A NOVEL CRYPTOCHROME-DEPENDENT OSCILLATOR IN NEUROSPORA CRASSA

A Dissertation

by

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ABSTRACT

Circadian clocks are composed of molecular oscillators and are found in most eukaryotes and some prokaryotes. The fungus *Neurospora crassa* is a leading model for studying the clock. In *N. crassa*, the well-described FRQ/WCC Oscillator (FWO) consists of a molecular feedback loop involving the negative element FRQ, the blue-light photoreceptor WC-1, and WC-2. WC-1 and WC-2 form a complex called WCC, which functions as the positive element in the feedback loop. The FWO is considered to be the core oscillator regulating overt rhythmicity. However, several labs have shown that rhythms can persist in the absence of a functional FWO under certain growth conditions and genetic backgrounds, suggesting the presence of additional oscillators in the cell. Using genetic approaches to identify components of these putative oscillators, we uncovered a mutant strain, called light-mutant 1 (LM1) that is robustly rhythmic in constant light, and in strains carrying deletions of FWO components; both of which are conditions that abolish FWO function. The oscillator (called the Light Mutant Oscillator, LMO) revealed in the LM1 mutant strain meets two of the three criteria for a circadian oscillator. The LMO has a free running period of ~ 24h, and it is temperature-compensated. However, while the LMO can respond to light cues, WC-1 is required for circadian entrainment to 24-h light cycles. The response of LM1 cells lacking the circadian blue-light photoreceptor WC-1 to blue-light suggested that alternate light inputs function in LM1 mutant cells. I show that the blue light photoreceptors VIVID and CRY compensate for each other, and for WC-1, in LMO light responses. Importantly, I show that deletion of the *cryptochrome* (*cry*) gene abolishes rhythmicity

in an LM1 strain, providing evidence for a role for CRY in the clock mechanism. The LM1 mutation is recessive, suggesting loss of function. Therefore, we hypothesize that the LM1 gene encodes a protein that negatively regulates the activity of the LMO. Our mapping and sequencing data have placed the LM1 mutation on the left arm of chromosome I, near the mating type locus; however, the identity of the mutated gene remains elusive.

DEDICATION

This work is dedicated to my family, especially to the memory of the following:

- my strict disciplinarian and bibliophilic father for being the first to sell me the idea of a Ph.D., albeit in Mathematics
- my maternal grandparents and paternal grandmother for their selfless contributions towards my intellectual development
- my mother-in-law for her rare love

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NOMENCLATURE

μ	micro (10^{-6})
Δ	delta (deletion)
ATP	adenosine triphosphate
bd	*band*
bp	base pairs
CAMK-1	calcium/calmodulin-dependent kinase-1
C-box	clock-box
ccg	clock-controlled gene
CDO	choline deficiency oscillator
CK	casein kinase
CT	circadian time
DAG	diacylglycerol
DD	constant darkness
DNA	deoxyribonucleic acid
FFC	FRQ/FRH complex
FGSC	Fungal Genetic Stock Center
FLO	FRQ-less oscillator
FRH	FRQ-interacting RNA helicase
FRP	free-running period
frq	*frequency*

FWD-1	F-box/WD-40 repeat-containing protein-1
G	gram
h	hour
kb	kilobases
LD	light/dark cycles
LL	constant light
LM	Light Mutant
LMO	Light Mutant Oscilllator
min	minute
mRNA	messenger ribonucleic acid
nano	Nanometer (10-9)
NRA	nitrate reductase activity
NRO	nitrate reductase oscillator
nt	nucleotide
ORF	open reading frame
PAS	PER-ARNT-SIM
PCR	polymerase chain reaction
RNA	Ribonucleic Acid
Rpm	Rotation per minute
SCN	suprachiasmatic nucleus
UV	ultraviolet

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CHAPTER I

INTRODUCTION AND LITERATURE REVIEW

The Circadian Clock

Day and night cycles caused by the Earth's rotation have had a major impact on the evolution of rhythmicity in living organisms. As a result, almost every organism on Earth has developed an internal circadian clock for measuring biological time (Dunlap et al., 2004). This self-sustained endogenous circadian clock is not a mere response to environmental changes, but a beneficial adaptation in organisms to their various niches that allows them to anticipate recurring environmental changes (Edery, 2000; Woelfle et al., 2004).

Circadian rhythms are endogenous biological oscillations generated in organisms that occur approximately every 24 hours, and persist under constant conditions(Bruce and Pittendrigh, 1957). Daily rhythms in behavior, physiology, and gene expression are widespread in cyanobacteria, fungi, plants, and animals (Young and Kay, 2001). Some examples of rhythmic processes include nitrogen fixation in cyanobacteria (Golden et al., 1997), sexual and asexual development in fungi (Bobrowicz et al., 2002; Dunlap and Loros, 2004), photosynthesis in plants (Hennessey and Field, 1991), and the sleep-wake cycle in humans (Czeisler and Gooley, 2007).

A disrupted clock in humans can be the result of mutations in clock genes, or can be due to our behavior, including shift work and travel across time zones. The effects of disruption of the human clock range from irregular sleep patterns, jet lag, headaches, epilepsy, and other metabolic and neurological disorders (Turek et al., 2001). An understanding of the molecular basis of circadian rhythms is being applied in sleep and shift work medicine, chemotherapy, and other types of drug therapy. New fields of medicine have emerged, such as chrono-pathology and chronotherapy that incorporate results from circadian research to achieve maximum efficiency in therapies. Examples include the consideration of circadian timing in drug dosage to enhance targeting of tumors over normal tissues in cancer treatment (Hrushesky, 1985; Mormont et al., 2002), and melatonin treatments to reset the clock in the blind, in shift workers, and in individuals traveling across time zones (Sack and Lewy, 1997; Sack et al., 1991).

Circadian rhythms are distinguished from other biological oscillations by having the following properties:

1) Free running period: the rhythm peaks and troughs with a period of approximately 24h in a self-sustained manner under constant environmental conditions.

2) Entrainment: environmental cues reset the phase of the rhythm to 24 h. The endogenous clock runs with a period that is not exactly 24 h, and needs to be reset to the 24-h period of the external cycle in order to be predictive. For an oscillator to be indeed entrained by an environmental cue, its FRP must match the period of the entraining cycle with a stable phase relationship, and the phase imposed on it by the cue must be

maintained when the organism is shifted to constant conditions. Light and temperature are the major environmental cues that reset the clock.

3) Temperature and nutritional compensation: the rhythms are maintained with approximately the same period over a wide range of physiologically relevant temperatures and nutritional conditions. Q_{10} is the standard measure of the temperature dependency of a biological process. For a process to be considered temperature-independent, the Q_{10} should be approximately equal to 1.0. Circadian rhythms meet the requirement of temperature independence with Q_{10} values ranging from 0.8-1.3, whereas for most biochemical reactions, the Q_{10} ranges from 2 - 3. The Q_{10} for circadian rhythms is calculated as the ratio of the inverse of the period (the period rate) at a higher temperature to the inverse of the period at 10°C lower (Dunlap et al., 2004).

The Classic Circadian Clock Model

Circadian rhythms are produced by a timing mechanism, composed of input pathways, output pathways and oscillators (Figure 1-1).

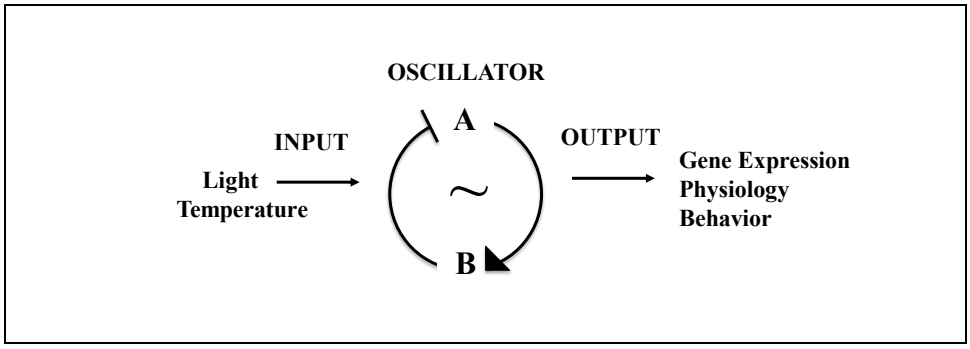

Figure 1-1. A simplified version of the circadian clock system. Refer to the text for details of the model.

Input pathways detect environmental cues (zeitgebers) and synchronize rhythms with external signals (Figure 1-1). At the molecular level, photoreceptors involved in entrainment have been identified in *Neurospora*, plants, *Drosophila*, and mammals, but not in cyanobacteria (Mackey et al., 2009). Once the photoreceptor is activated by light, it effects transduction of the signal to a component of the circadian oscillator and changes its levels or activity. This in turn causes a phase shift of the circadian oscillator (Crosthwaite et al., 1995).

An oscillator is made up of components that function at regular intervals to generate a rhythm. A circadian oscillator produces and maintains a rhythm with a period of about 24 hours. A pacemaker is an oscillator that can maintain its oscillations, and drive other oscillators, and control outputs (Bell-Pedersen et al., 2005). The molecular components

of eukaryotic circadian oscillators consist of positive elements and negative elements that interact to form an auto-regulatory negative feedback loop (Figure 1-1) (Baker et al., 2012).

The output pathways couple a circadian oscillator to clock-controlled genes (*ccgs*) and other circadian behaviors. In flies and mammals, overt activity rhythms are commonly monitored circadian outputs (Panda et al., 2002b). The most commonly assayed circadian output in *N. crassa* is the rhythm in asexual spore development (conidiation). Ccgs are genes whose time of day expression is controlled by a circadian oscillator and the term was first used in *N. crassa* (Loros et al., 1989). Thus, *N. crassa* was the pioneer organism for understanding clock regulation of rhythmic gene expression. The first set of rhythmically expressed genes, *ccg1* and *ccg2* in *N. crassa* were isolated by subtractive hybridization (Loros et al., 1989). Genes classified as *ccgs* are distinguished from oscillator genes, since clock function is not abolished in *ccg* deletion strains (Bell-Pedersen et al., 1992). Thereafter, microarray technology provided a more robust means for screening rhythmically expressed genes, where it was found that about 15% of the genome is clock-controlled at the level of transcript abundance (Correa et al., 2003; Nowrousian et al., 2003). Estimates in different organisms and tissues suggest that anywhere from 2-35% of the genome is under control of the clock (Covington et al., 2008; Panda et al., 2002a).

Molecular Mechanism of the Circadian Oscillator

Auto-regulatory molecular feedback loops comprise eukaryotic circadian oscillators (Aronson et al., 1994b; Lakin-Thomas, 2000). The components of the *Drosophila* and mammalian oscillators are conserved; however, with the exception of having shared domains, the components of the fungal clock are unique (Table 1-1) (Dunlap and Loros, 2004; Young and Kay, 2001). This intracellular network is a transcription/translation feedback loop (TTFL) involving clock genes that are either acting as transcription factors (positive elements), or biochemically as negative elements (Dunlap, 1999). The rise in the level of the negative elements, once each day through the activity of the positive elements is followed by down-regulation of positive elements by the negative elements. Subsequently, degradation of negative elements and new synthesis of positive elements restart the cycle. The positive elements in eukaryotic circadian oscillators are *N. crassa* WHITE COLLAR proteins WC-1 and WC-2, Drosophila CLOCK (CLK) and CYCLE (CYC), and mammalian CLOCK and BMAL1 (brain and muscle ARNT-like protein). FREQUENCY (FRQ) in *N. crassa*, PERIOD (Per) and TIMELESS (Tim) of *Drosophila*, and the mammalian CRYPTOCHROME (CRY) and PERIOD (PER) proteins are the negative elements of the oscillator (Bell-Pedersen et al., 2005) (Table 1-1).

The positive elements from these different classes of eukaryotes show evolutionary relatedness by possessing PAS motifs. The PAS domain was identified and named after a common domain found in *Drosophila* protein period (Per) vertebrate aryl hydrocarbon

nuclear translocator (ARNT) and *Drosophila* single-minded (Sim) (Nambu et al., 1991). In prokaryotes, a specialized PAS domain called the light, oxygen, voltage domain (LOV) allows proteins with the LOV domain to respond to these signals. In both pro- and eukaryotes, PAS domains are involved in protein-protein interactions (Huang et al., 1993; Taylor and Zhulin, 1999). In fact, all of the positive elements in eukaryotic circadian oscillators consist of heterodimers composed of two different proteins that interact via their PAS domains (Crosthwaite et al., 1997; Dunlap, 2008; King et al., 1997; Young and Kay, 2001). Furthermore, one of the two proteins is constitutively expressed, while the other is rhythmically expressed. Circadian output is derived, at least in part, from the action of the positive elements on the rhythmic expression of downstream targets of the oscillator (Rey et al., 2011; Smith et al., 2010a).

Neurospora crassa, a Model Organism for the Study of Circadian Clocks

Neurospora became a prominent tool in molecular genetics after being a successful experimental system for the establishment of "one gene-one enzyme hypothesis" by George Beadle and Edward Tatum (Beadle and Tatum, 1941). Since then, *N. crassa* has played a significant role in the fields of genetics, biochemistry, molecular biology, and circadian rhythms (Galagan et al., 2003). This haploid filamentous bread mold is now a model of choice for investigating a variety of biological problems, including circadian clocks, because of its simplicity (Bell-Pedersen et al., 1996), rapid growth rate, ease of transformation, availability of its genome sequence (Borkovich et al., 2004; Dunlap et al., 2007), and its innate ability to tell time (Pittendrigh et al., 1959). In addition,

Knockout (KO) strains are now available for almost every gene in the organism (Colot et al., 2006), reporter strains have been developed to assay gene expression (Freitag et al., 2004; Morgan et al., 2003), and regulatable promoters are available to study over expression or low level expression of a target gene (Aronson et al., 1994b; Freitag et al., 2004; Hurley et al., 2012).

N. crassa aerial hyphae grow and mature into bright orange spores called macroconidia (Springer, 1993). When the hyphae receive an environmental signal including illumination, desiccation, and carbon dioxide, or a signal from its endogenous clock, they form conidiophores that ultimately break off into macroconidia (Springer and Yanofsky, 1989) (Yoshida et al., 2008). The macrocondia will germinate to form new hyphae when they land on an appropriate substrate. This developmental rhythm provided an easy assay for circadian clock function and led to genetic analysis of the clock (Feldman and Hoyle, 1973).

The *N. crassa* Clock

N. crassa's role in chronobiology dates back to 1950's with reports of rhythms in conidiation that meet all of the criteria for a circadian rhythm (Pittendrigh et al., 1958; Sargent et al., 1966). Since this discovery, there has been tremendous progress in understanding its clockworks. The dissection of the *N. crassa* circadian system has yielded significant contributions to our current understanding of eukaryotic clock mechanisms, including how the feedback loop functions, light and temperature resetting, and temperature compensation of the clock (Baker et al., 2012; Dunlap, 1999; Heintzen and Liu, 2007; Liu and Bell-Pedersen, 2006).

The *N. crassa* conidiation rhythm is commonly assayed using special growth tubes called race tubes (Figure 1-2). Race tubes are hollow glass tubes about 40 cm long, 16mm in diameter, bent at both ends to hold agar medium. Wild- type cultures of *N. crassa* grow across the agar medium at a constant growth rate of about 3-4 cm a day, depending on temperature and nutritional factors (Bell-Pedersen et al., 1996a). Race tubes are inoculated with mycelia or conidia, and allowed to grow for one day under constant illumination (LL) and constant temperature. The growth front is marked before transfer of race tubes to constant darkness (DD), and marked subsequently at 24h intervals under a safe red light (the oscillator(s) responsible for the developmental rhythm is not responsive to red light). In DD, the growth front alternates between a band of conidia and hyphae every 22h. One can calculate the period length of the rhythm as the distance between the peaks of conidiation, and phase of the rhythm of conidiation by the position of the band relative to the initial light to dark transfer (Bell-Pedersen, 1998)

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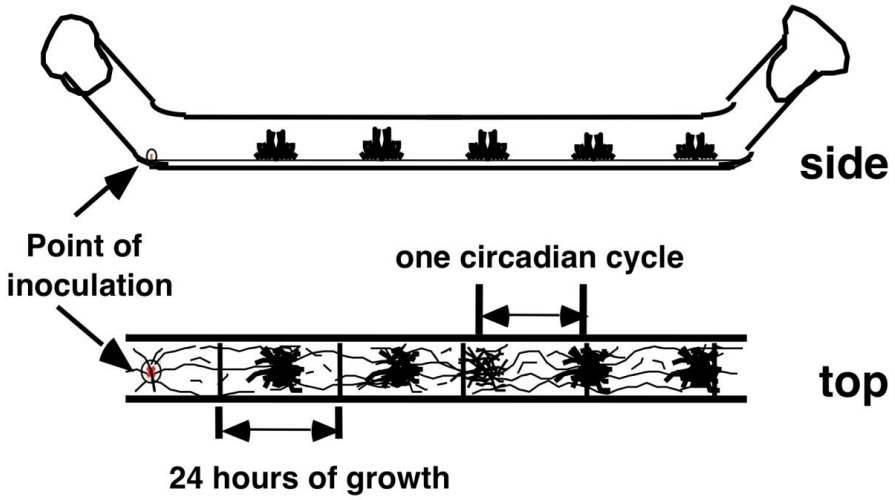

Figure 1-2. The race tube assay for circadian rhythms of development in *N. crassa* (Bell-Pedersen, 1998)***.** See the text for details of the figure.

