-1* strains are due to the presence of WC-1, we generated double photoreceptor mutants in the *cog-1* mutant background and examined rhythms in LL and responses to light in LD cycles at 25°. Consistent with a requirement for CRY in CDO function, all of the *cog-1* double photoreceptor mutants that harbor *cry* deletions were arrhythmic in LL (Figure 6A and Table 2). Of the double photoreceptor mutants, only the Δ*vvd*, Δ*wc-1*, *cog-1* strain displayed rhythms in LL. The period of the rhythm of Δ*vvd*, Δ*wc-1*, *cog-1* cells was similar to Δ*wc-1*, *cog-1* (Figure 2), but was significantly longer than Δ*vvd*, *cog-1* (Figure 5A and Table 2), suggesting that while VVD may modify the period of the CDO, VVD is not necessary for CDO rhythms. In LD cycles, light responses were observed in all of the double photoreceptor mutant strains that harbored the *cog-1* mutation (Figure 6B), and, consistent with our earlier results, entrainment to light required WC-1, but not VVD and CRY (Figure 6C).

We next generated strains that lacked all three blue-light photoreceptors, with and without the *cog-1* mutation, and assayed the developmental rhythm in LL and LD cycles at 25° (Figure 6, A and B; Table 2). Independent of the *cog-1* mutations, the triple photoreceptor deletion mutant strains were

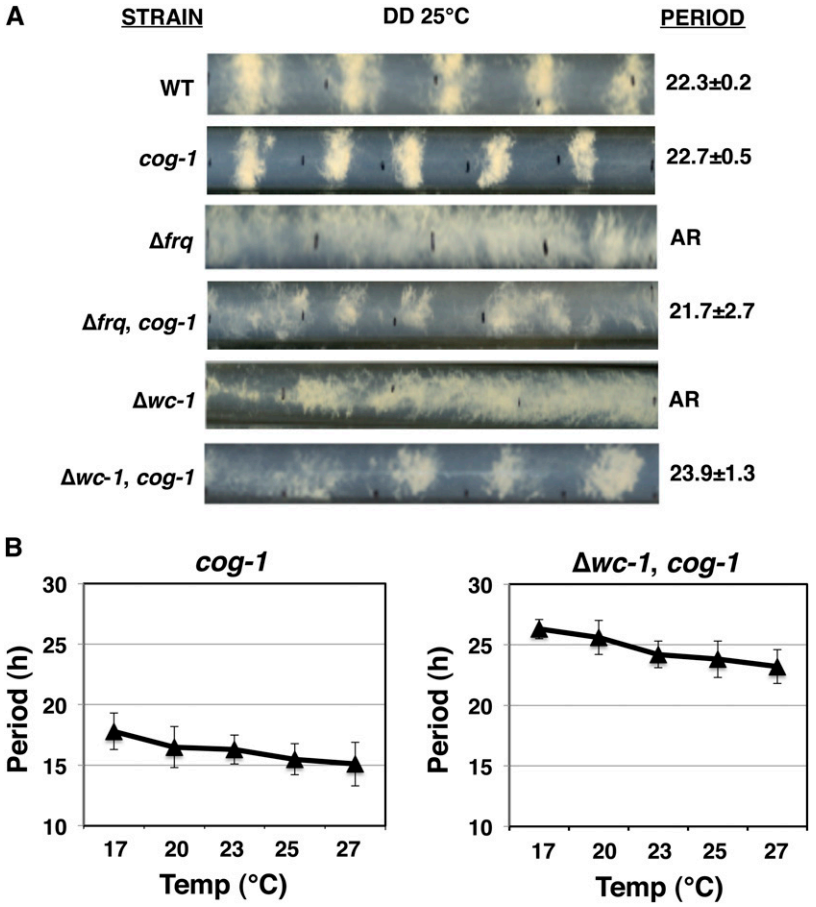

Figure 3 The CDO cycles in DD and is temperature-compensated. (A) The *cog-1* mutation restores circadian rhythms in development to *frq*-null and *wc-1*-null strains in DD. Representative race-tube pictures of the indicated strains are shown from cultures grown in DD at 25° and labeled as in Figure 2. (B) *cog-1* rhythms are temperature-compensated. Plots of the period (h) *vs.* temperature (°C) are shown for the indicated strains (±SD, $n \geq 60$).

arrhythmic under both lighting conditions. Arrhythmicity in the triple photoreceptor mutant cells may be due to a lack of synchronization of the clock by light in the culture or to loss of function of the oscillators. To distinguish these possibilities, we used a temperature shift from 30° to 25° to synchronize the cells (Gooch *et al.* 1994; Liu *et al.* 1998). In support of loss of clock function in the triple photoreceptor mutant, the strain was arrhythmic following a temperature shift (Figure S5). Together, these data indicated that any of the three blue-light photoreceptors are able to substitute for each other to drive rhythms in LD cycles in strains containing the *cog-1* mutation; however, in all cases, WC-1 was required for normal circadian entrainment to light.

To further examine the requirement for CRY in the function of the CDO, we assayed the photoreceptor mutant strains in DD at 25°. In strains where the FWO was functional, rhythms with periods close to WT were observed independently of *cry* or *cog-1* (Figure 7A and Table 2). In *wc-1* deletion strains, development is arrhythmic in DD as expected, but the *cog-1* mutation rescued rhythmicity (Figure 3). Consistent with a central role for CRY in the CDO, rescue of rhythmicity in Δ*wc-1* cells by *cog-1* depended upon CRY, as evidenced by arrhythmic development in the Δ*cry*, Δ*wc-1*, *cog-1* strain (Figure 7A). Together, these data suggest that CRY is necessary for the function of the CDO in LL (Figure 5A) and in DD (Figure 7A).

The *cry* promoter is bound by the WCC after a light pulse (Smith *et al.* 2010), providing a direct link between the FWO and the CDO. To examine this link further, we assayed the levels of CRY protein in WT and Δ*wc-1* strains with or without the *cog-1* mutation in DD and LL (Figure 7B). In cells grown for 24 h in LL, CRY levels were increased relative to the DD samples in WT, and *cog*-1 cells, but not in Δ*wc-1*, and Δ*wc-1*, *cog-1* cells, consistent with previous data demonstrating a role for WC-1 in CRY light responses (Froehlich *et al.* 2010). Low levels of CRY protein were also observed in Δ*wc-1* and Δ*wc-1*, *cog-1* cells grown in LL and harvested at different times of the day (Figure S6). Developmental rhythms persist in *cog-1* and Δ*wc-1*, *cog-1* cells in LL, but the levels of CRY protein varied widely between these strains, with very low levels in Δ*wc-1*, *cog-1* cells and high levels in *cog-1* cells (Figure 7B and Figure S6). Thus, the levels of CRY protein do not appear to correlate with rhythmicity of the strains in LL. We also examined WC-1 steady-state levels in the same cells. WC-1 levels were increased in cells grown in LL for 24 hr as compared to the 24-hr DD samples in WT, *cog-1*, and Δ*cry* cells and were slightly elevated in Δ*cry*, *cog-1* cells (Figure 7C). Similar results were observed in cells grown for 12, 15, and 18 hr in DD and LL (Figure S7). Consistent with previous data, we observed that WC-1 levels were elevated in Δ*cry* cells as compared to WT cells in DD (Olmedo *et al.* 2010). Surprisingly, this increase in WC-1 levels was not observed