On race tubes, rhythms in asexual spore formation in wild-type strains are thought to be masked due to a buildup of carbon dioxide. The *band* (*bd)* mutant strain helps to clarify the conidiation rhythm despite the CO_2 build up without affecting clock properties (Sargent et al., 1966). This mutation is widely used in studies of circadian rhythms. The *bd* mutation was recently determined to be a dominant allele of the *ras-1* gene (Belden et al., 2007). Petri plates and capped 150mm test tubes are also used to assay conidiation rhythms (Loros and Dunlap, 2001).

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*Fig. 1-2 Reprinted with permission from "Keeping pace with Neurospora circadian rhythms" by Bell-Pedersen, 1998. Microbiology, Volume 144, Pt.7: 1699-1711.

The FRQ/WCC oscillator (FWO) has long been considered to be the core oscillator in *N. crassa* (Figure 1-3; Table 1-2). Its major components are the negative element FRQ, and the positive elements WC-1 and WC-2. These components meet the criteria of clock components, as mutations or alterations in amounts of the components change the period length of the developmental rhythms, and abrupt changes in amounts of the components can reset the clock to a new phase (Aronson et al., 1994a; Bell-Pedersen et al., 2001). Null mutations of *frq*, *wc-1*, and *wc-2* abolish developmental rhythms under standard growth conditions (Lakin-Thomas, 2006b) .

In *N. crassa*'s molecular oscillator, WC-1 and WC-2 form a heterodimer through their PAS domains called the White Collar Complex (WCC) (Ballario et al., 1996; Talora et al., 1999). In DD, the WCC binds the Light Responsive Elements (LRE) of the *frq* promoter, which leads to the activation of *frq* transcription and a gradual increase in the levels of FRQ protein (Figure 1-3) (Froehlich et al., 2002). FRQ dimerizes and forms a complex with FRH (FRQ-interacting RNA helicase) (Cheng et al., 2005; Cheng et al., 2001a). The FRQ/FRH complex binds to, and promotes the phosphorylation of, the WCC by recruiting several kinases, CASEIN KINASE I (CKIa) and CASEIN KINASE II (CK II) (Yang et al., 2003), and the priming kinase, PROTEIN KINASE A (PKA) (He et al., 2005a; He and Liu, 2005; Huang et al., 2007). Once WCC is hyperphosphorylated, its activity is down-regulated such that it is unable to bind DNA and activate *frq* transcription. This leads to a decrease in *frq* transcript levels and a drop in FRQ protein production. Phosphorylation of FRQ by several kinases, including CKI, CKII, CAMK,

PKA, and PRD4, induces degradation of FRQ (Pregueiro et al., 2006; Yang et al., 2001). In addition, phosphorylated FRQ's interaction with FWD-1 (an ortholog of Drosophila's Slimb protein) leads to its ubiquitination and subsequent degradation by the proteaseome (He et al., 2003).

FRQ degradation is accompanied by dephosphorylation of WCC by phosphatases PP1 and PP2A. (Table 1-2) (Liu, 2005). Together, these events lead to the reactivation of *frq* transcription, allowing the cycle to restart the next day. In a positive feedback loop, FRQ maintains the levels of WC-1 and WC-2 proteins through phosphorylation. FRQ-induced phosphorylation of the WC proteins leads to increased stability of the proteins (Cheng et al., 2002; Querfurth et al., 2007). Thus, phosphorylated WCC is more stable, but unable to activate FRQ transcription.

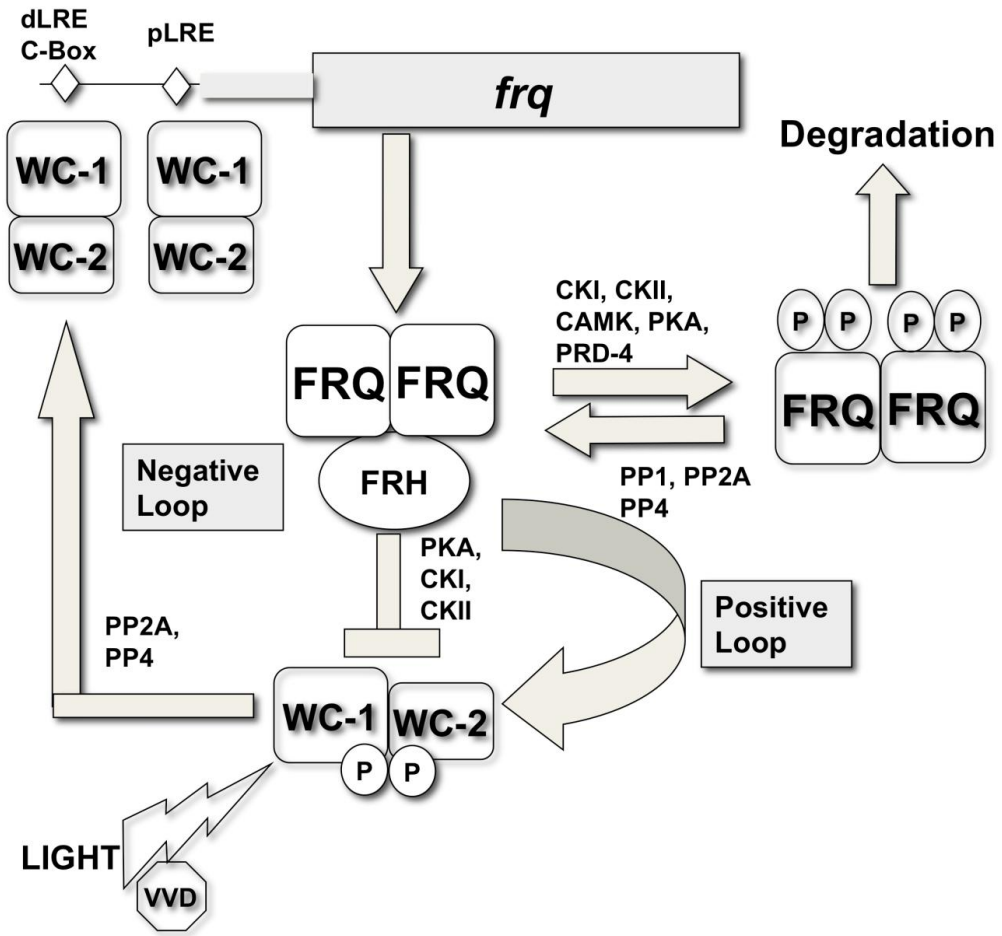

Figure 1-3. Model of the FWO. See the text for details of the model Vitalini *et al*., 2010. (Borkovich and Ebbole, 2010)*.

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Oscillator Complexity in *N. crassa* and Other Organisms

Several lines of evidence support the idea that multiple oscillators populated by multiple feedback loops exist within cells of unicellular and multicellular organisms (Young and Kay, 2001). It has been shown that overt rhythms are generated by individual cells in simple systems like *Gonyaulax* and *N. crassa*, as well as molecular rhythms in complex systems (Lakin-Thomas and Brody, 2004; Welsh et al., 1995). Early work by Pittendrigh and Bruce 1959 proposed a two-oscillator model to explain pupal eclosion rhythm in *Drosophila*. In this system, the oscillator responsible for the eclosion rhythm is driven by a light-sensitive temperature-compensated daytime oscillator (Pittendrigh, 1993). The presence of multiple coupled oscillators was also proposed in rodents to explain the splitting phenomenon (Pittendrigh and Daan, 1976). Splitting was first reported in rodents subjected to constant light (Pittendrigh, 1960). During splitting, the behavioral rhythm dissociates into two components, which become stably coupled in an antiphase relationship to each other. The discovery of splitting led to a model in which the clock is composed of two coupled distinct oscillators, an evening (E) and morning (M) oscillator that together provide a beneficial adaptation of the circadian system to different photoperiods (Daan et al., 2001).

In the unicellular marine alga, *Gonyaulax*, it's been shown that there are at least two independent oscillators with different light spectral sensitivities that control different outputs (aggregation and bioluminescence). The rhythm in aggregation is controlled by the A oscillator that responds to both blue and red light, whereas the B oscillator that

drives the bioluminescence rhythm is sensitive to only blue light. Consequently, the period length and the phase of these two distinct outputs are different from each other. In addition to the periods of these two outputs being independently altered by the same light pulse, the oscillators are also reset differently in the presence of a nutrient, creatine (Ronneberg and Morse, 1993; Morse et al, 1994; and Ronneberg, 1996). In plants, different outputs having different free-running periods have also been reported, further supporting the presence of multiple oscillators in organisms (Hotta et al., 2008).

There are distinct oscillators in different tissues in *Drosophila*, birds and mammals that can autonomously drive rhythmic output. For example, in *Drosophila*, the makeup of the peripheral oscillators differs among tissues (Bell-Pedersen et al., 2005; Giebultowicz, 2001). The mammalian circadian system is hierarchical with the suprachiasmatic nucleus (SCN) serving as the pacemaker (Ko and Takahashi, 2006). The light-entrainable SCN coordinates the timing of slave oscillators in other parts of the brain, and in peripheral organs, such as the liver and kidney (Yamazaki et al., 2000). Interestingly, these slave oscillators can sustain endogenous rhythms without input from the SCN for a few days provided they are synchronized by some exogenous signal. While some of the peripheral oscillators are identical to the pacemaker, it is not known if all peripheral oscillators are built using the same components as the SCN oscillator.

In *N. crassa*, several studies have shown that rhythms can persist in the absence of a functional FWO under certain growth conditions and genetic backgrounds, providing evidence for the existence of additional oscillators in the cell. These reports of residual rhythmic conidiation and gene expression in strains that lack FWO components suggest that the FWO is not the only oscillator driving rhythms in the fungus. The term FLO (*frq*-less-oscillator) was coined to refer to the class of putative oscillators that drive rhythms when the FWO is impaired (Iwasaki and Dunlap, 2000). It is not clear if there is one or multiple FLO's. Furthermore, most of the rhythms attributed to FLOs lack one or more fundamental clock properties (Dunlap and Loros, 2004). In most cases, the rhythms are only observed under specific growth or genetic conditions (Lakin-Thomas et al., 2011). These data led to the idea that the FWO serves as a pacemaker in *N. crassa* cells, driving rhythms in downstream "slave" oscillators. In this model, the slave oscillators are intrinsically rhythmic, but require the FWO for full circadian properties. However, the role of the FLO's in the circadian system is only speculative, as components have not yet been identified (Liu and Bell-Pedersen, 2006) .

A number of genetic backgrounds and growth conditions have been shown to enhance FLO developmental rhythms in FWO mutant strains (Aronson et al., 1994a; Loros and Feldman, 1986). First, strains that lack *frq* were found to show occasionally rhythmic behavior on race tubes. This rhythm in null strains became more robust when the cultures were given 12-h temperature cycles (Merrow et al., 1999). However, a question of whether the rhythm in temperature cycles was entrained (Roenneberg et al., 2005)

versus being driven by environmental signals (Pregueiro et al., 2005) arose. Studies from (Lakin-Thomas, 2006a) indicated both entrained and driven components of *frq* null strains in temperature cycles supporting the idea of FLOs in *N. crassa*. WC mutants have also been reported to exhibit a rhythm in development with a period of ~24h on sugarless medium in DD (Dragovic et al., 2002). Other examples of nutritional conditions that uncover FWO-independent rhythms include, single null *frq,* wc-*1,* and *wc-2* strains grown on media supplemented with farnesol, geraniol (Granshaw et al., 2003) or menadione (Brody et al., 2010). In addition, double mutants Δ*frq* Δ*cel* strains grown in media containing fatty acids, and Δ*frq* Δ*chol* strains grown under choline starvation (Lakin-Thomas and Brody, 2000) display rhythmic conidiation with long periods. A new mutation, UV-90 required for a functional FLO has been uncovered in the Δ*frq* Δ*chol* strain. In the UV-90 mutant, rhythmicity is abolished under low choline conditions, and the amplitude of the heat-entrainable oscillator is reduced grown in the presence of choline (Lakin-Thomas, 2011). Δ*frq*Δ*sod-1 (Yoshida et al., 2008)* and *ult* mutant strains in various FWO null-mutant backgrounds (Lombardi et al., 2007) have also been shown to restore conidiation rhythms. In all of these cases, the conidiation rhythms were assayed in DD.

Rhythmic conidiation has recently been reported in *N. crassa* in LL. This FLO was uncovered in *vvd* mutant strains. The rhythms were observable only when the strain was grown in Petri plates, and not visible in race tubes. The period of the rhythm was highly variable ranging from 6-21 h (Schneider et al., 2009). Recently, (Hunt et al., 2012)

observed that a multiple deletion strain carrying null mutations of *frq*,*wc-1*, *wc-2* and *vvd* responded to temperature cycles, but required components of the FWO for robust rhythms and full circadian properties.

Another category of FLOs regulating molecular rhythms include the Nitrate Reductase Oscillator (NRO) that drives circadian rhythms in nitrate reductase activity (NRA) (Christensen et al., 2004) and rhythms in the diacylglycerol levels in *frq* null strains. The NRO is nutritionally induced by growing *N. crassa* cells in media containing nitrate as the sole source of nitrogen. The NRO rhythms, observed in LL and DD, are more robust in a *frq*-null mutant strain than in a Δ*wc-1* mutant strain. A report of the rhythms in the levels of ccgs in *frq* null strains (Correa et al., 2003) was followed by the discovery of the WC-FLO that regulates rhythms in expression of the *ccg-16* gene (de Paula et al., 2006). The WC-FLO is the only FLO known to possess all the canonical circadian properties, but it also appears to be directly linked to the FWO in that it requires WC-1 and WC-2 (but not FRQ) for rhythmicity. Together this work points to the presence of other oscillators in *N. crassa*; however, molecular details of the oscillators are lacking.

The presence of multiple oscillator loops in the circadian system of organisms is thought to ensure robustness and stability (Cheng et al., 2001b; de Paula et al., 2007; Stelling et al., 2004) as well as precision and flexibility of biological rhythms (Locke et al., 2006). Robustness refers to the system being unaffected by endogenous and exogenous disturbances (Kitano, 2004). Flexibility allows different clock components to respond to

different environmental signals (Rand et al., 2004) which can either decrease or increase the robustness of the system (Kitano, 2004). Mathematical modeling of circadian oscillators demonstrated that greater flexibility can enhance the robustness of the circadian clock in *N. crassa* (Akman et al., 2010). Thus, the presence of multiple oscillators in *N. crassa* is consistent with a robust circadian clock that responds to several input signals.

Photoreception in *N. crassa*

Light is the most important cue for growth, development and ultimately, the survival of most organisms. *N. crassa* is only known to sense blue light (Ballario et al., 1996; Cheng et al., 2003b), although the genome sequence revealed the presence of red and green photoreceptors (Borkovich et al., 2004). The blue light-induced processes in *N. crassa* include carotenoid biosynthesis, hyphal branching, conidiation, protoperithecia development, and photo entrainment of the circadian clock (Lauter, 1996; Liu, 2003). In addition to the role of WC-1 and WC-2 in the circadian feedback loops, these two transcription factors are essential for *N. crassa* blue light responses (Ballario et al., 1996; Linden and Macino, 1997; Liu, 2003). WC-1 is the main blue-light receptor in *N. crassa*. The LOV domain of WC-1 binds the flavin chromophore FAD (Froehlich et al., 2002; He et al., 2002). Based on studies of LOV domains in plant and fungal photoreceptors (Crosson et al., 2003; Zoltowski and Crane, 2008), binding of FAD to WC-1 likely results in a conformational change in WC-1, which may open up target sites for kinases and phosphatases. In support of this model, both WC-1 and WC-2 undergo light-induced

changes in phosphorylation status (Schwerdtfeger and Linden, 2000; Talora et al., 1999). Light-induced changes in phosphorylation of WC-1 may in turn lead to a change in the ability of WC-1 to interact with WC-2, a change in WC-1 interaction with downstream signaling components, and/or a change in the stability of WC-1. Consistent with these ideas, several studies have suggested that WC-1 phosphorylation in response to light triggers its degradation (He et al., 2005b; Lee et al., 2000; Talora et al., 1999). WC-2 is also required for all light responses; however, the role of WC-2 in light responses is not as a photoreceptor, but it probably functions in light responses through its ability to stabilize WC-1 (Liu, 2003). The *wc* genes are required for light induction of *frq*, demonstrating an essential role for the WC proteins in light input to the clock (Crosthwaite et al., 1997). After light exposure, a large WCC (L-WCC) is activated through the WC-1 photosensory LOV domain (Froehlich et al., 2002). This light-activated L-WCC binds 2 elements in the *frq* promoter, dLRE and pLRE (Figure 1-3**),** leading to light-induced activation of *frq* transcription at any time of day (Froehlich et al., 2002; He et al., 2005b).

VVD is another blue-light photoreceptor in *N. crassa,* and noncovalently binds a flavin adenine dinucleotide (FAD) (Schwerdtfeger and Linden, 2003). *vvd* mutants are phenotypically distinguished from the WT strain because of their bright orange mycelia in LL due to overproduction of carotenoids. VVD is a protein with a single LOV domain (Heintzen, Loros et al. 2001) that shares a 42% sequence identity and 72% similarity to the LOV domain of WC-1 (Schwerdtfeger and Linden 2003). When the WCC LOV

domain is substituted with the VVD LOV domain, partial light sensing function is retained (Cheng et al., 2003a). In *vvd* loss of function mutants, WCC-dependent blue light responses are elevated and photoadaptation is lost, indicating that VVD functions as a general repressor of light responses through interactions with the WCC (Figure 1- 3) (Schwerdtfeger and Linden, 2001; Shrode et al., 2001). Previous work had indicated that VVD was localized in the cytoplasm, while WCC in the nucleus (Schwerdtfeger and Linden, 2003). However, *recent* studies on understanding the molecular mechanism of VVD's repressor function on WCC have shown that there is a direct physical interaction between WCC and VVD (Chen et al., 2010). At night, VVD inhibits WCC light responses by serving as a molecular memory of the brightness of the preceding day light and quells low light intensity from the moon. When daylight returns, VVD is degraded and WCC is switched on again. VVD inhibits. Thus, the mechanism for inhibition of WCC light responses by VVD is that VVD prevents the circadian clock from mixing up day and night (Malzahn et al., 2010). *vvd* transcripts are also clock-controlled. Whereas, VVD is not essential for the circadian clock to run in DD, *vvd* mutants exhibit an increased resetting response to light pulses. These data demonstrate that VVD functions as an output of the clock, and in turn provides a "gate" mechanism for light input to the clock (Heintzen et al., 2001).

N. crassa's completed genome sequence revealed the presence of other putative photoreceptors with similarity to bacterial and eukaryotic photoreceptors. These putative proteins include the blue-light photoreceptor cryptochrome (CRY), two red-light

phytochromes (PHY-1 and PHY-2) and a green light opsin (NOP-1). The potential roles for these additional photoreceptors in light sensing are not known (Borkovich et al., 2004; Galagan et al., 2003; Purschwitz et al., 2006). KO's of *phy-1*, *phy-2* and *cry* exhibit phenotypes similar to wild type (Chen and Loros, 2009; Purschwitz et al., 2006); while a *nop-1* mutation affects the expression levels of some developmentally-regulated genes during the later stages conidiation (Bieszke et al., 2007).

N. crassa's CRYPTOCHROME belongs to the DASH-type cryptochrome class (Daiyasu et al., 2004). It has an FAD binding domain, a methenylhydrofolate binding domain, and shares sequence similarity with DNA photolyase. However, its DNA photolyase ability does not include DNA repair. The expression of *cry* transcript and CRY protein are blue - light- regulated in *N. crassa* and dependent on WCC. Deletion of the *cry* gene does not disturb the conidiation rhythm, suggesting that it is not a clock component (Froehlich et al., 2010). In *Drosophila*, cryptochrome functions as circadian photoreceptor, and it is a part of the circadian feedback loop functioning as repressor of Tim (Emery et al., 1998). Mammalian cryptochromes are essential core clock components (Harmer et al., 2001; Young and Kay, 2001) (Table 1-1). *Arabidopsis* has multiple cryptochromes with circadian function (Somers et al., 1998). *N. crassa*'s CRY is light-regulated and is a direct target of the WCC. The deletion of cry does not affect light regulated genes (Froehlich et al., 2010).

Evidence for the Novel Light Mutant Oscillator (LMO) in *N. crassa*

To identify components of the FLO's, a Δ*frq;wc-2*^{234}*w* strain that lacked a functional FWO was mutagenized by UV light and screened in T cycles for loss of rhythmicity (Figure 1-4) (K. Suk Seo and D. Bell-Pedersen, unpublished). While, mutants that were arrhythmic were identified in the screen, most appeared to have developmental defects that were not associated with the clock. Fortuitously, all of the mutant strains were also grown in LL for a couple of days. Two mutants were identified that were rhythmic in LL, Light Mutant 1 (LM1) and LM2. The rhythm in LM1 was found to be more robust than LM2, and therefore most of my efforts have focused on characterizing this mutant. The LM1 mutant strain displays robust conidiation rhythms in LL, independent of whether or not the FWO is functional. Our studies also demonstrated that the LM1 mutation is a recessive mutation, suggesting (but not proving) that it is a loss of function allele. If indeed it is a loss of function mutation, this would suggest that the gene specified by the LM1 mutation encodes a protein that directly or indirectly negatively regulates the LMO, rather than functions as a component of the LMO.

Steps towards fully characterizing the novel LMO include: 1) testing the LMO for circadian properties, 2) identifying and characterizing the gene that is mutated in the LM1 strain, 3) testing a model that the FWO activates LM1 and represses the activity of the LMO, and 4) assessing the role of candidate photoreceptors in LMO light responses. This study will enhance our understanding of how multiple oscillators interact and integrate within cells to control circadian rhythms.

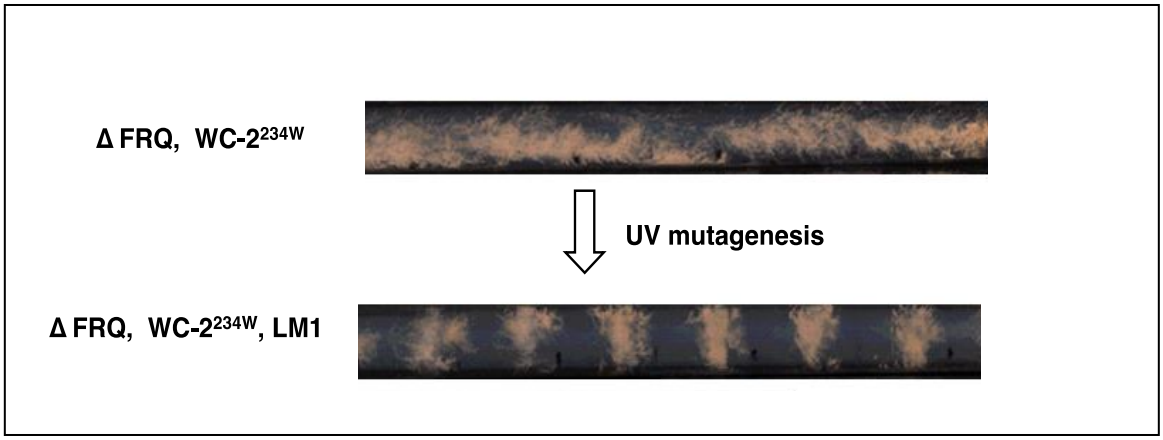


Figure 1-4. Identification of the LM1 mutation uncovers the LMO. A *bd*, *wc-2*^{234w}, *frq*^{10} strain was UV mutagenized and the resulting strains were assayed in LL for developmental rhythms on race tubes. The mutant strains, LM1 has a period of about a day in constant bright light (1200lux). *frq*^{10} is a *null* allele of *frq*, and *wc-2*^{234w} generates a truncated and non-functional WC-2 protein (Linden and Macino, 1997).

Table 1-1. Canonical clock genes in diverse organisms. [a]

Organism	Clock Gene	Protein Component	Role in Clock
Synechococcus elongatus strain PCC 7942	*kaiA*	KaiA	Positive element
	kaiC	KaiC	Negative element
Neurospora crassa	*frq*	FRQ	Negative element
	*wc-1**	WC-1*	Positive element/ Photoreceptor
	*wc-2**	WC-2*	Positive element
Drosophila melanogaster	*per*	PER	Negative element
	tim	TIM	Negative element
	*clk**	CLK*	Positive element
	*cyc**	CYC*	Positive element
	cry	CRY	Photoreceptor
Mouse/Humans	*mPer 1, mPer 2, mPer 3*	PER	Negative elements
	*Clk**	CLOCK*	Positive element
	Bmal1(MOP3)*	BMAL1*	Positive element
	Cry 1, Cry 2	CRY 1, CRY2	Negative elements

[a] *Adapted from Lakin-Thomas, 2000.*
**contains PAS domain*

Table 1-2. Some essential and non-essential *Neurospora* FRQ/WCC Circadian Clock Components [a,b]

Protein name	Clock-related role
WC-1	Required for circadian rhythms. Positive element in the feedback loop. Circadian photoreceptor and regulates blue- light responses in *N. crassa* . Physically interacts with WC-2 to form the White Collar Complex (WCC).
WC-2	Required for circadian rhythms. Heterodimerizes with WC-1 to form the WCC. Functions with WC-1 in activating *frq* transcription.
FRQ	Required for circadian rhythms. FRQ forms homodimers and also binds to FRH. It negatively regulates its own transcription by repressing WCC activity.
FRH (FRQ -interacting RNA helicase)	Required for circadian rhythmicity. Dimerizes with FRQ to repress WCC activity.
VVD	Not required for circadian rhythmicity. Required for photoentranment and maintaining proper phase at different temperatures. Photoreceptor that gates WCC activity and is responsible for photo adaptation.
CKIa (Casein Kinase Ia)	Binds and phosphorylates FRQ to promote its degradation. Mediates FRQ-dependent WC phosphorylation.
CKII (Casein Kinase II)	Complex is made up of CKA, CKB1 and CKB2. Required for circadian rhythmicity. Regulates FRQ stability and its repressor activity. Mediates FRQ-dependent WC phosphorylation.
CAMK-1	Kinase that phosphorylates FRQ *in vitro*. Mutations lead to changes in light-induced phase shifts and a slight change in period.
PRD4	Checkpoint kinase that phosphorylates FRQ in response to DNA damage. Not required for clock function.
PP1	Phosphatase that regulates FRQ stability probably through dephosphorylation.
PP2A	Phosphatase, its regulatory subunit, RGB-1 is important for closing the negative feedback loop.
PP4	Phosphatase, role in dephosphorylation of WCC, which promotes its nuclear localization.

[a] Adapted from Heintzen and Liu, 2007.

[b] Adapted from Vitalini *et al* ., 2010 (Borkovich and Ebbole, 2010)

CHAPTER II

A NOVEL CRYPTOCHROME-DEPENDENT OSCILLATOR IN NEUROSPORA CRASSA

Introduction

Circadian clocks, composed of molecular 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 in order 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 (Cambras et al., 2007; Morse et al., 1994; Sai and Johnson, 1999). Second, residual rhythmicity is found in some strains defective in known oscillator components (Collins et al., 2005; Emery et al., 2000; Loros and Feldman, 1986; Stanewsky et al., 1998). Third, some tissue-specific oscillators are constructed differently from core oscillators located in the brains of insects and animals (Collins et al., 2005; Emery et al., 2000; Ivanchenko et al., 2001; Krishnan et al., 2001; Stanewsky et al., 1998). Thus, multiple oscillators may exist both within, and among, cells in organisms with differentiated tissues.

The fungus *Neurospora crassa* is a leading model for studying the clock (Baker et al., 2012; Bell-Pedersen, 2000; Heintzen and Liu, 2007; Lakin-Thomas et al., 2011) and

light signaling (Bell-Pedersen et al., 2001; Chen and Loros, 2009; Linden et al., 1997; Liu, 2003; Merrow et al., 2006). In *N. crassa*, the core FRQ/WCC oscillator (FWO) consists of a transcriptional/ translational feedback loop involving the negative element FREQUENCY (FRQ), 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. The WCC binds the *frq* promoter and directly activates transcription of the *frq* gene (Froehlich et al., 2003). As FRQ protein accumulates it interacts with itself and FRQ-interacting RNA helicase (FRH) (Cheng et al., 2005; Cheng et al., 2001a), and then binds to, and promotes the phosphorylation, and inactivation, of the WCC (He et al., 2006; Schafmeier et al., 2005). This inhibition of the WCC 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. One of the most easily observed rhythms in *N. crassa* is asexual spore development (conidiation), which is measured using the race tube assay, and has a period in constant conditions of about 22 h (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 for the existence of additional oscillators in *N. crassa* cells (Aronson et al., 1994a; Brody et al., 2010; Christensen et al., 2004; Correa et al., 2003; de Paula et al., 2006; Dragovic et al., 2002; Granshaw et al., 2003; He et al., 2005a; Hunt et al., 2012;

Loros and Feldman, 1986; Merrow et al., 1999; Ramsdale and Lakin-Thomas, 2000). The term FLO (*frq*-less-oscillator) was coined to collectively describe these putative circadian and/or non-circadian oscillators (Iwasaki and Dunlap, 2000). Indeed, in most cases, the 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 about 24 h in the absence of environmental cues, entrainment of the free-running rhythm to 24 h by environmental cues, and temperature compensation of the clock (Baker et al., 2012; Lakin-Thomas, 2000). 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 means that the oscillator runs with a similar rate 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. While studies have been undertaken to identify molecular components of the FLO(s) (Hunt et al., 2012; Lakin-Thomas et al., 2011; Lombardi et al., 2007; Schneider et al., 2009; Shi et al., 2007; Yoshida et al., 2008), little is understood regarding their nature and function in the circadian system.

In an attempt to identify key components of the FLO(s), we carried out a genetic screen for mutations that enhance rhythmicity in strains that lack both the positive and negative components of the FWO. We identified a mutation called Light Mutant 1 (LM1) that displayed robust rhythms in conidiation in LL. The oscillator controlling these rhythms, called the Light Mutant Oscillator (LMO), fulfills two of the three criteria for a circadian oscillator; it free runs in constant conditions with a period close to a day, and is temperature compensated. However, while LM1 mutant strains are synchronized by LD cycles independent of WC-1, circadian entrainment in LD requires WC-1. Finally, I show that the blue-light photoreceptor CRYPTOCHROME (CRY) is necessary for LMO activity.

Materials and Methods

Strains

The *N. crassa* strains used in this study are listed in Table 2-1, and their period lengths under different growth conditions in Table 2-2. The *vvd* knockout (KO) strain was obtained from Dr. Christian Heintzen (Heintzen et al., 2001). The *Δwc-1::hph* stain was obtained from Dr. Jay Dunlap, and *Δwc-1::bar* was generated in our lab (Bennett et al., 2013) the *cry* KO (FGSC 12981) was generated by the *N. crassa* KO project (Colot et al., 2006) and obtained from the Fungal Genetics Stock Center (FGSC). All strains used in this study carry the ras^{bd} mutation, which clarifies the conidiation rhythm on race tubes (Belden et al., 2007; Sargent et al., 1966), and serves as the clock wild type control

strain. To generate the LM1 mutation, a strain defective in the positive and negative arm of the FWO (*wc-2*^{234W}, *ras*^{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 are described in Figure 2-9. To generate double and triple KO strains, a new *wc-1* deletion strain was generated by replacing the *wc-1* ORF with the *bar* gene conferring resistance to glufosinate (Bennett et al., 2013), and this construct was verified by PCR. The *Δcry*, LM1 strain was cotransformed with a 4.9 kb PCR fragment containing the entire *cry* gene and pBARGPE1 that contains the *bar* gene (Pall and Brunelli, 1993). To determine if the LM1 mutation is dominant or recessive, a heterokaryon of strain **(***ras*^{bd}**,** *arg-5*, *mat a*) and *ras*^{bd}, *his-3*, *lm1*, was generated. Heterokaryons were selected by growth on minimal media as described (Davis and de Serres, 1970). Heterokayotic strains with nuclear ratios of about 1:1 were further examined for rhythmicity on race tubes.

PCR was used to verify the genotypes of double and triple photoreceptor mutant strains using standard techniques. Genomic DNA was isolated from mycelia from 7-day old slant cultures as described (Jin et al., 2007). The PCR primers used to verify the KO strains are listed in Table 2-3.

Growth Conditions

All vegetative cultures were maintained on 1X Vogel's, 2% glucose, minimal medium with the appropriate supplements as required (Davis and de Serres, 1970; Vogel, 1956).

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 200ug/ml hygromycin B (Sigma Aldrich, St. Louis, MO). KO strains containing the *bar* gene were maintained on nitrate free Vogel's medium supplemented with glufosinate (Sigma Aldrich, St. Louis, MO). The composition of race tube media was 11.5 ml of 1X 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 allowed to grow for one day at 25°C, 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. Light intensity was measured with a VWR Scientific Dual Range Light Meter (Radnor, PA), and maintained at 1200 lux in all light experiments. The temperature of the chamber was monitored using an RH/Temp Data Logger (MicroDAQ.com Ltd., Contoocook, NH). The growth fronts of the race tubes were marked at 24h intervals using a red light for cultures in DD, and at lights on for cultures in light/dark (LD) cycles. Race tubes were scanned with an EPSON scanner (Long Beach, CA), and growth rates and periods were calculated from at least 12 replicate tubes per strain per condition. In our experiments, n = the number of conidial bands measured on replicate race tubes.

Table 2-1. List of strains used in this study.