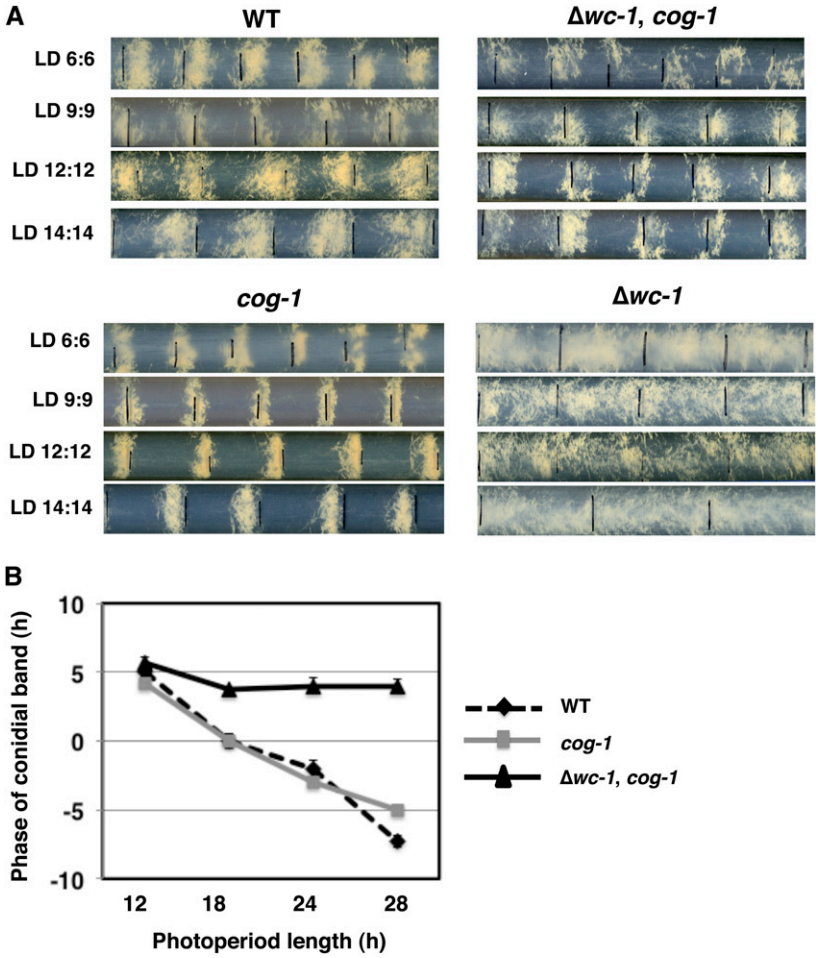

Figure 4 WC-1 is required for light entrainment of the circadian clock. (A) Race-tube cultures were exposed to different LD photoperiods (h). For example, LD 6:6 indicates a 12-hr photoperiod with cycles of 6 hr light and 6 hr dark. Representative race-tube pictures are shown for the indicated strains. Black lines on the race tubes denote when the light was turned on. (B) Plot of the phase of the conidiation band in relation to lights on in strains that showed light responses in each of the photoperiods from A (±SEM, $n \geq 5$). A positive number indicates that the conidiation band occurred after lights on, and a negative number indicates that the band occurred prior to lights on. In some cases, the error bar is smaller than the symbol.

in Δ*cry*, *cog-1* cells in DD (Figure 7 and Figure S6), suggesting the possibility that COG-1 functions as a CRY-dependent activator of the WCC. However, similar to CRY protein levels, no correlations between the levels of WC-1 protein and rhythmicity were observed.

CDO activity reduces growth rate

To determine if activity of the CDO has an affect on *Neurospora* cells, we compared the growth rates of representative strains with variations in FWO and CDO function in DD and LL (Table 3). Under both conditions, CDO activity reduced the growth rate as compared to WT cells. Cells with a *cry* deletion had a slightly slower growth rate in DD. In cells that lack both FWO and CDO activity in LL and DD, the growth rate was increased as compared to WT cells, suggesting that, while the clock provides an overall adaptive advantage to organisms in natural LD cycles, its activity negatively affects growth of the fungus in DD or LL, similar to results obtained in cyanobacteria (Ma *et al.* 2013).

Discussion

To investigate the organization of the *N. crassa* circadian system, we identified the *cog-1* mutation that restores rhythms to strains that lack a functional FWO in DD and LL. While the oscillator that drives this rhythm free-runs with a circadian period under constant conditions, and is temperature-compensated in LL, development in strains expressing the CDO, but lacking WC-1, were driven by light, rather than entrained by LD cycles. Thus, the CDO, similar to other reported FLOs (Dunlap and Loros 2004), lacks full circadian properties. In addition, these data revealed that blue-light photoreceptors in addition to WC-1 are capable of perceiving light information to ultimately drive development in the fungus. Surprisingly, any of the three blue-light photoreceptors—VVD, CRY, or WC-1—can compensate for each other in *cog-1* mutant light responses. These data support the notion that the photoreceptors function in multiprotein complexes, providing opportunities for crosstalk in response to light (Bayram *et al.* 2010)

The *cog-1* mutation is recessive (Figure S2), suggesting that *cog-1* is most likely a loss of function mutation. While we have not yet identified the defective gene product in the *cog-1* mutant strain, these data indicate that the gene specified by the *cog-1* mutation encodes a protein that either directly or indirectly reduces the activity of the CDO when it is present, rather than as a component of the CDO that is necessary for its function. There are several possible reasons for why the CDO might be repressed via *cog-1*. For example, the CDO may be required only under certain growth conditions,

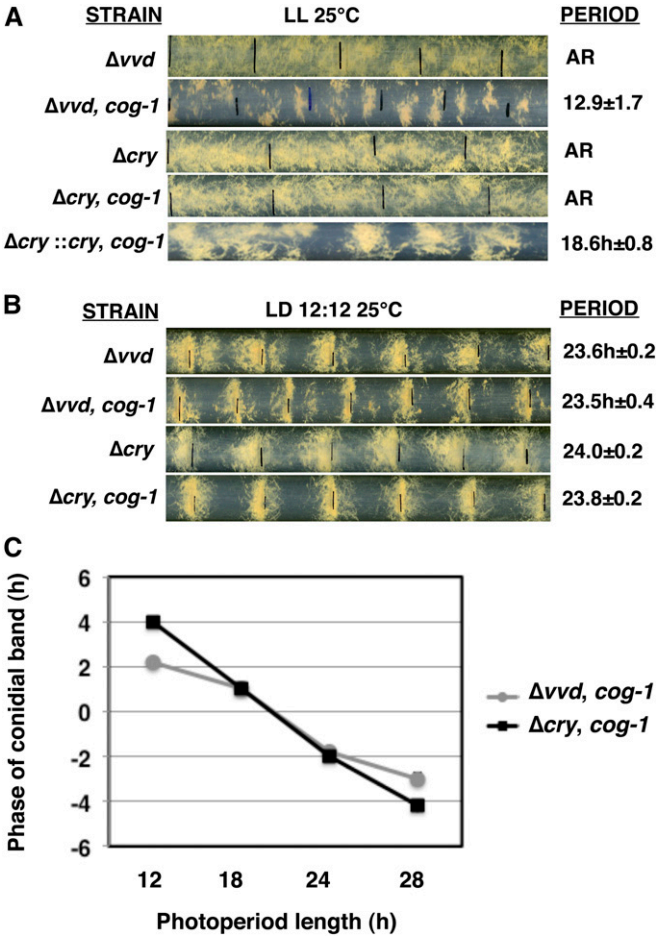

Figure 5 The blue-light photoreceptor CRY, but not VVD, is necessary for CDO function, and other photoreceptors can compensate for rhythms in LD cycles. Representative race-tube photographs of the indicated photoreceptor mutant strains are shown in LL at 25° (A) and in LD 12:12 cycles at 25° (B) and labeled as in Figure 2. (C) Plot of the phase of the conidiation bands in relation to lights on for the indicated strains (±SEM, $n \geq 5$).

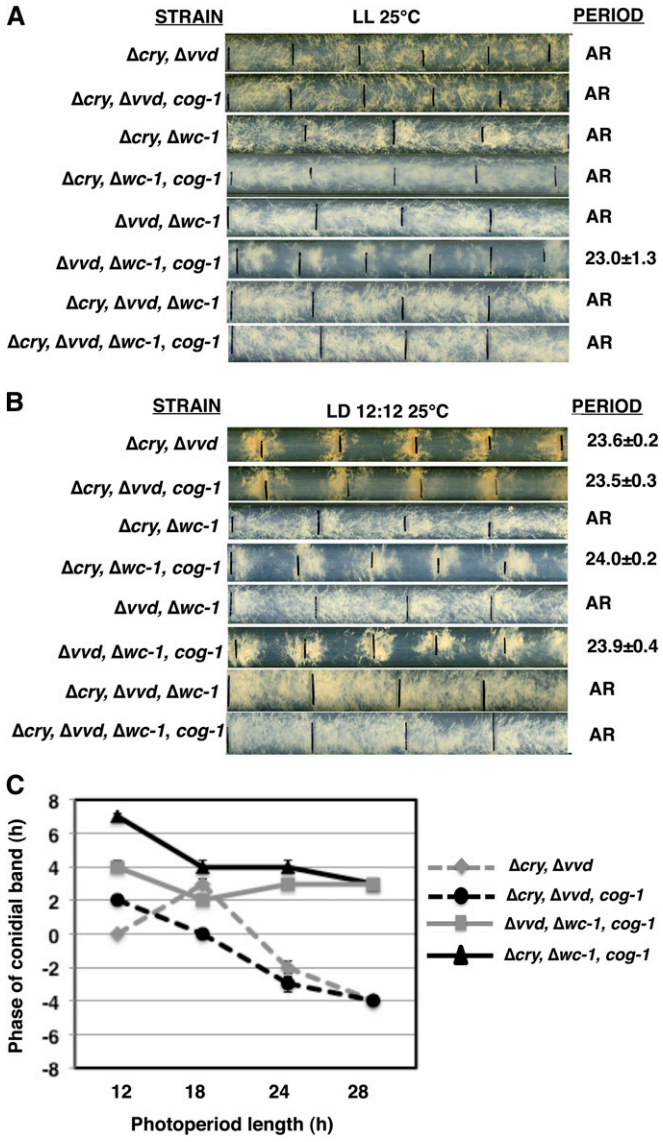

Figure 6 CRY and VVD are not required for light entrainment of the circadian clock. (A) Race-tube cultures of the indicated photoreceptor mutant strains were grown in LL and (B) were exposed to different LD photoperiods (h) and labeled as in Figure 2. (C) The phases of the conidiation band in relation to lights on were plotted (±SEM, $n \geq 5$) as in Figure 4B.

and shutting off the CDO when those conditions are not met may provide a growth advantage to the organism. Consistent with this hypothesis, we observed that strains with a functional CDO had a reduced growth rate (Table 3). Alternatively, the CDO may be an ancient oscillator that was shut down to allow a more efficient oscillator, the FWO, which fully entrains to environmental conditions, to take over and provide a mechanism for anticipation of environmental cycles.

Our data are consistent with a role for CRY in the function of the CDO, as CRY deletion strains are arrhythmic under conditions in which the CDO is normally expressed (Figure 5, Figure 6, and Figure 7). In plants and insects, CRY is necessary for light entrainment of the circadian clock (Emery *et al.* 1998, 2000; Stanewsky *et al.* 1998), and in animals, CRY 1 and CRY 2 function as negative components of the core circadian oscillator (Reppert and Weaver 2002). Some insects, such as the monarch butterfly, have both *Drosophila* and mammalian versions of CRY, supporting an ancestral-like clock mechanism that involves both light sensing and transcriptional repressor roles for CRY (Zhu *et al.* 2008). CRY in *N. crassa* is a member of the CRY-DASH family of proteins, and while it can bind chromophores, it does not appear to have photolyase activity typical of CRY-DASH proteins (Froehlich *et al.* 2010). Both *cry* messenger RNA (mRNA) and protein are induced by light, and light induction requires WC-1, consistent with direct binding of the WCC to the *cry* promoter (Smith *et al.* 2010). Furthermore, *cry* mRNA accumulates with a low-amplitude circadian rhythm, peaking in the nighttime (Froehlich *et al.* 2010). The *cry* deletion strains show a small decrease in amplitude of a few light-induced genes and a slight phase delay in LD

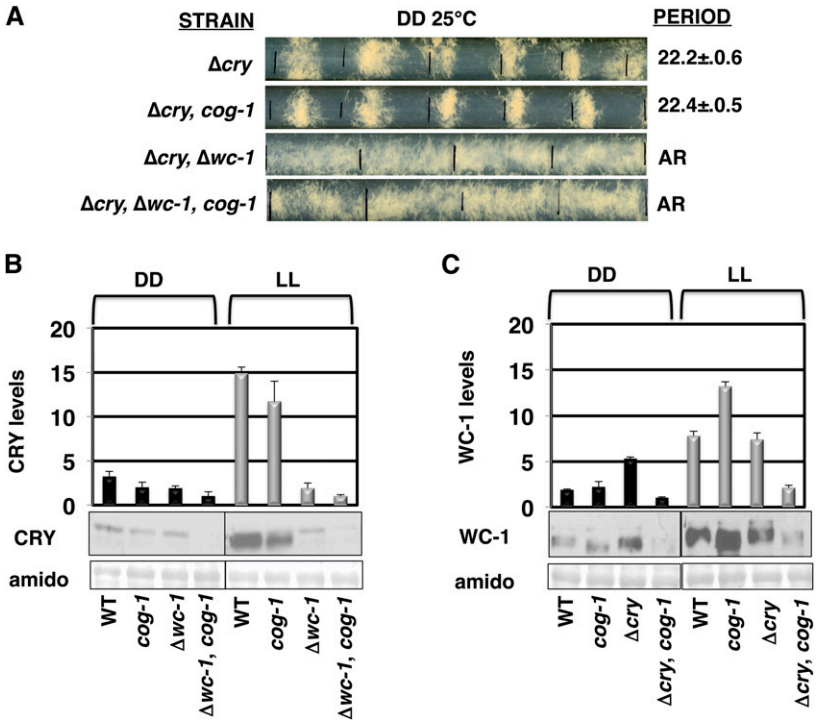

Figure 7 CRY, but not VVD or WC-1, is required for CDO-generated rhythms in DD. (A) Representative race tubes of the indicated strains labeled as in Figure 2. (B and C) Representative Western blots of CRY protein (B) and WC-1 protein (C) in the indicated strains harvested following 24 hr of growth in DD or LL and plotted above (±SEM, $n = 3$). The amido-stained membrane is shown below and was used to normalize protein loading.

entrainment in otherwise WT strains (Chen *et al.* 2009). Given the central role of CRY in the insect and animal clockworks, the lack of pronounced circadian or light phenotypes in CRY deletion mutants in *Neurospora* has, until now, been surprising (Chen and Loros 2009; Froehlich *et al.* 2010; Olmedo *et al.* 2010). While our data strongly support a central role for CRY in the CDO, we were surprised to find that steady-state levels of CRY protein do not correlate with the rhythmicity of the strains. For example, the levels of CRY protein are low in Δ*wc-1*, *cog-1* and Δ*wc-1* cells in LL, but only the Δ*wc-1*, *cog-1* displays rhythms in development (Figure 2, Figure 7, and Figure S6). The data also pointed to the possibility that *cog-1* functions both as a CRY-dependent activator of WC-1 (as evidenced by the low levels of WC-1 protein in Δ*cry*, *cog-1* cells as compared to Δ*cry* cells in LL) and as a negative regulator of CDO activity. Taken together, CDO rhythms appear in cells that lack a functional FWO, have low levels (or activity) of CRY protein, and carry the *cog-1* mutation. Furthermore, in *cog-1* strains in LL, the period is 17 hr and the levels of CRY are high, whereas in Δ*wc-1*, *cog-1*, cells the period is 25 hr and the levels of CRY are low. These data suggest that low levels (or activity) of CRY correlate with a longer CDO period. These data, together with our results showing no detectable consequences in rhythms in DD in strains completely lacking CRY (and thus a functional CDO), support the idea that the CDO functions downstream of the FWO and is not necessary for developmental rhythms.

Similar to circadian oscillators, we predict that the CDO functions as an autonomous molecular feedback loop, although this still needs to be determined. Because the CDO does not have full circadian properties, requiring WC-1 for LD entrainment, we surmise that it lies downstream of the FWO and may function as a slave oscillator to enhance rhythmic outputs, such as the development rhythm. This observation is reminiscent of previous studies demonstrating that WCC plays a central role in generating FLO-like oscillatory behavior in development in FWO-deficient strains in temperature cycles (Hunt *et al.* 2012). In LL and naturally occurring LD cycles, the CDO may take on a more prominent role to maintain rhythms during long periods of light when the FWO would normally break down. This idea is congruent with previous suggestions characterizing the role of VVD in LD entrainment (Elvin *et al.* 2005), in which the FWO oscillator is predicted to fully function in the night, whereas a slave oscillator, such as the CDO, may function during periods of light to complete the downstream rhythmic events. Our observation that the CDO is less robust in DD (Figure 3) also fits with this hypothesis. Additional evidence points to an interaction between the FWO and the CDO, including differences in the period of the rhythm in the *cog-1* strains FWO-deficient *vs.* FWO-sufficient strains grown in LL (Figure 2), and the demonstration that the *cry* promoter is a direct target of the WCC (Smith *et al.* 2010). However, because the phase of the rhythm in *cry* (nighttime peak) does not match the morning activity of the WCC (Froehlich *et al.* 2010), it is possible that other components of the CDO control *cry* rhythmicity.