Strain name	Genotype	Lab Strain number	Source/Reference
ras^{bd}, *A*	*ras-1*^{bd}, *A*	DBP 369	FGSC 1858
ras^{bd}, *a*	*ras-1*^{bd}, *a*	DBP 368	FGSC 1859
LM1, *A*	*ras-1*^{bd}, LM1, *A*	DBP 694	this study
LM1, *a*	*ras-1*^{bd} , LM1, *a*	DBP 695	this study
Δ*frq*, *a*	*a*, *ras-1*^{bd}, *frq*^{10}	DBP 287	Aronson et al., 1994
Δ*frq*, *A*	*A*, *ras-1*^{bd}, *frq*^{10}	DBP 776	FGSC 7490
Δ*frq*, *LM1*, *A*	LM1, *A*, *ras-1*^{bd}, *frq*^{10}	DBP 831	this study
Δ*wc-1*, *A*	*A*, *ras-1*^{bd} , Δ*wc-1* ^{hyg}	DBP 580	Lee at al., 2003
Δ*wc-1*, LM1	LM1, *A*, *ras-1*^{bd} , *wc-1* ^{hyg}	DBP 696	this study
Δ*wc-1*, *A*	*A*, *ras-1*^{bd}, Δ*wc- 1*^{bar}	DBP 1223	Laboratory stock
Δ*wc-1*, LM1, *a*	LM1, *a*, *ras-1*^{bd}, Δ*wc-1*^{bar}	DBP 1369	this study (DBP 1223 x DBP 695)
Δ*vvd*, *a*	*a*, *ras-1*^{bd}, Δ*vvd* ^{hyg}	DBP 693	Heintzen et al., 2001
Δ*vvd*, *A*	*A*, *ras-1*^{bd}, Δ*vvd* ^{hyg}	DBP 1634	this study (DBP 369 x DBP 693)
Δ*vvd*, LM1, *A*	LM1, *A*, *ras-1*^{bd}, Δ*vvd* ^{hyg}	DBP 1335	this study (DBP 693 x DBP 694)
Δ*vvd*, LM1, *a*	LM1, *a*, *ras-1*^{bd}, Δ*vvd* ^{hyg}	DBP 1638	this study (DBP 695 x DBP 1335)
Δ*cry*, *a*	*a*, Δ*cry* ^{hyg}, *ras-1*^{bd}	DBP 963	Laboratory stock
Δ*cry*, LM1, *A*	LM1, *A*, *ras-1*^{bd}, Δ*cry* ^{hyg}	DBP 1022	this study (DBP 963 x DBP 694)
Δ*cry*, Δ*wc-1*, *A*	*A*, *ras-1*^{bd}, Δ*cry* ^{hyg} , Δ*wc-1*^{bar}	DBP 1598	this study (DBP 963 x DBP 1223)
Δ*cry* ,Δ*wc-1*, *a*	*a*, *ras-1*^{bd}, Δ*cry* ^{hyg}, Δ*wc-1*^{bar}	DBP 1599	this study (DBP 963 x DBP 1223)
Δ*cry*, Δ*wc-1*, LM1, *A*	LM1, *A*, *ras-1*^{bd} , Δ*cry* ^{hyg} , Δ*wc-1*^{bar}	DBP1645	this study (DBP 1022 x DBP 1369)
Δ*cry*, Δ*vvd*, *a*	*a*, *ras-1*^{bd}, Δ*cry* ^{hyg} , Δ*vvd* ^{hyg}	DBP 1640	this study (DBP 693 x DBP 963)
Δ*cry*, Δ*vvd*, *A*	*A*, *ras-1*^{bd}, Δ*cry* ^{hyg}, Δ*vvd* ^{hyg}	DBP 1600	this study (DBP 693 x DBP 963)
Δ*cry*, Δ*vvd*, LM1, *A*	LM1, *A*, *ras-1*^{bd}, Δ*cry*^{hyg} , Δ*vvd* ^{hyg}	DBP1601	this study (DBP 1022 x DBP 1638)
Δ*vvd*, Δ*wc*-1, *A*	*A*, *ras-1*^{bd}, Δ*vvd* ^{hyg}, Δ*wc- 1*^{bar}	DBP 1639	this study (DBP 693 x DBP 1223)
Δ*vvd*, Δ*wc-1*, *a*	*a*, *ras-1*^{bd}, Δ*vvd* ^{hyg}, Δ*wc-1* ^{bar}	DBP 1637	this study (DBP 693 x DBP 1223)
Δ*vvd*, Δ*wc-1*, LM1, *A*	LM1, *A*, *ras-1*^{bd} , Δ *vvd* ^{hyg} , Δ *wc-1*^{bar}	DBP 1635	this study (DBP 1335 x DBP 1369)
Δ*vvd*, Δ*wc-1*, LM1, *a*	LM1, *a*, *ras-1*^{bd}, Δ*vvd* ^{hyg,}, Δ*wc-1*^{bar}	DBP 1636	this study (DBP 1335 x DBP 1369)
Δ*cry*, Δ*vvd*, Δ*wc-1*, *a*	*a*, *ras-1*^{bd}, Δ*cry* ^{hyg}, Δ*vvd* ^{hyg} ,*wc-1* ^{bar}	DBP 1593	this study (DBP 1599 x DBP 1634)
Δ*cry*, Δ*vvd*, Δ*wc-1*, *A*	*A*,*ras-1*^{bd}, Δ*cry* ^{hyg}, Δ*vvd* ^{hyg} , Δ*wc-1* ^{bar}	DBP 1592	this study (DBP 1599 x DBP 1634)
Δ*cry*, Δ*vvd*, Δ*wc-1*, LM1, *a*	LM1, *a*, *ras-1*^{bd} ,Δ*cry* ^{hyg} , Δ*vvd* ^{hyg} , Δ*wc-1* ^{bar}	DBP 1597	this study (DBP1022 x DBP 1636)
Δ*cry*, Δ*vvd*, Δ*wc-1*, LM1, *A*	LM1, *A*, *ras-1*^{bd}, Δ*cry* ^{hyg}, Δ*vvd* ^{hyg} , Δ*wc-1* ^{bar}	DBP 1596	this study (DBP1022 x DBP 1636)

Results

The LMO functions independently of the FWO

In attempts to identify components of *N. crassa* FLO's, 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 Δ*frq*, *wc-2*^{234W} strain was mutagenized by UV light to 50% survival, and a mutant strain, called Light Mutant 1 (LM1) was identified that had robust rhythms in LL (Figure 2- 1, Table 2-2). The LM1 mutant strain was outcrossed multiple times to the WT clock strain *ras*^{bd} to isolate the LM1 mutation from all other mutations. The resulting *ras*^{bd}, LM1 strain (called LM1) was used for all subsequent analyses.

To confirm that the LM1 mutation rescued developmental rhythms in strains that lack a functional FWO, the LM1 strain was crossed to *ras*^{bd}, *frq*-null (Δ*frq*) and *ras*^{bd}, *wc-1*-null (Δ*wc-1)* strains. Progeny that were Δ*frq*, LM1 and Δ*wc-1*, LM1 were isolated and examined, along with control sibling strains, for rhythmicity in LL (Figure 2-2 and Table 2-2). Consistent with previous observations, the *ras*^{bd} 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 LM1 mutation. In the absence of the FWO components, the period of the LM1 rhythm is near 24 h, whereas LM1 strains with a functional FWO had a shorter period. These data suggested that the FWO and the LM

oscillator (LMO) genetically interact in LL. While all LM1 and Δ*wc-1,* LM1 replicate race tube cultures displayed rhythms in LL (100%), the number of rhythmic race tubes of Δ*frq*; LM1 was reduced to 75%.

Figure 2-1. Identification of the LM1 mutant strain that uncovers the LMO. A FWO mutant strain (*wc-2*^{234W}, Δ*frq*) was mutagenized by ultraviolet (UV) light, and the resulting strains were assayed in constant light (LL) (1200 lux) at 25°C for rescue of developmental rhythms on race tubes. The LM1 mutation displayed rhythmic development under these conditions, whereas the parental strain was arrhythmic. The direction of growth is from left to right, and the solid black lines correspond to 24 h of growth.

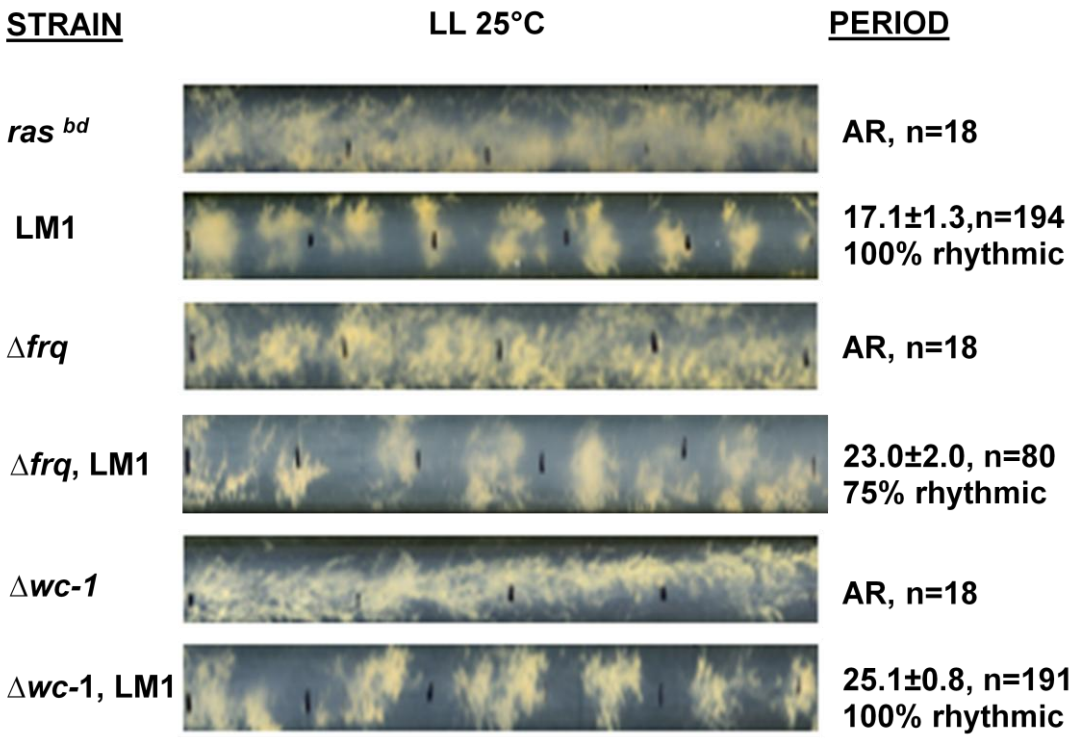


Figure 2-2. The LM1 mutation restores rhythms in strains that lack a functional FWO in LL**.** Representative photographs of race tubes of the indicated strains in LL (1200 lux) at 25°C. Black lines on the tubes represent 24 h growth fronts. The period lengths of the rhythmic strains are calculated ± standard deviation (SD) from the number of conidial bands (n) indicated for each strain. AR indicates that the strain was arrhythmic. The % rhythmic indicates the number of race tube cultures that were rhythmic, divided by the total number of race tubes assayed, for each rhythmic strain.

Table 2-2. Period lengths of wild type (WT) and LM1 mutants, single, double and triple photoreceptor mutants in constant bright light (LL), constant darkness (DD) and light/dark cycles (LD) cycles.

	LL (1200lux)			DD				LD 6:6		LD 9:9		LD 12:12		LD 14:14	
	bd	*bd, LM1*		*bd*		*bd, LM1*		*bd*	*bd, LM1*	*bd*	*bd, LM1*	*bd*	*bd, LM1*	*bd*	*bd, LM1*
Genotype	Period (h)		% rhythmic	Period (h)	% rhythmic	Period (h)	% rhythmic	Period (h)		Period (h)		Period (h)		Period (h)	
WT	AR (n=18)	17.1±1.3 (n*=194)	100%	22.3±0.8 (n*=60)	100%	22.7±0.5 (n*=77)	100%	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*cry*	AR	AR	–	22.2±0.5 (n*=60)	100%	22.4±0.5 (n*=60)	100%	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*	AR	12.9±1.7 (n*=47)	100%	21.7±0.4 (n*=60)	100%	23.2±0.9 (n*=77)	100%	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*wc-1*	AR (n=18)	25.1±0.8 (n*=191)	100%	AR	–	23.9±1.3 (n*=77)	40%	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*vvd*Δ*wc-1*	AR	23.0±1.3 (n*=62)	100%	AR	–	21.3±1.8 (n*=44)	60%	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	AR	–	AR	–	AR	–	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*	AR	AR	–	22.2±0.5 (n*=60)	100%	22.2±0.5 (n*=60)	100%	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 (n*=60)
Δ*cry*Δ*vvd*Δ*wc-1*	AR	AR	–	AR	–	AR	–	AR	AR	AR	AR	AR	AR	AR	AR
Δ*frq*	AR (n=18)	23.2±2.0 (n*=80)	100%	AR (n=12)	–	21.7±2.7 (n*=34)	50%	ND	ND	ND	ND	ND	ND	ND	ND

Cultures were grown on 1X Vogels, 0.1% glucose, 0.17% arginine monochloride and 1.5% agar. Race tubes were kept at 25^{0}C under different conditions. n represents the number of bands per strain used to calculate period. n* represents the number of race tubes used to calculate growth rate. Values are represented as mean + S.D. AR – arrhythmic. .ND-not determined.

The LMO oscillator free runs in constant conditions and is temperature compensated

Using heterokaryon analyses, we determined that the LM1 mutation was recessive (Figure 2-10), suggesting the LM1 is a loss of function mutation that uncovers an autonomous LMO. To determine if the LMO has properties of a circadian oscillator, LM1 strains were further examined for free-running rhythms in DD, light entrainment, and temperature compensation. Consistent with the LL data, independent of the FWO, strains containing the LM1 mutation are rhythmic in DD. However, the robustness of the rhythms (% rhythmic) in LM1 strains that also lacked components of the FWO was decreased in DD, as compared to LL (Figure 2-3A) suggesting that in DD, the FWO overrides, or enhances, the LMO rhythms. Furthermore, the LM1 period was similar to the ras^{bd} strain in DD, suggesting that FWO components function in LL to shorten the LMO period.

To determine if the LMO rhythm is temperature compensated, we assayed the developmental rhythm in LM1 and Δ*wc-1*, LM1 strains in LL, conditions in which the FWO is not rhythmic, but the LMO 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 0.95 - 1.21 (Sargent et al., 1966). As expected, the period of the rhythm differed between the LM1 and Δ*wc-1*, LM1 strains, and the Q_{10} values measured for both strains between 17 and 27°C was 1.2, confirming that the LMO is temperature compensated (Figure 2-3B).

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 h (Johnson et al., 2003). If the rhythm entrains to different environmental cycles it will display a period that equals the environmental cycle, and have stable phase angles that differ in different 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. Thus, to investigate entrainment of the LMO, we examined strains that display the LMO in different light/dark (LD) cycle lengths (Figure 2-4). For *ras*^{bd}, LM1, and Δ*wc-1*, LM1 strains, the period of the rhythm matched the imposed LD cycle length. Strains with WC-1 displayed different phases of conidiation in the different duration LD cycles, consistent with entrainment. However, the phase angles in Δ*wc-1*, LM1 strains stayed relatively constant in the different LD cycles, indicative of an LD-driven rhythm (Figure 2- 4B).

To rule out the possibility that the synchronization of Δ*wc-1*, LM1 strains in LD cycles was due to changes in temperature, rather than 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°C in each cycle, and these small changes are not sufficient to drive the developmental rhythm, and several of our test strains did not show rhythmic development in LD (e.g. Figure 2-4). Therefore, we can rule out the possibility that the rhythms observed in Δ*wc-1*, LM1

strains in LD cycles was due to 0.5°C cycles in the incubators. Thus, these data demonstrated that WC-1 is required for stable entrainment of the circadian clock in *N. crassa*, and that photoreceptors other than WC-1 are capable of responding to light in the Δ*wc-1*, LM1 strain.

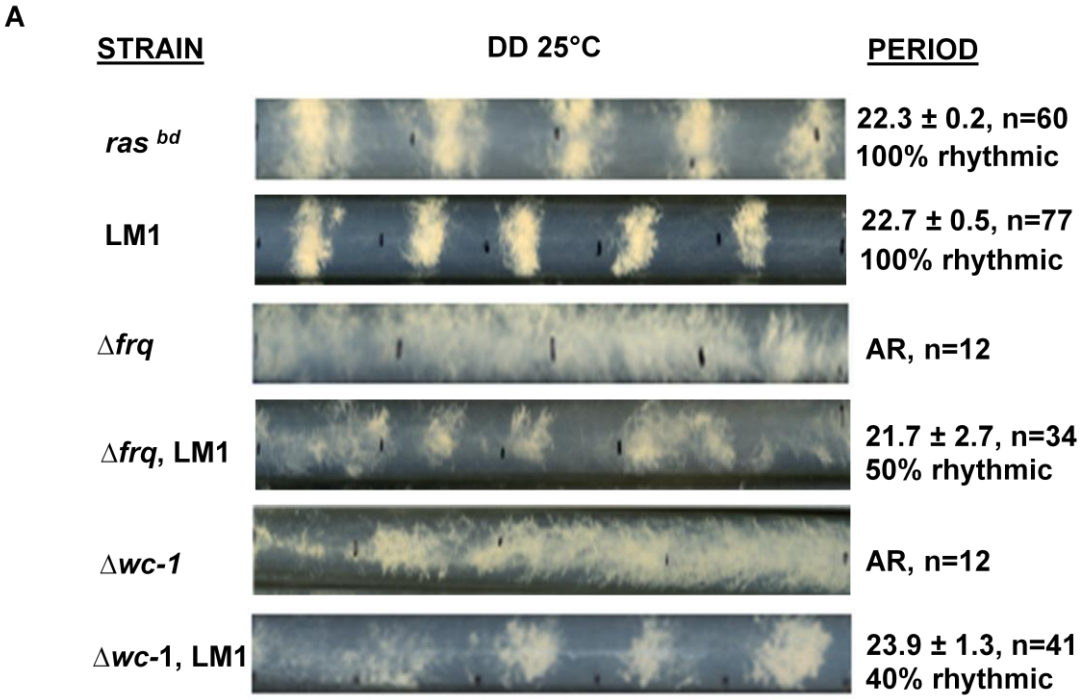


Figure 2-3. The LMO cycles in DD and is temperature compensated**.** (A) The LM1 mutation restores circadian rhythms in development to *frq*-null and *wc-1*-null strains in constant darkness (DD). Representative race tube pictures of the indicated strains are shown from cultures grown in DD at 25° C and labeled as in Figure 2-2.

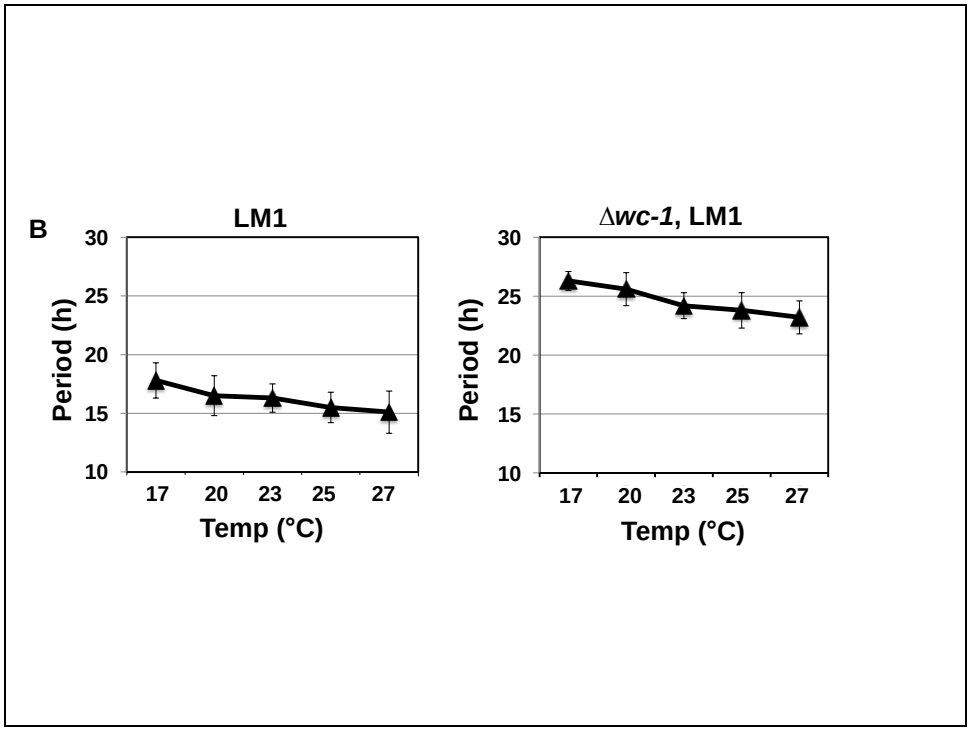


Figure 2-3 Continued.
(B) LM1 rhythms are temperature compensated. Plots of the period (h) versus temperature (°C) are shown for the indicated strains.

CRY is essential for LMO for rhythms in LL

The *N. crassa* clock is only known to be responsive to blue light (Sargent and Briggs, 1967). Therefore, to identify the photoreceptors responsible for light responses in the Δ*wc-1*, LM1 strain, we crossed the LM1 mutant strain to strains carrying deletions of the other known blue light photoreceptors in *N. crassa*, including VVD (Zoltowski et al., 2007) and CRY (Froehlich et al., 2010) to generate Δ*vvd*, LM1, and Δ*cry*, LM1 strains. These strains, and the control siblings, were assayed in LL and LD cycles at 25°C (Figure 2- 5). In the Δ*vvd*, LM1 strain in LL, rhythms in development were observed

(Figure 2-5A); however, the period of the rhythm was reduced, and more variable, as compared to LM1 (compare Figure 2-5B to Figure 2-2). The Δ*vvd*, LM1 strains also entrained to LD cycles (Figure 2-5B & 2-5C). Surprisingly, in Δ*cry*, LM1 strains grown in LL, the developmental rhythms were abolished. The rhythm was rescued by ectopic transformation of the *cry* gene into the Δ*cry*, LM1 strain (Δ*cry*, LM1::*cry)* (Figure 2-5A**).** In addition, the Δ*cry*, LM1 strain entrained normally to LD cycles (Figure 2-5B & 2-5C). Entrainment of both Δ*vvd*, LM1 and Δ*cry*, LM1 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 LMO in LL, and that both CRY and VVD are dispensable for the LM1 light responses. However, WC-1 photoreceptor likely compensates for the loss of individual VVD or CRY photoreceptors in these strains.

To determine if light responses in the Δ*vvd*, LM1 and Δ*cry*, LM1 strains are due to the presence of WC-1, we generated double photoreceptor mutants in the LM1 mutant background, and examined rhythms in LL and responses to light in LD cycles at 25°C. Consistent with a requirement for CRY in LMO function, all of the LM1 double photoreceptor mutants that harbor *cry* deletions were arrhythmic in LL (Figure 2-6A). Only the Δ*vvd*, Δ*wc-1,* LM1 strain displayed rhythms in LL. The period of the rhythm of the Δ*vvd*, Δ*wc-1,* LM1 strain was similar to the Δ*wc-1,* LM1 strain (Figure 2-2), but was significantly longer than the Δ*vvd,* LM1 strain (Figure 2-5A), suggesting that *vvd* does not directly function in the LMO. In LD cycles, light responses were observed in all of the double photoreceptor mutant strains that harbored the LM1 mutation (Figure 2-5B),

and consistent with previous results, entrainment required WC-1, but not VVD and CRY (Figure 2-5C).

We next generated strains that lacked all 3 blue light photoreceptors, with and without the LM1 mutation, and assayed the developmental rhythm in LL and LD cycles at 25°C (Figure 2-5D & 2-5E). Independent of the LM1 mutations, the triple photoreceptor deletion mutant strains were arrhythmic under both lighting conditions. 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 LM1 mutation; however, in all cases, WC-1 is required for normal circadian entrainment.

To further examine the requirement for CRY in the function of the LMO, we also examined the photoreceptor mutant strains in DD at 25°C. In DD, all strains harboring WT WC-1 were rhythmic regardless of VVD, CRY of LM1 (Figure 2-7). When *wc*-1 is deleted, strains are normally arrhythmic in DD, but the LM1 mutation rescues rhythmicity (Figure 2-3). Rescue of the Δ*wc-1* rhythmicity by LM1 depends upon CRY because the Δ*cry,* Δ*wc-1,* LM1 strain is arrhythmic, consistent with a central role for CRY in the LMO.

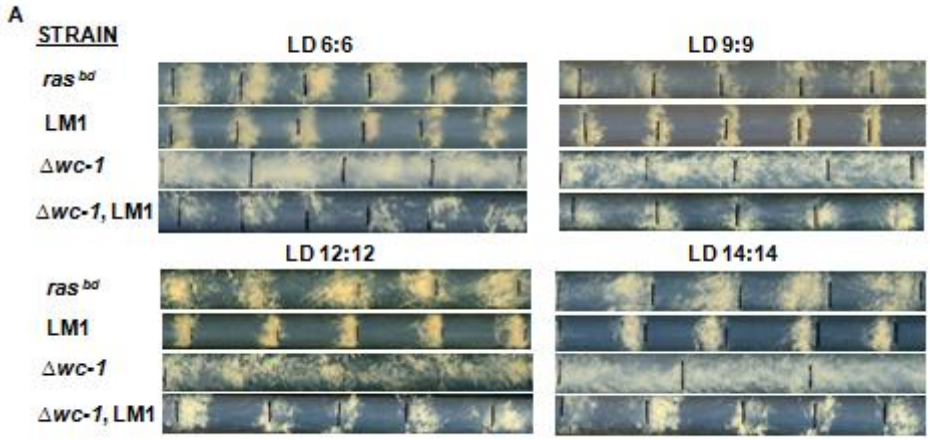


Figure 2-4. WC-1 is required for light entrainment of the circadian clock**.** (A) Race tube cultures were exposed to different light: dark (LD) photoperiods (h). For example, LD 6:6 indicates a 12 hour photoperiod with cycles of 6 h light and 6 h dark. Representative race tube pictures are shown for the indicated strains. Black lines on the race tubes denote when the light was turned on.

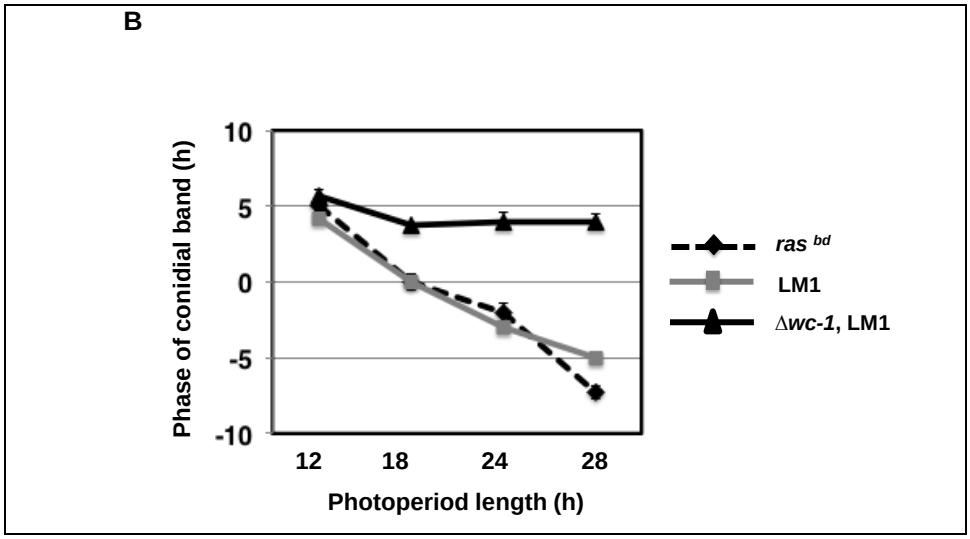
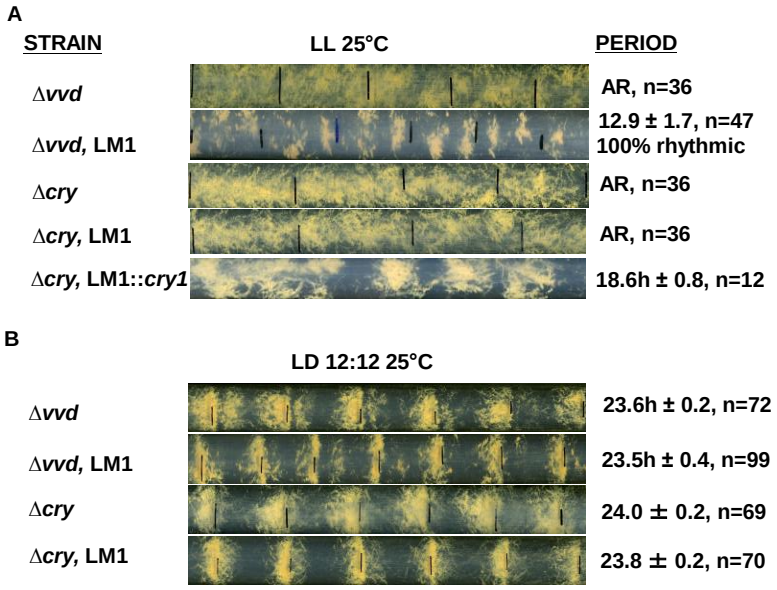


Figure 2-4 Continued.
(B) Plot of the phase of the conidiation band in relation to lights on in strains that showed light responses in each of the photoperiods from (A) (± SEM, n≥5). A positive number indicates that the conidiation band occurred after lights on, and a negative number indicates that the band occurred prior to lights on. In some cases, the error bar is smaller than the symbol.



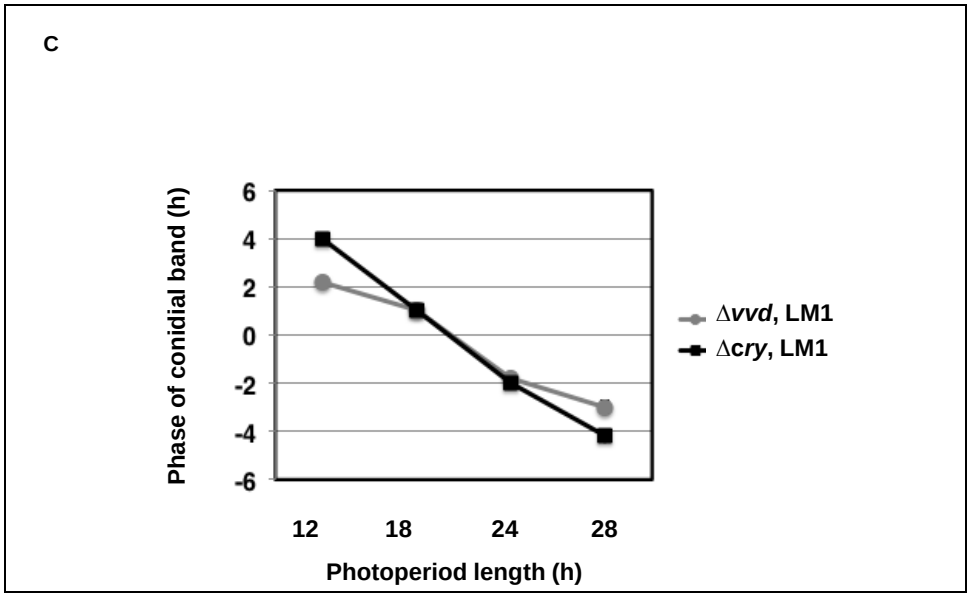


Figure 2-5. The blue-light photoreceptor CRY, but not VVD, is necessary for LMO function, and other photoreceptors can compensate for rhythms in LD cycles. Representative race tube photographs of the indicated photoreceptor mutant strains are shown in LL at 25°C (A) and in LD 12:12 cycles at 25°C in (B). (C) Plot of the phase of the conidiation bands in relation to lights on for the indicated strains. The figure is labeled as in Figures 2-2 and 2-4.

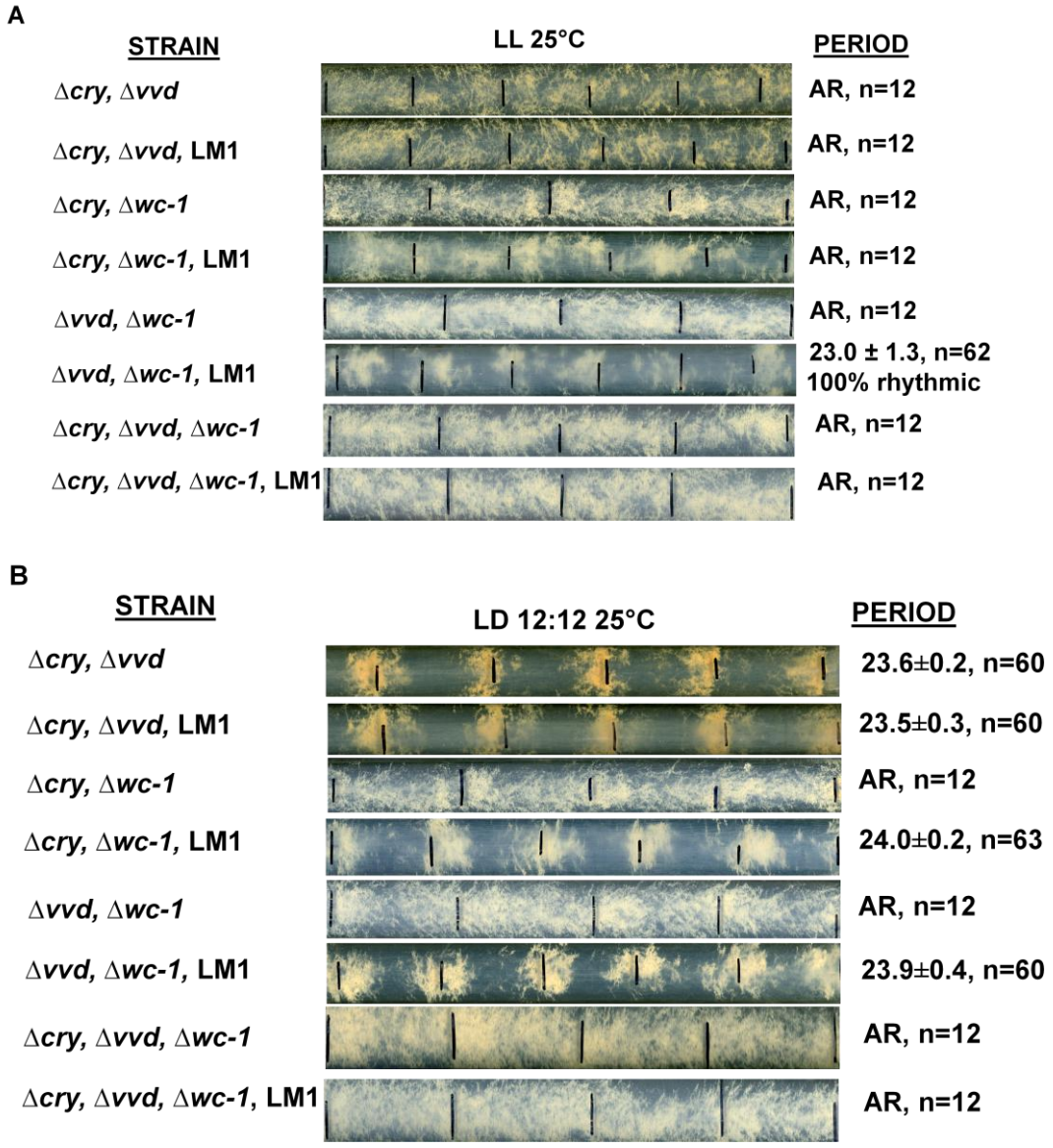


Figure 2-6. CRY and VVD are not required for light entrainment of the circadian clock. (A) Race tube cultures of the indicated photoreceptor mutant strains were grown in LL, and (B) were exposed to different LD photoperiods (h).