We propose a simple model to provide a framework for future tests of the complexity of the clock (Figure 8). In *cog-1*-deficient strains, and independent of the FWO, CRY is active and the CDO feedback loop oscillates. How *cog-1* represses the CDO under conditions in which the FWO is not operative (such as in mutants of the FWO or in LL) is not known. Light signals may directly affect CRY activity to synchronize the CDO rhythm in LD cycles. In LL, some mechanism

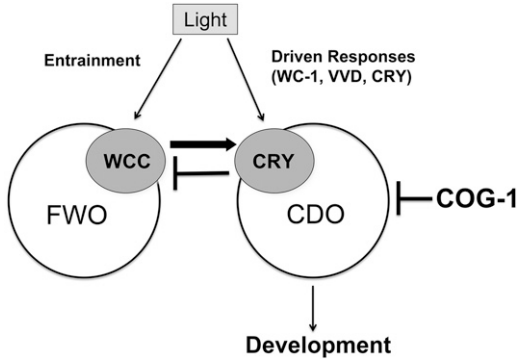

Figure 8 Model of the circadian clock composed of the FWO and CDO. See the text for a description of the model.

would be expected to exist to desensitize CRY activity during chronic light treatment. It has been shown that VVD functions in *N. crassa* to desensitize photo-transduction pathways during chronic light treatment and plays a role in establishing the phase of the clock in LD cycles (Elvin *et al.* 2005). Thus, VVD is a good candidate for desensitizing CRY to chronic light to promote CDO function. Consistent with a role for VVD in LL, the period of the CDO rhythm is significantly reduced in Δ*vvd*, *cog-1* strains (Figure 5). This model leads to several testable predictions: (i) that the activity of CRY would be increased in *cog-1* mutant strains and reduced in WC-1 deletions in LL; (ii) that the activity of CRY would cycle in LL and LD in the absence of WC-1; (iii) that Δ*vvd* will double the cycling of the CDO due to increased CRY activation in LL, which would be dependent on WC-1; (iv) that mutations in *cry*, or artificially increasing the levels of CRY in Δ*wc-1*, *cog-1* strains, would alter the period of the CDO rhythm; and (v) identification of CRY-interacting proteins would uncover additional components of the CDO. In any case, the clock system is likely to be even more complex than depicted here. For example, under specialized growth conditions, developmental rhythms with periods ranging between 6 and 21 hr were observed in *vvd* mutant alleles in LL that are dependent on WC-1, but not on FRQ (Schneider *et al.* 2009). In addition, rhythms in the expression of the *ccg-16* gene in *N. crassa* are controlled by a FLO that requires WC-1, but not FRQ, for activity. This FLO, called the WC-FLO, that appears to be both temperature-compensated and entrained to environmental cycles independently of WCC and FRQ (de Paula *et al.* 2006, 2007).

In summary, this work provides new insights into the complexity of the oscillator system in *N. crassa* and light responses. The identification of CRY as an CDO component, and the discovery of the *cog-1* mutation that uncovered the CDO, will undoubtedly aid in testing models of the CDO, its connections to the environment, and to the FWO. As CRY is considered to be an ancient photoreceptor that has different activities in diverse organisms, including DNA repair, light perception, and running of the circadian clock (Somers *et al.* 1998; Stanewsky *et al.* 1998; Reppert and Weaver 2002; Daiyasu *et al.* 2004; Froehlich *et al.* 2010), our finding of the CRY-dependent CDO may provide key insights into the evolution of the clock.

Acknowledgments

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Literature Cited

Aronson, B. D., K. A. Johnson, and J. C. Dunlap, 1994 Circadian clock locus frequency: protein encoded by a single open reading frame defines period length and temperature compensation. Proc. Natl. Acad. Sci. USA 91: 7683–7687.

Baker, C. L., J. J. Loros, and J. C. Dunlap, 2012 The circadian clock of *Neurospora crassa*. FEMS Microbiol. Rev. 36: 95–110.

Bayram, O., G. H. Braus, R. Fischer, and J. Rodriguez-Romero, 2010 Spotlight on *Aspergillus nidulans* photosensory systems. Fungal Genet. Biol. 47: 900–908.

Belden, W. J., L. F. Larrondo, A. C. Froehlich, M. Shi, C. H. Chen *et al.*, 2007 The *band* mutation in *Neurospora crassa* is a dominant allele of *ras-1* implicating RAS signaling in circadian output. Genes Dev. 21: 1494–1505.

Bell-Pedersen, D., 2000 Understanding circadian rhythmicity in *Neurospora crassa*: from behavior to genes and back again. Fungal Genet. Biol. 29: 1–18.

Bell-Pedersen, D., S. K. Crosthwaite, P. L. Lakin-Thomas, M. Merrow, and M. Okland, 2001 The Neurospora circadian clock: Simple or complex? Philos. Trans. R. Soc. Lond. B Biol. Sci. 356: 1697–1709.

Bennett, L. D., P. Beremand, T. L. Thomas, and D. Bell-Pedersen, 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.

Brody, S., K. Oelhafen, K. Schneider, S. Perrino, A. Goetz *et al.*, 2010 Circadian rhythms in *Neurospora crassa*: downstream effectors. Fungal Genet. Biol. 47: 159–168.

Cambras, T., J. R. Weller, M. Angles-Pujoras, M. L. Lee, A. Christopher *et al.*, 2007 Circadian desynchronization of core body temperature and sleep stages in the rat. Proc. Natl. Acad. Sci. USA 104: 7634–7639.

Chen, C. H., and J. J. Loros, 2009 Neurospora sees the light: light signaling components in a model system. Commun. Integr. Biol. 2: 448–451.

Chen, C. H., C. S. Ringelberg, R. H. Gross, J. C. Dunlap, and J. J. Loros, 2009 Genome-wide analysis of light-inducible responses reveals hierarchical light signalling in Neurospora. EMBO J. 28: 1029–1042.

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

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

Christensen, M. K., G. Falkeid, J. J. Loros, J. C. Dunlap, C. Lillo *et al.*, 2004 A nitrate-induced *frq*-less oscillator in *Neurospora crassa*. J. Biol. Rhythms 19: 280–286.

Collins, B. H., S. Dissel, E. Gaten, E. Rosato, and C. P. Kyriacou, 2005 Disruption of Cryptochrome partially restores circadian rhythmicity to the arrhythmic period mutant of Drosophila. Proc. Natl. Acad. Sci. USA 102: 19021–19026.

Colot, H. V., G. Park, G. E. Turner, C. Ringelberg, C. M. Crew *et al.*, 2006 A high-throughput gene knockout procedure for Neurospora reveals functions for multiple transcription factors. Proc. Natl. Acad. Sci. USA 103: 10352–10357.

Correa, A., Z. A. Lewis, A. V. Greene, I. J. March, R. H. Gomer *et al.*, 2003 Multiple oscillators regulate circadian gene expression in Neurospora. Proc. Natl. Acad. Sci. USA 100: 13597–13602.

Crosthwaite, S. K., J. J. Loros, and J. C. Dunlap, 1995 Light-induced resetting of a circadian clock is mediated by a rapid increase in frequency transcript. Cell 81: 1003–1012.

Daiyasu, H., T. Ishikawa, K. Kuma, S. Iwai, T. Todo *et al.*, 2004 Identification of cryptochrome DASH from vertebrates. Genes Cells 9: 479–495.

Davis, R. H., and F. J. de Serres, 1970 Genetic and microbiological research techniques for *Neurospora crassa*. Methods Enzymol. 17: 79–143.

de Paula, R. M., Z. A. Lewis, A. V. Greene, K. S. Seo, L. W. Morgan *et al.*, 2006 Two circadian timing circuits in *Neurospora crassa* cells share components and regulate distinct rhythmic processes. J. Biol. Rhythms 21: 159–168.

de Paula, R. M., M. W. Vitalini, R. H. Gomer, and D. Bell-Pedersen, 2007 Complexity of the *Neurospora crassa* circadian clock system: multiple loops and oscillators. Cold Spring Harb. Symp. Quant. Biol. 72: 345–351.

Dragovic, Z., Y. Tan, M. Gorl, T. Roenneberg, and M. Merrow, 2002 Light reception and circadian behavior in "blind" and "clock-less" mutants of *Neurospora crassa*. EMBO J. 21: 3643–3651.

Dunlap, J. C., and J. J. Loros, 2004 The Neurospora circadian system. J. Biol. Rhythms 19: 414–424.

Dunlap, J. C., and J. J. Loros, 2005 Analysis of circadian rhythms in Neurospora: overview of assays and genetic and molecular biological manipulation. Methods Enzymol. 393: 3–22.

Edgar, R. S., E. W. Green, Y. Zhao, G. van Ooijen, M. Olmedo *et al.*, 2012 Peroxiredoxins are conserved markers of circadian rhythms. Nature 485: 459–464.

Elvin, M., J. J. Loros, J. C. Dunlap, and C. Heintzen, 2005 The PAS/LOV protein VIVID supports a rapidly dampened daytime oscillator that facilitates entrainment of the Neurospora circadian clock. Genes Dev. 19: 2593–2605.

Emery, P., W. V. So, M. Kaneko, J. C. Hall, and M. Rosbash, 1998 CRY, a Drosophila clock and light-regulated cryptochrome, is a major contributor to circadian rhythm resetting and photosensitivity. Cell 95: 669–679.

Emery, P., R. Stanewsky, J. C. Hall, and M. Rosbash, 2000 A unique circadian-rhythm photoreceptor. Nature 404: 456–457.

Froehlich, A. C., J. J. Loros, and J. C. Dunlap, 2003 Rhythmic binding of a WHITE COLLAR-containing complex to the *frequency* promoter is inhibited by FREQUENCY. Proc. Natl. Acad. Sci. USA 100: 5914–5919.

Froehlich, A. C., C. H. Chen, W. J. Belden, C. Madeti, T. Roenneberg *et al.*, 2010 Genetic and molecular characterization of a cryptochrome from the filamentous fungus *Neurospora crassa*. Eukaryot. Cell 9: 738–750.

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

Gooch, V. D., R. A. Wehseler, and C. G. Gross, 1994 Temperature effects on the resetting of the phase of the Neurospora circadian rhythm. J. Biol. Rhythms 9: 83–94.

Granshaw, T., M. Tsukamoto, and S. Brody, 2003 Circadian rhythms in Neurospora crassa: farnesol or geraniol allow expression of rhythmicity in the otherwise arrhythmic strains *frq*^{10}, *wc-1*, and *wc-2*. J. Biol. Rhythms 18: 287–296.

Griffin, Jr., E. A., D. Staknis, and C. J. Weitz, 1999 Light-independent role of CRY1 and CRY2 in the mammalian circadian clock. Science 286: 768–771.

He, Q., P. Cheng, and Y. Liu, 2005 The COP9 signalosome regulates the Neurospora circadian clock by controlling the stability of the SCFFWD-1 complex. Genes Dev. 19: 1518–1531.

He, Q., J. Cha, H. C. Lee, Y. Yang, and Y. Liu, 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., and Y. Liu, 2007 The *Neurospora crassa* circadian clock. Adv. Genet. 58: 25–66.

Heintzen, C., J. J. Loros, and J. C. Dunlap, 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.

Hunt, S., M. Elvin, and C. Heintzen, 2012 Temperature-sensitive and circadian oscillators of *Neurospora crassa* share components. Genetics 191: 119–131.

Ivanchenko, M., R. Stanewsky, and J. M. Giebultowicz, 2001 Circadian photoreception in Drosophila: functions of cryptochrome in peripheral and central clocks. J. Biol. Rhythms 16: 205–215.

Iwasaki, H., and J. C. Dunlap, 2000 Microbial circadian oscillatory systems in Neurospora and Synechococcus: models for cellular clocks. Curr. Opin. Microbiol. 3: 189–196.

Jin, Y., S. Allan, L. Baber, E. K. Bhattarai, T. M. Lamb *et al.*, 2007 Rapid genetic mapping in *Neurospora crassa*. Fungal Genet. Biol. 44: 455–465.

Johnson, C. H., J. A. Elliott, and R. Foster, 2003 Entrainment of circadian programs. Chronobiol. Int. 20: 741–774.

Krishnan, B., J. D. Levine, M. K. Lynch, H. B. Dowse, P. Funes *et al.*, 2001 A new role for cryptochrome in a Drosophila circadian oscillator. Nature 411: 313–317.

Lakin-Thomas, P. L., 2000 Circadian rhythms: new functions for old clock genes. Trends Genet. 16: 135–142.

Lakin-Thomas, P. L., D. Bell-Pedersen, and S. Brody, 2011 The genetics of circadian rhythms in Neurospora. Adv. Genet. 74: 55–103.

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

Li, S., K. Motavaze, E. Kafes, S. Suntharalingam, and P. Lakin-Thomas, 2011 A new mutation affecting FRQ-less rhythms in the circadian system of *Neurospora crassa*. PLoS Genet. 7: e1002151.

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

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

Liu, Y., M. Merrow, J. J. Loros, and J. C. Dunlap, 1998 How temperature changes reset a circadian oscillator. Science 281: 825–829.

Lombardi, L., K. Schneider, M. Tsukamoto, and S. Brody, 2007 Circadian rhythms in *Neurospora crassa*: clock mutant effects in the absence of a *frq*-based oscillator. Genetics 175: 1175–1183.

Loros, J. J., and J. C. Dunlap, 2001 Genetic and molecular analysis of circadian rhythms in Neurospora. Annu. Rev. Physiol. 63: 757–794.

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

Ma, P., M. A. Woelfle, and C. H. Johnson, 2013 An evolutionary fitness enhancement conferred by the circadian system in cyanobacteria. Chaos Solitons Fractals 50: 65–74.

Merrow, M., M. Brunner, and T. Roenneberg, 1999 Assignment of circadian function for the Neurospora clock gene *frequency*. Nature 399: 584–586.

Merrow, M., C. Boesl, J. Ricken, M. Messerschmitt, M. Goedel *et al.*, 2006 Entrainment of the Neurospora circadian clock. Chronobiol. Int. 23: 71–80.

Morse, D., J. W. Hastings, and T. Roenneberg, 1994 Different phase responses of the two circadian oscillators in Gonyaulax. J. Biol. Rhythms 9: 263–274.

Olmedo, M., C. Ruger-Herreros, E. M. Luque, and L. M. Corrochano, 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.

Pall, M. L., and J. P. Brunelli, 1993 A series of six compact fungal transformation vectors containing polylinkers with multiple unique restriction sites. Fungal Genet. Newsl. 40: 59–62.

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

Reppert, S. M., and D. R. Weaver, 2002 Coordination of circadian timing in mammals. Nature 418: 935–941.

Sai, J., and C. H. Johnson, 1999 Different circadian oscillators control Ca(2+) fluxes and lhcb gene expression. Proc. Natl. Acad. Sci. USA 96: 11659–11663.

Sargent, M. L., and W. R. Briggs, 1967 The effects of light on a circadian rhythm of conidiation in Neurospora. Plant Physiol. 42: 1504–1510.

Sargent, M. L., W. R. Briggs, and D. O. Woodward, 1966 Circadian nature of a rhythm expressed by an invertaseless strain of *Neurospora crassa*. Plant Physiol. 41: 1343–1349.

Schafmeier, T., A. Haase, K. Kaldi, J. Scholz, M. Fuchs *et al.*, 2005 Transcriptional feedback of Neurospora circadian clock gene by phosphorylation-dependent inactivation of its transcription factor. Cell 122: 235–246.