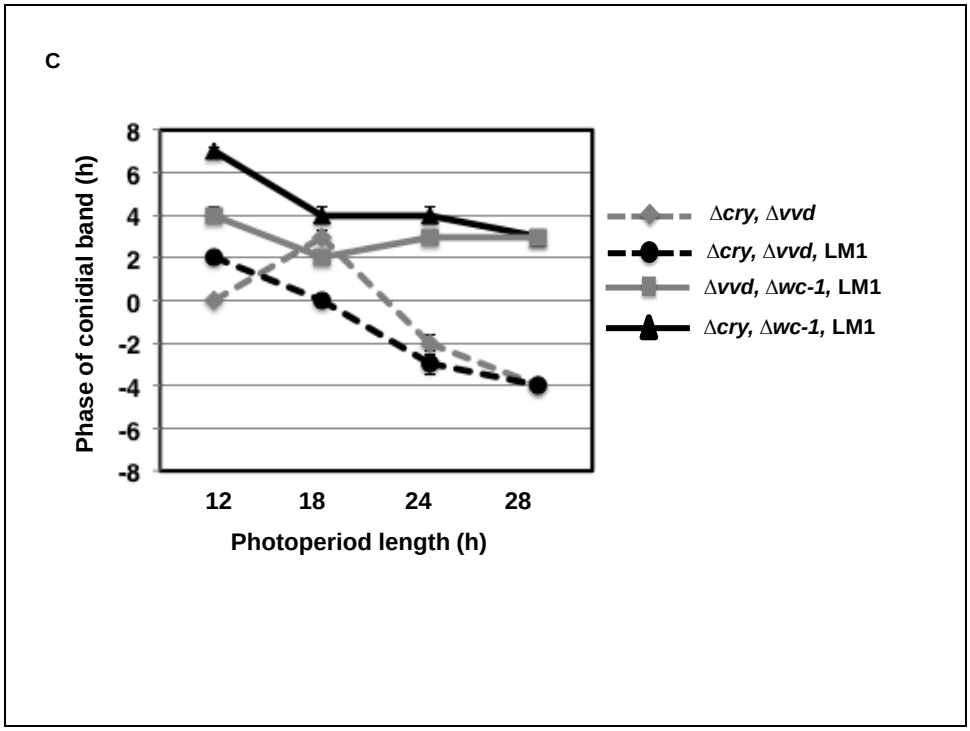


Figure 2-6 Continued.
(C) The phases of the conidiation band in relation to lights on were plotted (± SEM, n≥5) as in Figure 2-4B.

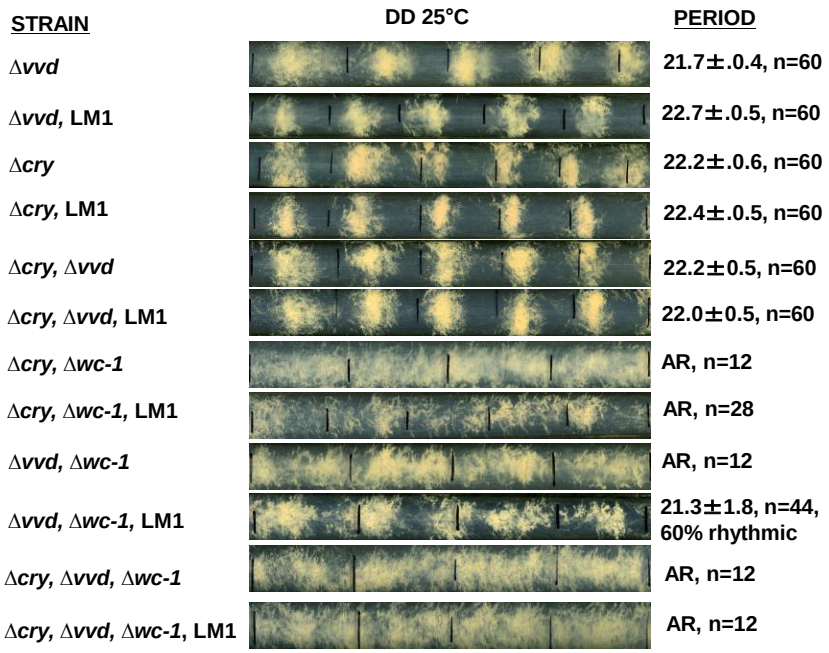


Figure 2-7. CRY, but not VVD or WC-1, is required for LMO-generated rhythms in DD. Representative race tube photographs of the indicated strains, and labeled as in Figure 2-2.

Discussion

To investigate the organization of the *N. crassa* circadian system, we identified the LM1 mutation that restores robust 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 in constant conditions, and is temperature compensated in LL, development in strains expressing the LMO, but lacking WC-1, were driven by light, rather than entrained by LD cycles. Thus, the LMO, similar to other reported FLO's (Dunlap and Loros, 2004), lacks full circadian properties. In addition, these data revealed that blue light photoreceptors besides 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 the LM1 mutant light response.

The LM1 mutation is recessive (Figure 2-9), suggesting that LM1 is most likely a loss of function mutation. While we have not yet identified the defective gene product in the LM1 mutant strain, these data indicate that the gene specified by the LM1 mutation encodes a protein that either directly or indirectly reduces the activity the LMO when it is present, rather than as a component of the LMO that is necessary for its function. Why would the cell want to shut off the LMO? It is possible that the LMO is only required under special growth conditions. It is possible that the LMO represents an ancient oscillator that was shut off in order to allow for the expression of the FWO that has all of the core circadian properties, including entrainment. Answers to these questions will await what the consequences are to the cell when the LMO is rendered non-functional.

In this regard, our data are consistent with a role for CRY in the function of the LMO, as CRY deletion strains are arrhythmic under conditions in which the LMO is normally expressed (Figure 2-5, 2-6 &2-7). In plants and insects, CRY is necessary for light entrainment of the circadian clock (Emery et al., 1998; Emery et al., 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* 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., 2010a). Furthermore, *cry* mRNA accumulates with a circadian rhythm, peaking in the nighttime. The *cry* deletion strains show a small decrease in amplitude of a few light induced genes, and a slight phase delay in LD entrainment. The period of the Δ*frq*, LM1 and Δ*wc-1*, LM1 in LL is roughly 24 h. Considering that *cry* is a direct target of the WCC (Smith et al., 2010a), one might expect the and Δ*wc-1*, LM1 strain to be arrhythmic. However our data suggest that a sufficient amount of *cry* is produced in this strain to allow the LMO to function. Experiments are currently in progress to examine the levels of CRY in these strains. In addition, the similar period of the two strains in LL is consistent with FRQ positively controlling the levels of WCC as had been previously reported. Furthermore, these data suggest that the lower the levels of CRY, the longer the LMO period. In considering what is already known about positive and negative elements of core circadian oscillators in which decreasing the levels of a positive element lengthens period, our data suggests CRY functions as appositive element. Given the central role of CRY in the insect and animal clockworks, the lack of a pronounced circadian or light phenotype in CRY mutants in *N. crassa* has been surprising (Chen and Loros, 2009; Froehlich et al., 2010; Olmedo et al., 2010). However, our data suggests that light responses in CRY mutants can be restored by one of the other blue light photoreceptors.

Finally, it is of interest to determine if there any consequences of the fitness of cells that have a fully functional FWO, but lack the LMO as observed in the Δ*cry*, LM1 strain.

Similar to circadian oscillators, we predict that the LMO functions as an autonomous molecular feedback loop, although this still needs to be determined. Because the LMO does not have full circadian properties, requiring WC-1 for LD entrainment, we surmise that it lies downstream of the FWO, and normally functions as a slave oscillator to enhance rhythmic outputs, such as the development rhythm. This observation is reminiscent of studies by Hunt et al., 2012, who demonstrated that WCC plays a central role in generating FLO-like oscillatory behavior in development in FWO-deficient strains in temperature cycles. In LL and naturally occurring LD cycles, the LMO 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, and whereas a slave oscillator, such as the LMO, may function during periods of light to complete the downstream rhythmic events. Our observation that the LMO rhythms lack full circadian properties, and is less robust in DD (Figure 2- 3), also fits with this hypothesis. Additional evidence points to an interaction between the FWO and the LMO, including differences in the period of the rhythm in LM1 strains FWO-deficient versus FWO-sufficient strains grown in LL (Figure 2-2). Furthermore, ChIP-seq analyses revealed that the *cry* promoter is a direct target of the WCC (Smith et al., 2010a). 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 LMO control *cry* rhythmicity.

We propose a simple model to provide a framework for future tests of the complexity of the clock (Figure 2-8). In LM1 deficient strains, and independent of the FWO, CRY is active and the LMO feedback loop oscillates. How LM1 represses the LMO 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 LMO rhythm in LD cycles. In LL, some mechanism 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 LMO function. Consistent with a role for VVD in LL, the period of the LMO rhythm is significantly reduced in Δ*vvd*, LM1 strains (Figure 2-5). This model leads to several testable predictions; 1) that the activity of CRY would be increased in LM1 mutant strains, and reduced in WC-1 deletions in LL, 2) the activity of CRY would cycle in LL and LD in the absence of WC-1, and 3) that Δ*vvd* will double the cycling of the LMO due to increased CRY activation in LL, which would be dependent on WC-1. In any case, the clock system is likely to be even more complex than depicted here, as rhythms

in the expression of the *ccg-16* gene in *N. crassa* are controlled by a FLO that does not require FRQ or WC-1 for activity, and this FLO, called the WC-FLO, that appears to be both temperature compensated and entrained to environmental cycles independent of WCC and FRQ (de Paula et al., 2006; de Paula et al., 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 LMO component, and the discovery of the LM1 mutation that uncovered the LMO, will undoubtedly aid in testing models of the LMO, and its connections to the environment and 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 (Daiyasu et al., 2004; Froehlich et al., 2010; Reppert and Weaver, 2002; Somers et al., 1998; Stanewsky et al., 1998), our finding of the CRY-dependent LMO may provide key insights into the evolution of the clock.

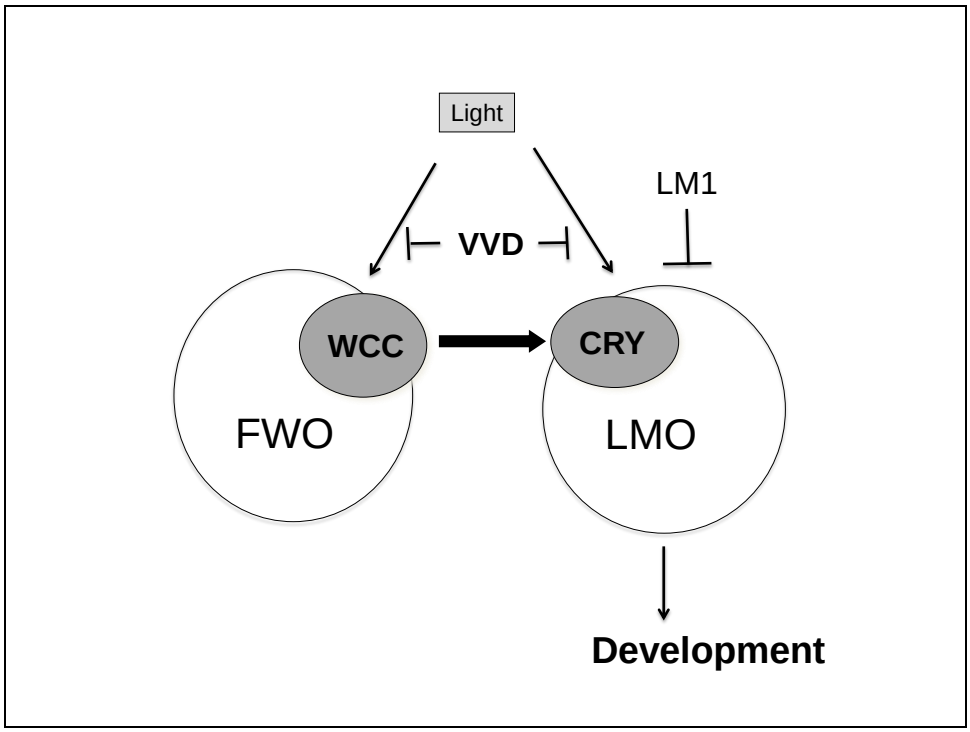


Figure 2-8. Model of the circadian clock composed of the FWO and LMO. See the text for a description of the model.

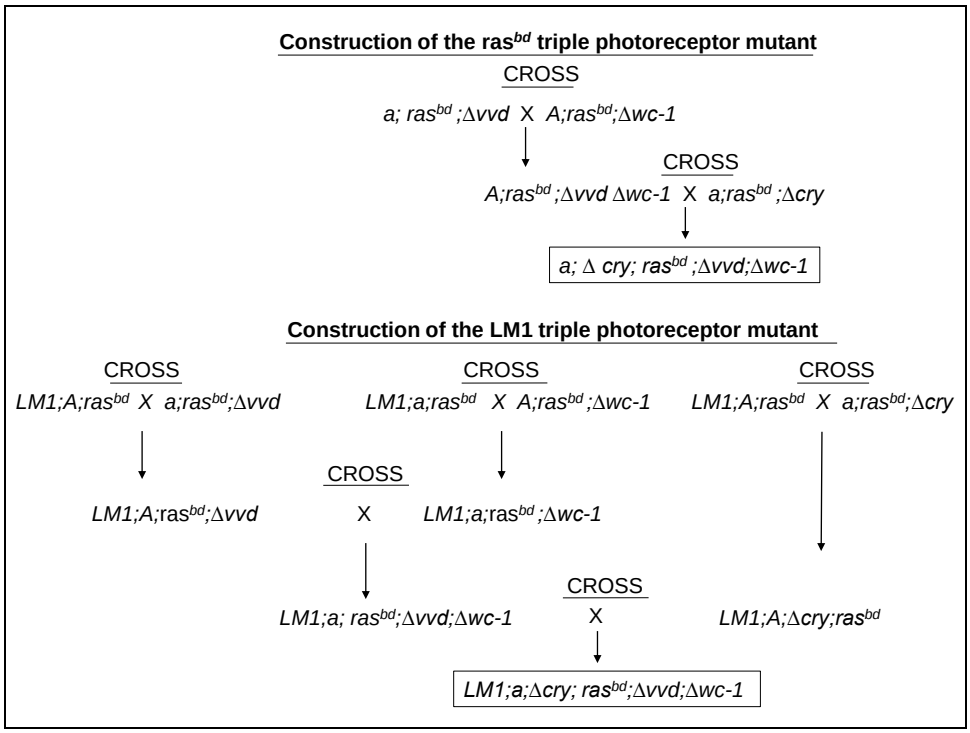


Figure 2-9. Flowchart for construction of ras^{bd} and LM1 triple photoreceptor mutant strains. The indicated strains were crossed (X) to generate the desired mutant strains.

Table 2-3. List of primers used for strain verification

Name	Primers
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'TGTATGTAGTGTACGGGGCAAA 3'
YG	3'CATCAGGTCGGAGACGCTGTCG 5'
VVD ORF forward	5'GAG CTC CAT CTC ATC CTC 3' (Hunt et al., 2012)
VVD ORF reverse	5'GCC CAA TCGCAG AAT AAG ACG 3' (Hunt et al., 2012)
ΔWC-1::bar check forward	5'CGT TCG ATA GAC GCA ACG TCA 3' (Bennett et al., 2013)
bar check 5' reverse	5'GTTGCG TGC CTT CCA GGG ACC 3' (Bennett et al., 2013)

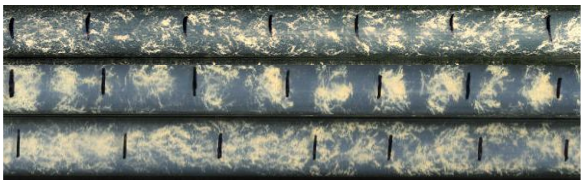


Figure 2-10. The LM1 mutation is recessive. Race tube cultures of *ras*^{bd} , *his-3*, LM1, *mat a*, supplemented with histidine, and *ras*^{bd}, *arg-5*, *mat a* supplemented with arginine, and a heterokaryon formed between the two strains and grown on minimal media in LL are shown. The solid lines indicate 24 h of growth in LL, and growth is from left to right.

Table 2-4. Growth rates of wild type and LM1 mutants, single, double and triple photoreceptor mutants in constant bright light (LL) and constant darkness (DD) and light/dark (LD) cycles.