Schneider, K., S. Perrino, K. Oelhafen, S. Li, A. Zatsepin *et al.*, 2009 Rhythmic conidiation in constant light in *vivid* mutants of *Neurospora crassa*. Genetics 181: 917–931.

Shi, M., L. F. Larrondo, J. J. Loros, and J. C. Dunlap, 2007 A developmental cycle masks output from the circadian oscillator under conditions of choline deficiency in Neurospora. Proc. Natl. Acad. Sci. USA 104: 20102–20107.

Smith, K. M., G. Sancar, R. Dekhang, C. M. Sullivan, S. Li *et al.*, 2010 Transcription factors in light and circadian clock signaling networks revealed by genomewide mapping of direct targets for Neurospora White Collar Complex. Eukaryot. Cell 9: 1549–1556.

Somers, D. E., P. F. Devlin, and S. A. Kay, 1998 Phytochromes and cryptochromes in the entrainment of the Arabidopsis circadian clock. Science 282: 1488–1490.

Stanewsky, R., M. Kaneko, P. Emery, B. Beretta, K. Wager-Smith *et al.*, 1998 The cryb mutation identifies cryptochrome as a circadian photoreceptor in Drosophila. Cell 95: 681–692.

Sweeney, B. M., and J. W. Hastings, 1960 Effects of temperature upon diurnal rhythms. Cold Spring Harb. Symp. Quant. Biol. 25: 87–104.

Vitalini, M. W., R. M. de Paula, W. D. Park, and D. Bell-Pedersen, 2006 The rhythms of life: circadian output pathways in Neurospora. J. Biol. Rhythms 21: 432–444.

Vogel, H. J., 1956 A convenient growth medium for Neurospora (medium N). Microb. Genet. Bull 13: 2–43.

Westergaard, M., and H. K. Mitchell, 1947 Neurospora V. A synthetic medium favoring sexual reproduction. Am. J. Bot. 34: 573–577.

Yoshida, Y., T. Maeda, B. Lee, and K. Hasunuma, 2008 Conidiation rhythm and light entrainment in superoxide dismutase mutant in *Neurospora crassa*. Mol. Genet. Genomics 279: 193–202.

Zhu, H., I. Sauman, Q. Yuan, A. Casselman, M. Emery-Le *et al.*, 2008 Cryptochromes define a novel circadian clock mechanism in monarch butterflies that may underlie sun compass navigation. PLoS Biol. 6: e4.

Zoltowski, B. D., C. Schwerdtfeger, J. Widom, J. J. Loros, A. M. Bilwes *et al.*, 2007 Conformational switching in the fungal light sensor VIVID. Science 316: 1054–1057.

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A Novel Cryptochrome-Dependent Oscillator in *Neurospora crassa*

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Table S1 List of primers

NAME	PRIMER SEQUENCE
CRY ORF Forward	5'TTG ACC AGA CGA TCC CAA AA 3'
CRY ORF Reverse	5'TGG TTT CTT CGT TTA TGG GC 3'
ΔCRY::hyg Check Forward	5'TGT ATG TAG TGT ACG GGG CAA A 3'
YG	5’GCT GTC GCA GAG GCT GGA CTA C 3’
VVD ORF Forward	5'GAG CTC CAT CTC ATC CTC 3'
VVD ORF Reverse	5'GCC CAA TCGCAG AAT AAG ACG 3'
ΔWC-1::bar Check Forward	5'CGT TCG ATA GAC GCA ACG TCA 3'
Bar Check 5' Reverse	5'GTTGCG TGC CTT CCA GGG ACC 3'
VVD KO	5’ GTT GTG TCC TCT GAG TTT CC 3’
HY	5’GTT GGT CAA GAC CAA TGC GGA GCA 3’

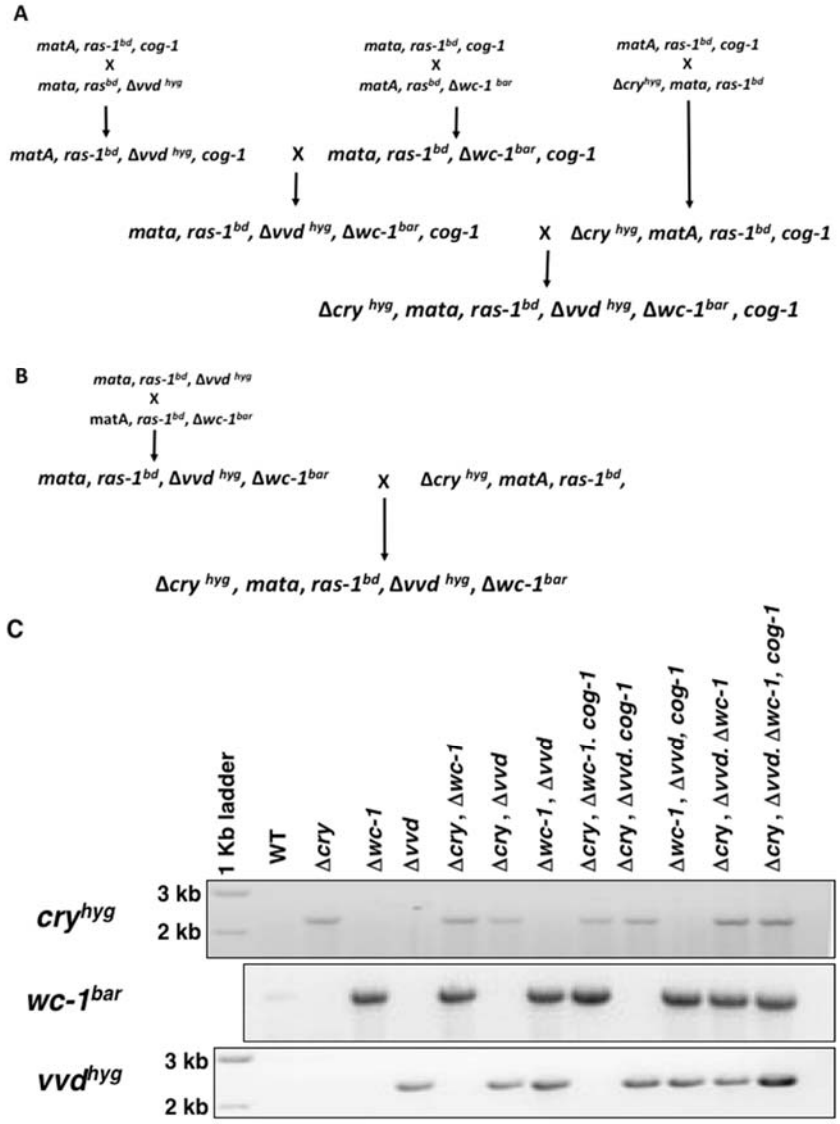

Figure S1 Construction and confirmation of triple photoreceptor mutant strains. Triple photoreceptor deletion strain construction in the *cog-1*$^{-}$ (A) and *cog-1*$^{+}$ backgrounds. The indicated strains were crossed (X) to generate the desired progeny. See **Table 1** for strain numbers. C) Strain validation by PCR. PCR was carried out to amplify regions of the genome from strains containing the indicated deletions. To identify strains with the *cry*^{hyg} deletion, primers CRY ORF forward and YG were used (**Table S1**), to identify the *wc-1*^{bar} deletion, primers barcheck 5' reverse and Δ*wc-1*::*bar* check forward were used (**Table S1**), and to identify the *vvd*^{hyg} deletion, primers *vvd* KO and HY were used (**Table S1**). Amplified products are only observed, as expected, in the appropriate KO alleles.

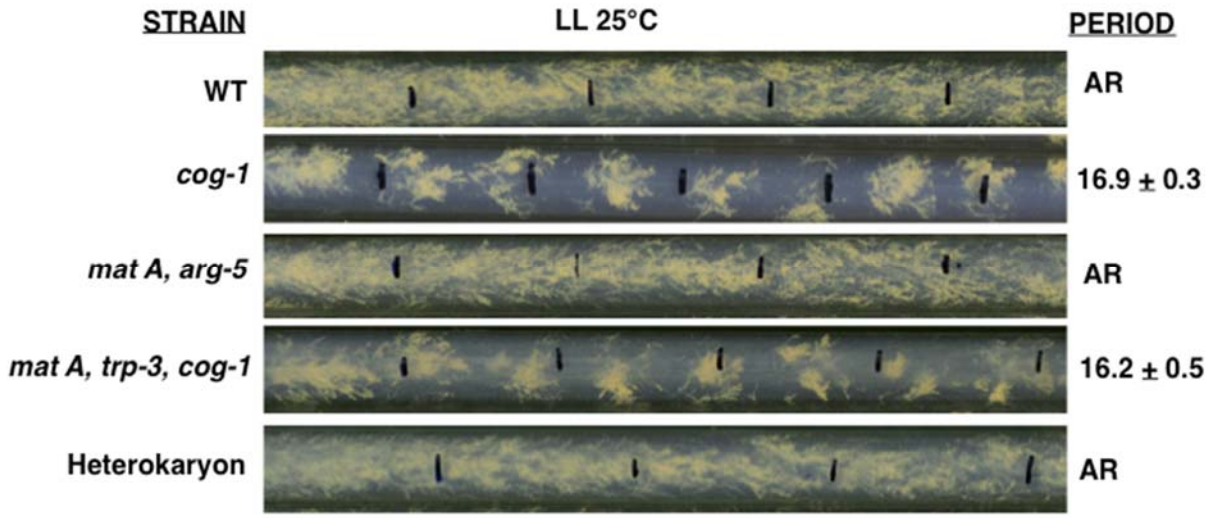

Figure S2 The *cog-1* mutation is recessive. Race tube cultures of *matA, trp-3, cog-1*, supplemented with tryptophan, *mata, arg-5,* supplemented with arginine, and a heterokaryon formed between the two strains and grown on minimal media in LL are shown, along with WT and *cog-1* controls. The solid lines indicate 24 h of growth in LL, and growth is from left to right. Periods are given on the right (± S.D, n≥30). AR = arrhythmic.

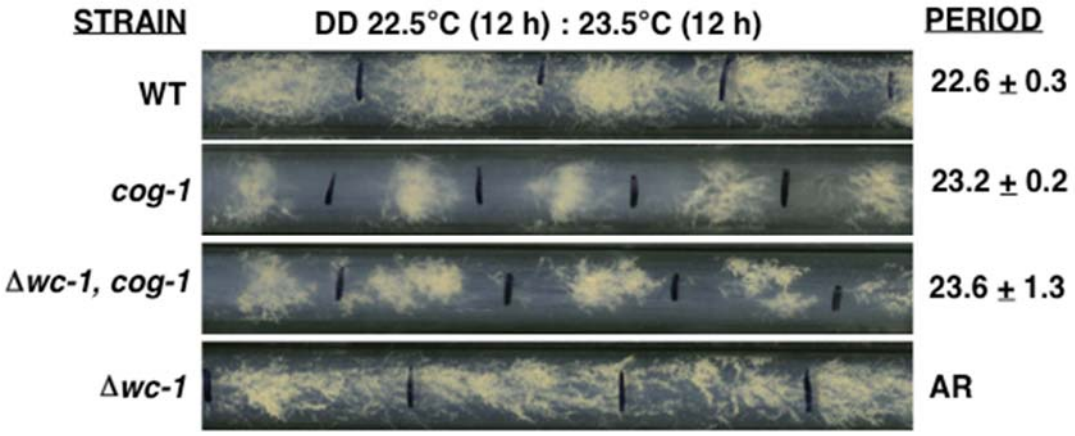

Figure S3 A 1°C temperature change is not sufficient to drive the developmental rhythm in clock deficient cells. Representative tubes of the indicated strains were incubated in DD and given a 1°C temperature shift (22.5°C for 12 hrs- 23.5°C for 12 hrs). The black lines represent daily growth fronts. Periods are given on the right (± S.D, n≥80). AR = arrhythmic.

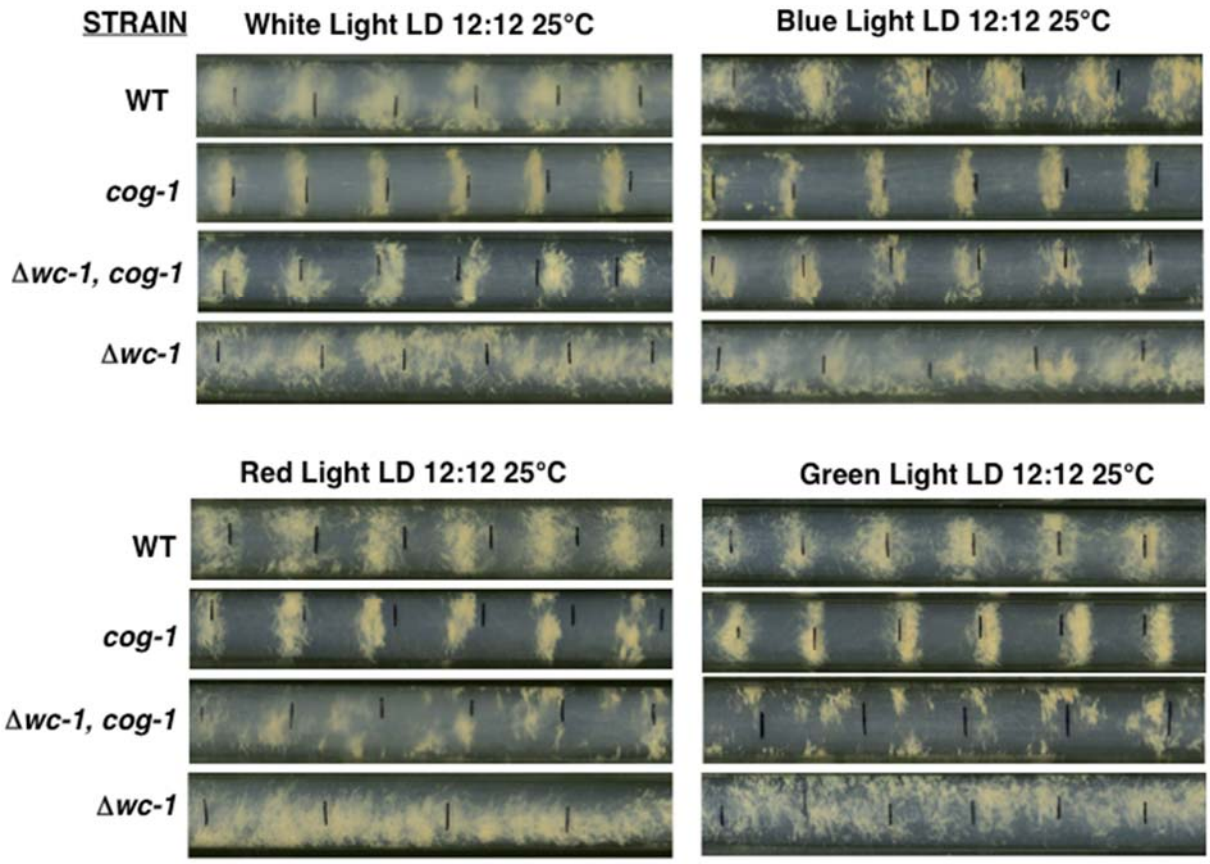

Figure S4 Conidiation rhythms under different light wavelengths. Representative tubes of indicated strains were incubated in 12:12LD cycles at 25°C using different wavelengths of light as indicated. The light intensity was as follows: white light (72 lux), red light (60 lux), green light (64 lux), blue light (70 lux). Black lines on the race tubes denote when the light was turned on. Note that Δ*wc-1, cog-1* cells are responsive to white and blue light, but not to red or green light cycles.