	LL (1200lux)		**DD**		**LD 6:6**		**LD 9:9**		**LD 12:12**		**LD 14:14**	
	bd	***bd, LM1***	***bd***	***bd, LM1***	***bd***	***bd, LM1***	***bd***	***bd,LM1***	***bd***	***bd, LM1***	***bd***	***bd, LM1***
Genotype	**Growth Rate (cm/day)**											
WT	**4.0±0.2 (n=18)**	**3.5±0.2 (n=18)**	**4.0±0.2 (n=12)**	**3.3±0.3 (n=12)**	**1.6±0.2 (n=12)**	**1.6±0.2 (n=12)**	**3.1±0.2 (n=12)**	**3.0±0.2 (n=12)**	**3.4±0.2 (n=12)**	**3.5±0.2 (n=12)**	**4.1±0.2 (n=12)**	**3.8±0.2 (n=12)**
Δ*cry*	**4.5±0.4 (n=18)**	**4.8±0.4 (n=18)**	**3.8±0.4 (n=12)**	**3.8±0.4 (n=12)**	**1.8±0.2 (n=12)**	**1.9±0.2 (n=12)**	**3.6±0.2 (n=12)**	**3.4±0.2 (n=12)**	**3.8±0.3 (n=12)**	**3.9±0.3 (n=12)**	**4.6±0.2 (n=12)**	**4.6±0.1 (n=12)**
Δ*vvd*	**3.7±0.2 (n=18)**	**3.3±0.2 (n=18)**	**4.0±0.4 (n=12)**	**3.1±0.3 (n=12)**	**1.8±0.2 (n=12)**	**1.6±0.2 (n=12)**	**3.4±0.2 (n=12)**	**2.7±0.2 (n=12)**	**3.7±0.3 (n=12)**	**3.0±0.2 (n=12)**	**4.2±0.2 (n=12)**	**3.8±0.3 (n=12)**
Δ*wc-1*	**4.3±0.3 (n=18)**	**3.2±0.2 (n=18)**	**4.3±0.4 (n=12)**	**3.7±0.2 (n=12)**	**2.2±0.2 (n=12)**	**1.9±0.2 (n=12)**	**4.0±0.2 (n=12)**	**3.7±0.2 (n=12)**	**4.5±0.2 (n=12)**	**3.6±0.2 (n=12)**	**5.6±0.3 (n=12)**	**4.4±0.2 (n=12)**
Δ*vvd*Δ*wc-1*	**4.3±0.2 (n=12)**	**3.4±0.2 (n=12)**	**4.6±0.3 (n=12)**	**4.0±0.2 (n=12)**	**2.2±0.3 (n=12)**	**1.9±0.2 (n=12)**	**3.7±0.2 (n=12)**	**3.1±0.2 (n=12)**	**4.4±0.3 (n=12)**	**3.6±0.2 (n=12)**	**5.1±0.4 (n=12)**	**4.3±0.3 (n=12)**
Δ*cry*Δ*wc-1*	**4.3±0.2 (n=12)**	**3.7±0.4 (n=12)**	**4.8±0.3 (n=12)**	**4.1±0.2 (n=12)**	**2.2±0.2 (n=12)**	**1.9±0.2 (n=12)**	**3.5±0.2 (n=12)**	**3.0±0.2 (n=12)**	**4.7±0.2 (n=12)**	**3.6±0.2 (n=12)**	**5.4±0.3 (n=12)**	**4.4±0.3 (n=12)**
Δ*cry*Δ*vvd*	**3.8±0.2 (n=12)**	**3.8±0.3 (n=12)**	**4.0±0.3 (n=12)**	**3.9±0.2 (n=12)**	**1.7±0.2 (n=12)**	**1.9±0.2 (n=12)**	**2.8±0.2 (n=12)**	**2.8±0.2 (n=12)**	**3.7±0.2 (n=12)**	**3.6±0.2 (n=12)**	**3.8±0.3 (n=12)**	**4.2±0.3 (n=12)**
Δ*cry*Δ*vvd*Δwc-1	**4.4±0.3 (n=12)**	**4.4±0.3 (n=12)**	**4.6±0.3 (n=12)**	**4.9±0.2 (n=12)**	**ND**	**ND**	**ND**	**ND**	**4.5±0.3 (n=12)**	**4.8±0.2 (n=12)**	**ND**	**ND**
Δ*frq*	**3.6±0.3 (n=18)**	**3.0±0.2 (n=18)**	**3.6±0.2 (n=12)**	**3.2±0.2 (n=12)**	**ND**	**ND**	**ND**	**ND**	**ND**	**ND**	**ND**	**ND**

Cultures were grown on 1X Vogel's, 0.1% glucose, 0.17% arginine monochloride and 1.5% agar. Race tubes were kept at 25°C under different conditions. n represents the number of race tubes used to calculate growth rate. Values are represented as mean + S.D. ND-not determined.

CHAPTER III

IDENTIFICATION OF THE GENE SPECIFIED BY THE LM1 MUTATION

Introduction

Neurospora crassa has proven to be a premier model for understanding oscillator complexity, and for determining mechanisms by which oscillators interact (de Paula et al., 2007). The molecular feedback loop of the core FRQ-WCC Oscillator (FWO), which controls overt rhythmicity in *N. crassa* has been well characterized. In addition to the FWO, several labs, including ours, have shown that FRQ-less oscillators (FLOs) drive residual rhythms in the absence of a functional FWO (Lakin-Thomas et al., 2011). However, the identities of molecular components of FLOs are still largely unknown. Using genetic approaches, we uncovered a new FLO, called the LMO, in a Light Mutant 1 (LM1) strain. Interestingly, the LM1 mutation has a huge impact on both on the circadian clock and light input pathway in *N. crassa*. First, the LM1 strain is rhythmic in constant light (LL) and in strains carrying deletions of the FWO. Second, a putative blue light photoreceptor, CRY is required for the LMO rhythms. Third, the LM1 strain responds to light in the absence of the blue light photoreceptor, WC-1. To further characterize the LMO, it is therefore important to identify and clone the gene that is mutated in the LM1 mutant strain.

The LM1 mutation was isolated following UV irradiation of a strain lacking functional FWO (K. Suk Seo and D. Bell-Pedersen, unpublished data) and has been shown to be

recessive by forced heterokaryon analysis (Xiaoguang Liu and Bell-Pedersen, unpublished data). Initial Cleaved Amplified Polymorphic Sequence (CAPS) mapping placed the LM1 mutation on chromosome 1 (Xiaoguang Liu, Howard Huang and Bell-Pedersen, unpublished data). With a combination of CAPS markers and standard genetic mapping, the LM1 was mapped to a region covering about 186 kilobases (kb) around the mating type locus on the left arm of chromosome I (Figure 3-1) (Xiaoguang Liu and Bell-Pedersen, unpublished data). Mapping results from a 3-point cross placed LM1 between NCU02173 (nt 996277) and the mating type locus (*mat*) (nt 1857093) (Xiaoguang Liu and Bell-Pedersen, unpublished data). More refined mapping and sequencing studies suggested that the mutation is within a 55 kb region of chromosome I that contains 16 predicted open-reading frames (ORF). With the mapping data obtained, several different approaches were taken to try to identify and clone the gene specified by the LM1 mutation:

a. High throughput sequencing to sequence the entire genome of the LM1 strain and compare this to wild-type strains to identify the mutation.
b. Sequencing of individual candidate genes located within the mapped region from the LM1 strain.
c. A candidate gene approach, which examines if knockouts (KO) of genes in the mapped region have an LM1 phenotype.
d. Complementation of the LM1 mutation with cosmid clones containing the genes spanning the mapped region.

Materials and Methods

Strains

For PCR and sequencing, FGSC #1858, FGSC 1859, *bd*, LM1, *mat a (*DBP 695*)* and *bd* LM1, *mat A* (DBP 694) were used. Available KO strains of candidate genes in the mapped region were obtained from the Fungal Genetics Stock Center (FGSC) as indicated in Table 3-1. The *bd*, LM1, *mat a* (DBP 695) strain was used as recipient strains for complementation. Progeny were selected for hygromycin resistance and screened on race tubes for the LM1 phenotype.

Race tube assay

The composition of the race tube media was as described previously in Chapter II. Progeny from crosses of the candidate genes to the *bd* strain were screened for the LM1 phenotype, while transformants from the cosmid complementation were screened for arrhythmicity on race tubes in LL.

Genomic DNA isolation

For the isolation of genomic DNA, the method described by (Jin et al., 2007) was initially used. To obtain a higher yield of DNA, we later followed the method described in (Pomraning et al., 2009). The amount of DNA recovered was quantified by Nanodrop (Thermo Scientific Wilmington, DE). The genomic DNA was used as template for PCR reactions.

Cosmid DNA preparation

We obtained bacterial cells containing cosmid preparations from Dr. Michael Freitag (Oregon State University) and the FGSC. The cosmid DNA was extracted from overnight cultures grown in Luria Broth (LB) media using Qiagen Plasmid DNA Pure kit (Valencia, CA). All of the cosmids used were constructed with hygromycin as a selectable marker. The individual cosmid concentrations were quantified by Nanodrop (ThermoScientific, Wimington, DE).

PCR/primers/enzyme digestion conditions

The sequences of the ORF with an extra 1kb on either side of the candidate genes were downloaded from the Neurospora crassa database (http://www.broadinstitute.org/annotation/genome/neurospora/MultiHome.html). The PCR primers used for amplification and sequencing primers were designed using WebPrimer (http://www.yeastgenome.org/cgi-bin/web-primer). A few changes were made to the default parameters: Optimum Tm _60, Minimum Tm_55, Maximum Tm_ 62. Optimum percent GC content_ 50 Minimum GC _45 Maximum GC_60. Optimum primer length_ 20, Minimum length_18 Maximum length_ 22. If these criteria were too stringent to obtain valid primers, the settings were revised accordingly. The parameters for obtaining sequencing primers were as follows: Length of DNA in which to search for valid primers-50. Distance between sequencing primers_450. Optimum percent GC content_ 50 Minimum GC _45 Maximum GC_60. The primers were ordered from IDT (www.idt.dna.com) (Integrated DNA Technologies, Coralville, IA). Two PCR schemes

used either Ex-Taq (Clontech Laboratories, Mountain View, CA) or Phusion Hot Start Polymerase (NEW ENGLAND BioLabs Inc, Ipswich, MA) as described in their manuals.

For seven of the candidate genes, the sequencing was done by cloning the PCR product into Zero Blunt PCR Cloning Kit (Invitrogen), whereas four others were done by sequencing directly off the PCR product. Sequencing was performed using Perkin Elmer ABI Big Dye Reaction Mix (Waltham, MA). The conditions in the thermocycler were as follows: 96º C 2min, 96 ºC 30s, 48ºC 15s, 60ºC 4 min, for 99 cycles. Unincorporated dye terminators were removed using BioRadP-30 spin columns purchased from Gene Technology Lab (GTL) TAMU and the pellets were thoroughly dried. The sequencing was done at the GTL, TAMU (College Station, TX). Sequence data was analyzed by DNA Sequencher version 4.8 sequence analysis software, Gene Codes Corporation (Ann Arbor, MI). Restriction enzymes from New England Biolabs (Beverly, MA) were used.

High throughput sequencing

Whole genome sequencing was done by Dr. Michael Freitag (Oregon State University) using an Illumina HiSeq 200 platform. The *bd, A* strain was crossed to *bd,* LM1, *a* and *bd, a* strain was crossed to the *bd,* LM1, *A* strain. Thirty *bd* and 30 LM1 individual progeny were obtained from each cross. Each of the progenies was screened on race tubes to verify their phenotypes. Genomic DNA from 3 recombinant progeny from each cross were pooled and sequenced.

Transformation by electroporation

The electroporation of *N. crassa* conidia was carried out according to Dr. Wayne Versaw's modification of the procedure described by (Margolin et al., 1997). Conidia from 7-day old slants were inoculated on to 200 ml Vogel's minimal medium slants supplemented with 2% glucose in 500 ml flasks and incubated at 30 ºC for 18 days. Conidia were harvested by filtration using 35 ml of cold 1M sorbitol, and transferred into a 50 ml Falcon tube. A small volume of sample was removed for quantification of the conidia. The concentration of the sample was determined at OD of 420 nm. The conidia suspension was centrifuged for 5 min at 3,200 rpm at 4ºC using a Sorvall RT 6000B (Thermo Electron, Franklin, MA). The supernatant was decanted; 30 ml sorbitol was added to pellet, mixed and centrifuged. The wash step was repeated 4 times. The standard final density of the conidial suspension was 7.25 x 10^8conidia/ml.

The concentration of cosmid DNA used for transformation was 50ng/µl. All the reactions were set up in pre chilled Eppendorf tubes. To each tube, 70ul conidia was added to the tubes and placed on ice for 15 min. Using a Biorad Gene Pulser (Hercules CA), the pulse settings for a 1mm electroporation cuvettes (VWR, Radnor, PA) were as follows: voltage- 1.5 kilovolts (kV), resistance- 600 ohms, and capacitance- 25 microfarads.

Following electroporation, 500 µl of chilled sorbitol was added to each cuvette and the conidial suspension was removed. Another 500µl of sorbitol was added to each cuvette and the conidial suspension was removed. All of the tubes were placed on ice. Each transformation sample was added into 9ml Recovery Buffer in a 50 ml conical tube. Tubes were then placed in the 30ºC incubator with shaking for 2h. Two ml of 10XFGS (20% L-sorbose, 0.5% fructose, 0.5%glucose) was added to tube of 2% Top Agar (2% agar, 2M sorbitol, 1X Vogel's, 2X FGS). This mix was then poured into the screw cap tube containing sample buffer and recovery medium (1X Vogel's, 1X Histidine). Tubes were mixed quickly and poured over already prepared agar plates containing 200 ug/ml hygromycin. Once solidified, plates were placed in the 30º C incubator and observed for transformants.

Results

High throughput sequencing

Our initial sequencing of the LM1 strain was carried out using progeny from a cross between *bd; mat A* and *bd; LM1;mat a* and *bd; mat a* and *bd;LM1; mat A*. Thirty WT and 30 LM1 progeny were screened on race tubes to verify their phenotypes. Three *bd* and three *bd lm1* recombinant siblings were identified, genomic DNA isolated, and the DNA pooled and sequenced using Illumina next generation sequencing. The sequencing data suggested that the LM1 mutation was between NCU02017 at 1663103 and NCU02002 at 1719081. In the region around the mating type locus where LM1 maps, there is a high degree of sequence polymorphism, as compared to the sequenced WT

strain. Therefore, using only a small number of samples, it was impossible to pick out a single point mutation. However, a small region near where LM1 map was found to lack the polymorphisms, suggesting that this region is tightly linked to the mutation in LM1. The region contains the 16 candidate genes (Table 3-1). A second round of sequencing results suggested an area between nt 1459392and nt 1616166 might contain the LM1 mutation based on several LM1-specific polymorphisms. This region contains 40 ORFS.

Candidate gene approach

The 16 genes found between nt 1663103 and nt 1719081 on chromosome I and listed in Table 3-1 were our primary candidates for genes mutated in the LM1 strain. For each of the genes, I amplified the open reading frame (ORF) plus 1kb upstream and 1 kb downstream from two independent LM1 strains and control *bd* strain by PCR to allow coverage for non-coding and intergenic sequences. After the PCR fragments sizes were confirmed on agarose gels, the PCR products were cloned and sequenced. The LM1 and *bd* sequences were compared to the available *N. crassa's* genome sequence, no mutations were found.

In addition to the sequencing strategy, I tested available KOs of the candidate genes for the LM1 phenotype. At the time, the 13 available KO strains of the sixteen candidate genes were obtained from the Fungal Genetics Stock Center and were crossed to the *bd, A* (FGSC 1858) strain. Three KO strains NCU02005, NCU02007 and NCU02014 were unavailable. Progeny that carry the deletion, conferring hygromycin resistance and the

bd allele were tested on race tubes for rhythmic conidiation in LL. On the average, 25 *bd*, Δcandidate progeny from each of the 13 crosses were tested on race tubes. None displayed the LM1 phenotype in LL. The possible complication with this approach is that a KO might not have the same phenotype as the mutant LM1 allele.

Other candidate genes from the SNP analyses from the second round of sequencing that displayed LM1-specific polymorphisms were examined. These candidate genes were predicted to contain a stop codon, base substitution or a deletion located between nt 1459392 and nt 1616166. Candidate genes with potential SNPs were sequenced and compared with the *bd*sequence, no LM1-specific mutations were identified. The candidate genes that were sequenced are listed in Table 3-1. Finally, I also sequenced additional candidate genes, NCU01973, NCU07496, NCU00523, that are located near LM1 and encode proteins involved in chromatin remodeling. This work was motivated by unpublished findings by Dr. William Belden (Rutgers University), who showed that a mutation in a histone acetylase was rhythmic in LL.

Cosmid complementation

Based on the genetic mapping, the position of the LM1 mutant strain was placed between two CAPS markers 1-59 and 1-85 (Figure 3-1). More refined mapping by a 3 point cross placed the LM1 mutation between NCU02173 and *mat*. Cosmids that extended between the 2 markers (nt 1518724 – nt 2087126) and beyond the 16 candidate genes were obtained for complementation, (Xiaoguang Liu and Bell-Pedersen). None of

these rescued the LM1 mutation. Based on the sequencing results suggesting that the mutation might be between NCU02017 at nt 1663103 and NCU02002 at nt 1719081, I tested cosmids (including overlapping ones) adequately covering this region (1651742-1741445) for complementation (Figure 3-2A). There was no cosmid coverage between 1705112 and 1711756 where 2 genes NCU02003 and NCU02004 are located. The *bd*, LM1 strain was transformed with the individual cosmids to see if any of the cosmids rescued the LM1 phenotype. On examination of several independent transformants, none of the cosmids complemented the mutation, as rhythmicity was not abolished in the LM1 strain in LL (Table 3-2).

Discussion

Despite our efforts, to identify the LM1 mutation by mapping, sequencing the LM1 genome, sequence candidate genes or by complementation, we have been unable to identify the LM1 mutation. We plan to sequence more LM1 siblings using paired end reads to search for deletions and inversions. Furthermore, we will increase the number of siblings of WT and LM1 from 3 to 30. By increasing the number of DNA samples, we expect to be able to identify a single mutation. DNA from 30 WT and 30 LM1 siblings will be used for sequencing. Once candidate mutations are identified, the same region of DNA will be independently sequenced from the LM1 strain for verification of the mutation, along with the control WT DNA. DNA from the candidate gene will be transformed into the LM1 strain to demonstrate complementation of the LM1 phenotype.

Finally, we are using the *N. crassa* KO collection and testing for linkage between the KOs and LM1 in attempts to more finely map the LM1 mutation.