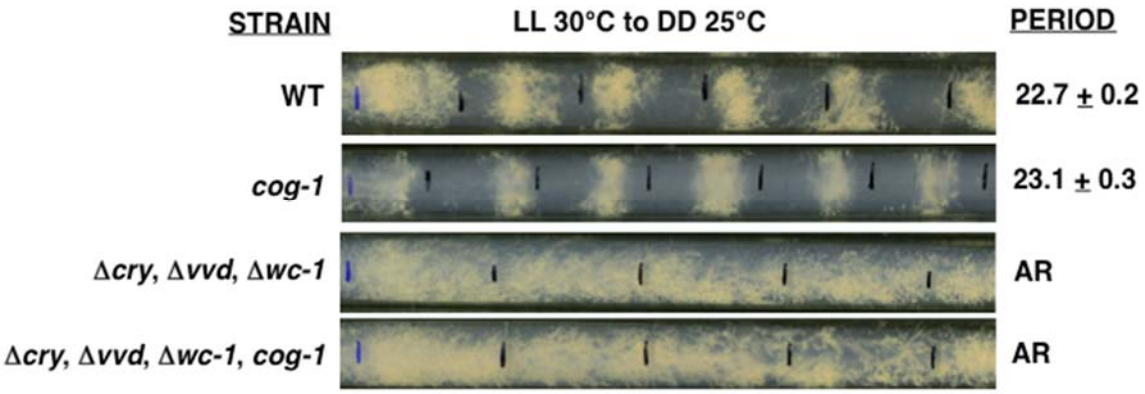

Figure S5 The *cog-1* triple photoreceptor mutant is arrhythmic following temperature synchronization. Representative tubes of the indicated strains in DD. The cultures were grown at 30°C LL for 24 hrs and then transferred to DD at 25°C to synchronize the clock. Black lines represent daily extension fronts. Periods are given on the right (± S.D, n≥30). AR = arrhythmic.

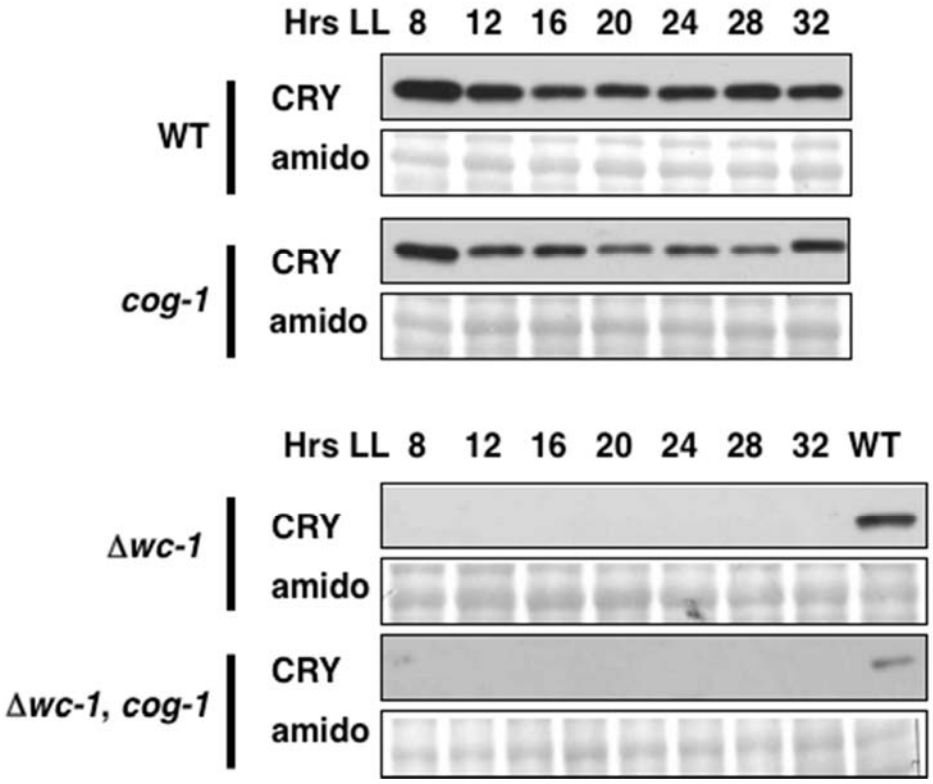

Figure S6 The levels of CRY protein are low in LL independent of time of day in *Δwc-1* and *Δwc-1, cog-1* cells. Western blots from the indicated strains grown in LL and probed with CRY antibody. In the *Δwc-1* and *Δwc-1, cog-1* western blots, WT is the wild type control harvested after 24 h of growth in LL.

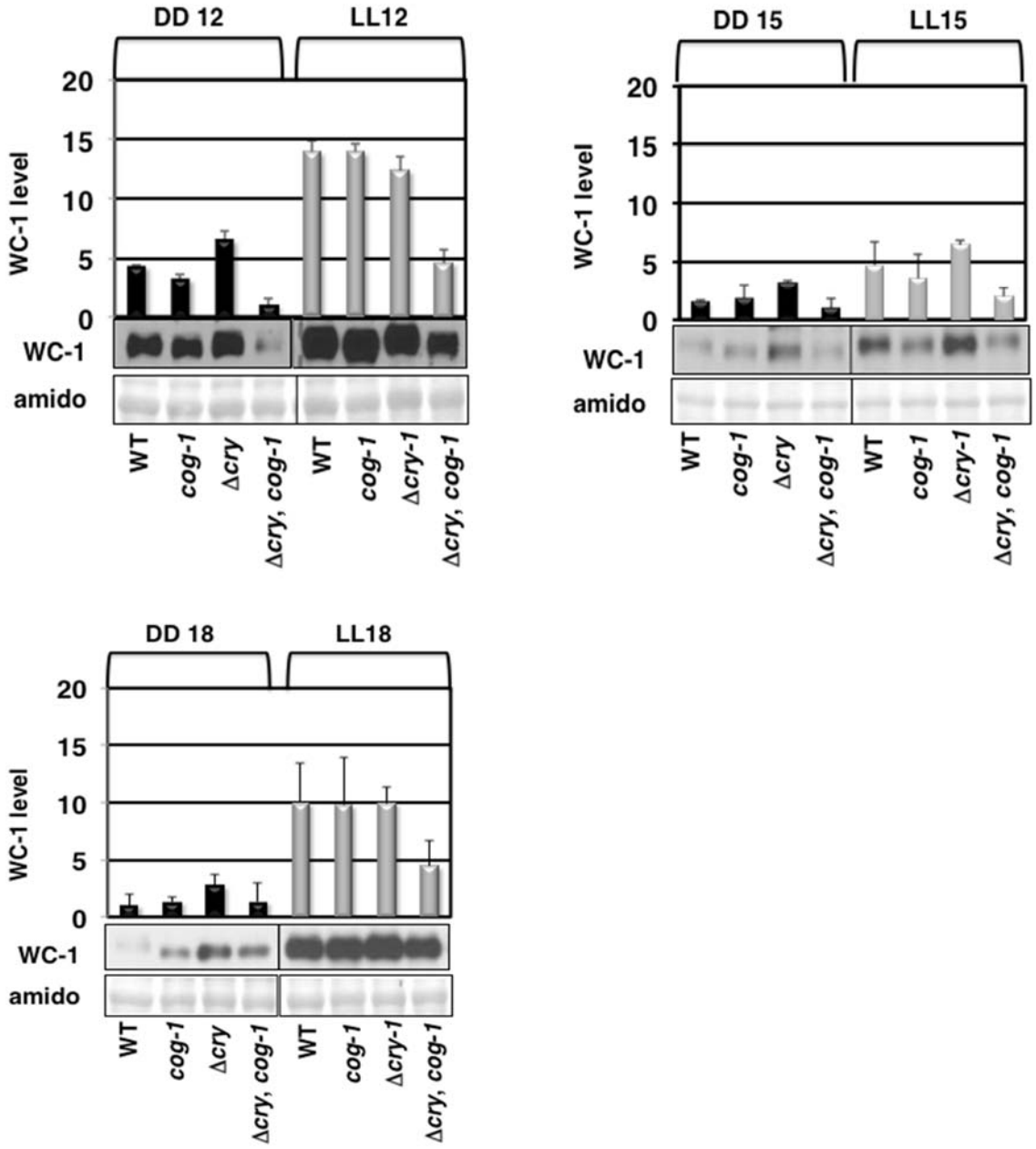

Figure S7 WC-1 protein levels are not increased in Δcry,cog-1 cells in DD. Western blots of WC-1 protein in the indicated strains harvested following 12, 15, or 18 h of growth in DD or LL, and plotted (± SEM. n=2). In each plot the lowest level was set to 1. The amido stained membranes are shown below, and were used to normalize protein loading.