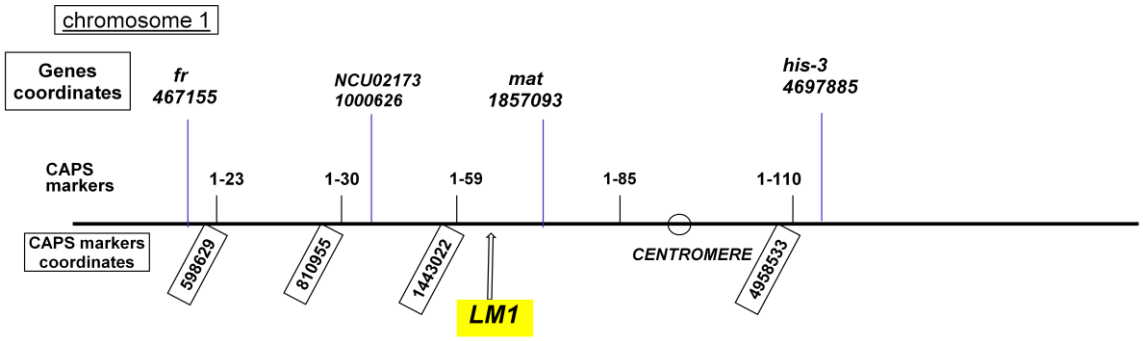


Figure 3-1. Mapping of the LM1 mutation. CAPS markers on chromosome I. LM1 has been mapped to chromosome I. Refined mapping of the LM1 mutation placed it on the left arm of chromosome I between CAPS markers 1-59 and 1-85.

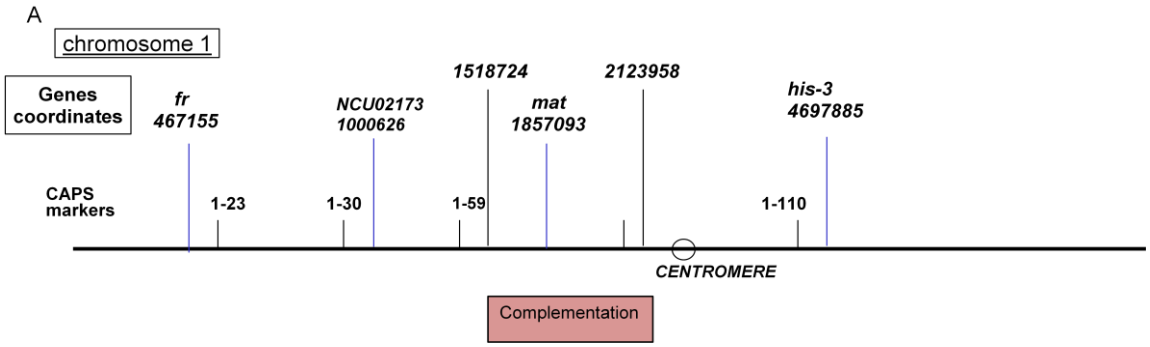


Figure 3-2. Identification of the LM1 mutation. A. Cosmid clones covering the region between nt 1518724 – nt 2123958 were tested for complementation.

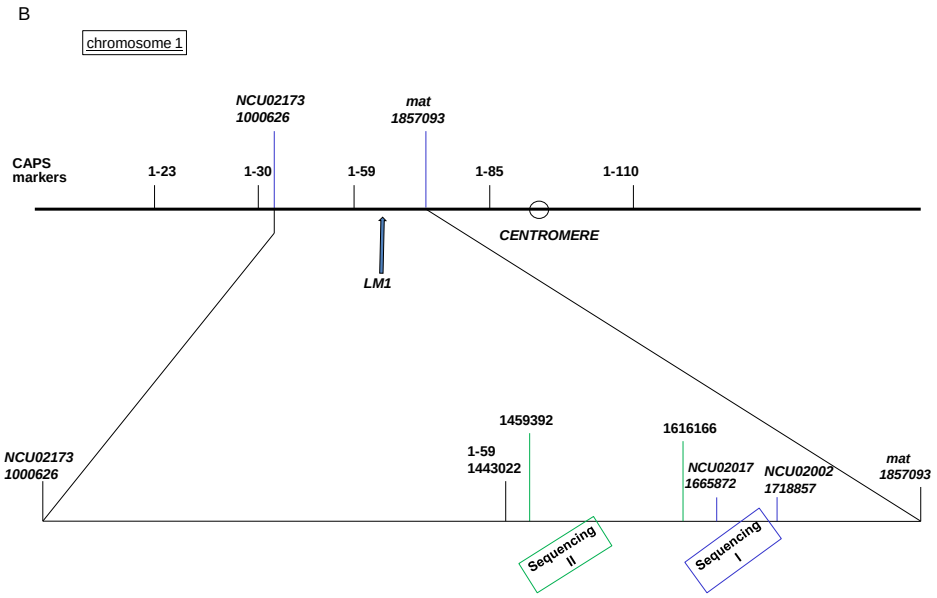


Figure 3-2 Continued.

B. High throughput sequencing results. Initial sequencing results suggested that the LM1 mutation resides in an area between 1663103-1719081. 16 genes in this region were our candidate genes. Other sequencing results suggested the LM1 mutation is located between nt 1459392 and nt 1616166.

Table 3-1. Candidate genes near the mating type locus on Linkage Group I.

Mutant *N. crassa* FGSC Strain #	Locus	Gene Name	Location N. crassa OR74A(NC10): Supercontig1		Mating type	Gene symbol	KO tested for LM1 phenotype
			Start	Stop			
12929	[*b]NCU02053.7	mutagen sensitive-42	1526760	1531289	*a*	*rev1*	yes
13282	[*b]NCU02051.7	hypothetical protein	1543429	1548853	*a*		yes
12853	[*b]NCU02046.7	hypothetical protein	1570281	1573326	*a*		yes
none	[*b]NCU02044.7	GTP-binding protein	1574297	1576855	*a*		no
11108	[a]NCU02017.7	all development altered-2	1663002	1665672	*a*	*ada-2*	yes
14113	NCU02016.7	hypothetical protein	1665463	1666966	*a*		yes
19235	NCU02015.7	Palmitoyltransferase ERF2	1667609	1671392	*a*		yes
22053	NCU02014.7	hypothetical protein	1671707	1673426	*a*		no
14112	NCU02013.7	hypothetical protein	1673395	1675076	*a*		yes
12852	[*a]NCU02012.7	hypothetical protein	1675354	1679439	*a*		yes
13281	NCU02011.7	importin subunit beta-1	1679855	1684385	*a*	*nup-8*	yes
13280	NCU02010.2	2-isopropylmalate synthase	1685685	1689164	*a*	*leu-4*	yes
13279	[*a]NCU02009.7	FreB	1690523	1694696	*a*		yes
13901	NCU02008.7	hypothetical protein	1696442	1697182	*a*		yes
22533	NCU02007.7	iron sulfur cluster biosynthesis protein Isd11	1697784	1698862	*a*		no
13467	NCU02006.7	hypothetical protein	1699141	1703074	*a*		yes
22829	NCU02005.7	phosphoadenosine phosphosulfate reductase	1702853	1704727	*a*	*cys-5*	no
13278	[*a]NCU02004.7	Phosphoserine phosphatase	1706234	1708298	*a*	*ser-3*	yes

Table 3-1 **(Continued)**.							
Mutant *N. crassa* FGSC Strain #	Locus	Gene Name	Location N. crassa OR74A(NC10): Supercontig1		Mating type	Gene symbol	KO tested for LM1 phenotype
			Start	Stop			
14111	NCU02002.7	hypothetical protein	1715205	1718857	*a*		Yes
18721	NCU02000.7	splicing factor 3B subunit 10	1720671	1721335	*a*		Yes
14110	NCU01999.7	hypothetical protein	1721690	1724492	*a*		Yes
13378	NCU01996.7	pH-response regulator protein palI/rim-9	1736763	1740530	*a*		Yes
14544	NCU01995	hypothetical protein	1741307	1743453	*a*		Yes
14454	*[c]NCU01973.7	set-domain histone methyltransferase-8	1813940	1818960	*a*	*set-8*	Yes
11182	*[c]NCU07496.7	set-domain histone methyltransferase-7	408524	415263	*a*	*set-7*	Yes
12078	*[c]NCU00523.7	NAD-dependent deacetylase sirtuin-2	8130238	8132369	*a*	nst-2	Yes
FGSC: Fungal Genetics Stock Center.							
Source: *N. crassa* genome at the Broad Institute. * indicates genes that were sequenced. [a] represents genes for initial sequencing results. [b] represents genes from second sequencing results. [c] represents chromatin remodelers							

Table 3-2. List of cosmids

pLORIST Cosmid	Lab strain#	Location	Restriction Enzyme	Total number of transformants		Total number of transformants tested with LM1 phenotype	
				50ng/ul uncut	cut	50ng/ul uncut	Cut
H046 B7	418	1672579-1711756	SwaI	30	25	0/30	0/25
H124 E11	419	1693963-1734914	SbfI	30	8	0/19	0/8
H60 C9	420	1655963-1698463	SwaI	30	_	0/22	_
H046 G8	421	1705112-1741445	SfiI	30	25	0/24	0/25
H103 B6	422	1651742-1691820	SwaI	30	16	0/30	0/16
H123 G6	426	1554168-1594467	FseI	30	10	0/29	0/10
H032 F9	427	1532857-1575345	PmeI	30	6	0/30	0/6

CHAPTER IV

SUMMARY

As a model organism, *Neurospora crassa* lends itself to understanding molecular details of oscillator complexity because it has a well defined core FRQ/WCC oscillator (FWO), that functions similarly to circadian oscillators in other eukaryotes (Dunlap, 1999), and FRQ-less oscillators (FLOs) that promote rhythmic behavior in the absence of a functional FWO (Lakin-Thomas et al., 2011). However, until this study, no components of the FLOs had been identified. Here I found that CRY is necessary for function of the LMO, suggesting it is a component of the Light Mutant Oscillator (LMO). Future work on the LMO requires; 1) confirmation of CRY as a component of the LMO, 2) the identification of other components of the LMO, 3) determining the molecular mechanisms for LMO function, and 4) discovering how the FWO and LMO communicate with each other. These data will help to further test the model that the LMO normally functions downstream of the FWO, but can respond directly to environmental signals such that under certain growth conditions, the LMO functions autonomously to drive overt rhythms.

The LM1 strains show rhythms in development independent of components of the FWO, in constant light (LL) and constant darkness (DD). The LMO has a free running period of about a day, is temperature compensated and drives light responses in the absence of the blue light photoreceptor, WC-1. However, while the LMO can respond to light, unlike the FWO, the LMO is not entrained to light: dark (LD) cycles. These data

suggested that the LMO is a slave oscillator to the FWO under normal growth conditions. However, it is possible that in LD cycles, or in other conditions, the LMO can function independent of the FWO. In this study, I also discovered that the blue light photoreceptors, WC-1, VVD, and CRY can compensate for one another in the LMO's light response, but only WC-1 confers circadian light entrainment.

The identification of CRY as a component of the LMO was a breakthrough in understanding oscillator complexity in *N. crassa*. Prior to this study, the role of the FLO's in the circadian system had not been well characterized, as no FLO component had been identified (Liu and Bell-Pedersen, 2006). The identification of CRY as a key component of the LMO opens up the possibility of determining the identity of other components of the LMO by finding proteins that interact with CRY, or proteins that control the expression of *cry* mRNA. In addition, we are now in a position to determine what happens, if anything, to the FWO when the LMO is unable to function. This information, along with the knowledge that the *cry* gene promoter is a direct target of the WCC, will help to establish whether or not the FWO and LMO are mutually coupled.

The organization of *N. crassa's* clock system is more like *Drosophila* and mammals than cyanobacteria, requiring a complex network of interconnected transcriptional and translational loops to drive rhythmic gene expression (Brunner and Schafmeier, 2006). In *Synechococcus elongatus*, transcription is not necessary for generating rhythms as self sustained rhythmicity has been shown to occur in the presence of the key clock proteins,

KaiA, KaiB, KaiC and ATP in a test tube (Nakajima et al., 2005). The molecular clocks of *Drosophila*, mammals, and plants consist of multiple interlocked feedback loops, and rhythms can be observed when components of the core feedback loops are disrupted (Hardin, 2004; Ko and Takahashi, 2006; McClung, 2006). Thus, similar to *N. crassa*, there appears to be multiple autonomous oscillators driving rhythmicity (Lakin-Thomas et al., 2011; Lakin-Thomas and Brody, 2004). Despite this evidence, the components of the oscillators are not known. Thus, our work in *N. crassa* may provide the foundation for understanding the nature of the other oscillators in higher eukaryotes. This information will be critical to understanding the mechanistic basis for what goes wrong during jet leg, shift work, or in genetic disorders that uncouple the communication between oscillators and lead to diseases such as metabolic disorder and cancer.

In mammals, the SCN requires photic input to regulate peripheral tissues (Ko and Takahashi, 2006). Light is also required to synchronize the endogenous clock to daily environmental cycles. Without light resetting, the endogenous clock will be out of phase with the environment and no longer be predictive. The master pacemaker sets the timing, ensures proper phasing, and entrains the peripheral oscillators to local time. It is still not altogether clear if the molecular make up of the peripheral oscillators is identical to the master pacemaker oscillator in the SCN, or if like the FWO and LMO, differ in composition. In any case, the mammalian clock system is reminiscent of the FWO providing entraining signals to the LMO. Also, similar to direct light input to the LMO, the peripheral oscillators in cell culture can be independently synchronized by serum

shock, and can free run under constant conditions. Thus, understanding how the FWO and LMO communicate in entrained conditions may lead to a better understanding of the hierarchical mammalian clock system.

In addition to the LMO requiring the FWO for entrainment, the discovery that CRY, a functionally diverse protein found in different phyla, is a component of the LMO, suggests the possibility that the LMO is an ancient oscillator. The role of CRY protein in *Drosophila* is different from its role in mammals. In *Drosophila*, CRY is a photoreceptor and it is required for circadian entrainment (Emery et al., 2000; Stanewsky et al., 1998), and it is also an oscillator component in some peripheral tissues (Ivanchenko et al., 2001), but not in other tissues (Ito et al., 2008). In mammals CRY functions as a negative component of the central oscillator mechanism (Reppert and Weaver, 2002). In zebra fish, there are three different types of CRY - one with resemblance to *Drosophila* CRY, one that is similar to the mammalian CRY (Kobayashi et al., 2000), and one that is a member of the CRY-DASH family (Daiyasu et al., 2004). CRY in *N. crassa*, Arabidopsis, and Cyanobacteria are most similar to CRY-DASH (Brudler et al., 2003). *N. crassa* CRY was found to bind both FAD and MTHF, confirming its role in photoreception (Froehlich et al., 2010). In addition, *N. crassa cry* mRNA and protein levels are induced by light, and this induction requires WC-1. Rhythms in *cry* mRNA are observed peaking in the evening, yet disruption of *cry* does not alter the developmental rhythm in cultures grown in DD, indicating that CRY is not necessary for the function of the FWO. In the future, it will be important to further validate that CRY is a component

of the LMO. To do this, one could generate a CRY over expression strain in an LM1 mutant background and examine these strains in LL for altered rhythmicity. In addition, I predict that similar to all other known circadian oscillators, CRY will be part of a negative feedback loop. I showed that when the levels of CRY in strains expressing the LMO are reduced, the period of the developmental rhythm is lengthened. Unlike the negative role for CRY in the mammalian clock, these data are consistent with CRY functioning in the LMO as a positive element in the feedback loop. Using our knowledge of core oscillator function to guide future experiments, I believe it is important to determine if CRY can function as a transcription factor in *N. crassa*, and if so, to identify the direct genetic targets of CRY using ChIP-seq. At least one of these targets should encode the negative elements in the feedback loop, and this can be determined by examining the levels of CRY protein in knockout mutations. The expectation would be that the levels of CRY would be higher at all times of day when the negative element(s) are disrupted. Finally, independent of CRY, other efforts to identify components of the LMO would be to carry out genetic screens for mutants that abolish rhythmicity in the LM1 mutant strain that also lacks a functional FWO.

Critical to this study is to determine the location of the LM1 mutation. Once identified, one can test whether the FWO and LMO are coupled through the LM1 gene. To establish if the FWO regulates the activity of the LMO via control of LM1, LM1 mRNA and protein levels can be examined in WT, ΔFRQ, and ΔWC-1 strains over a time course in DD and LL. If LM1 accumulates with a circadian rhythm, and if the rhythmic

accumulation of LM1 mRNA and protein levels is altered in mutants of the FWO, these data would support the hypothesis that LM1 links the two oscillators. Because CRY is a component of the LMO, one can also test FWO's regulation of the LMO by determining if deletion and over-expression of components of the FWO, and LM1, affect levels of *cry* mRNA and CRY protein. In WT strains, previous experiments have shown that mutations in *cry* do not alter rhythms in *frq* mRNA levels or FRQ protein levels (Froehlich et al., 2010).

We have shown that the LM1 mutation is recessive. Our working model of the *N. crassa* clock is that constitutively high levels of WC-1 induce CRY expression in LL. In an LM1 mutant strain, CRY may feedback to inhibit WC-1 levels resulting in LMO rhythms. On the other hand, when CRY is absent, overt rhythms are abolished. The LMO rhythms persist in strains lacking WC-1, suggesting that the amount of *cry* present in the absence of WC-1 is adequate to drive rhythms. To further test this model, it will be necessary to determine the levels of CRY protein in WT, LM1 and LM1 Δ*wc-1* strains. Finally, we predict that VVD functions in both the LMO and FWO to down regulate the ability of light to impact the oscillators (photoadaptation). This hypothesis can be directly tested by measuring the light responses of CRY (and other LMO components) in a VVD mutant strain. We would expect the levels of CRY (and other LMO components) to remain high in ΔVVD strains at all times in the light.

Once the LM1 gene is cloned, and with the validation that CRY is a component of the LMO, we can begin to address all of the outstanding questions regarding the importance of the LMO in controlling overt rhythmicity, and if and how the LMO interacts with the FWO. Our expectation is that this work will help to better define the circadian clock system not only in *N. crassa*, but also in mammals, which in turn may lead to better treatments for circadian disorders.

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