MAPPING CIRCADIAN OUTPUT PATHWAYS IN *Neurospora crassa*

A Dissertation

by

LINDSAY DANIELLE BENNETT

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Chair of Committee,	Deborah Bell-Pedersen
Committee Members,	Daniel Ebbole
	Wayne Versaw
	Terry Thomas
Head of Department,	Thomas McKnight

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ABSTRACT

Circadian clocks are ubiquitous in eukaryotic organisms, providing the ability to anticipate regularly occurring stressful environmental changes. The molecular clock leads to a change in physiology of the organism such that it is prepared for predictable changes. While the external signals detected by the clock, as well as the molecular mechanism of clock components have been extensively characterized, less is known about how the clock manifests time of day information to the organism as a whole. Our lab has focused on identifying output pathways from the clock, using the model organism *Neurospora crassa*.

We have previously demonstrated the circadian regulation of the conserved Mitogen Activated Protein Kinase (MAPK) OS-2 pathway, a homolog of the mammalian p38 pathway, and necessary for maintaining osmotic homeostasis in Neurospora. I present data indicating the circadian regulation of the 2 other MAPK pathways in Neurospora, the mammalian ERK1 and ERK2 like MAPKs, MAK-1 and MAK-2, and show that they are outputs of the clock. Furthermore, I identified around 500 genes that are mis-regulated when MAK-1 is deleted; greater than 25% of those genes are predicted to be clock-controlled. I demonstrated that the clock is signaling through the MAK-1 pathway to regulate 3 clock-controlled genes (ccgs) that encode proteins involved in several different biological processes including, stress response, cell wall formation, and mitochondrial phosphate transport.

I established the circadian regulation of the transcript levels of 2 of the MAK-1 cascade components, *mek-1* and *mak-1*. Additionally, I found that the accumulation of

MEK-1 protein is clock-controlled, suggesting this is one mechanism by which the clock regulates the activity of MAK-1. Additional studies were carried out to elucidate the proteins that directly regulate the expression of *mek-1* and *mak-1*; however, the mechanisms of direct clock control remain unclear and require further investigation.

The finding that the circadian clock regulates all MAPK pathways in Neurospora, combined with the conservation of both the circadian clock and MAPK pathways in mammals provide compelling evidence that mammalian MAPK pathways are also regulated as clock output pathways to control circadian physiology. There is a strong link between aberrations in mammalian clocks, MAPKs, and disease, and therefore, an urgent need to further characterize the circadian regulation of the MAPK families, which will reveal new avenues for therapeutic treatments.

DEDICATION

I dedicate this work to my parents, Daniel and Linda Bennett, without whom I would never be the person I am today.

Additionally, I would like to thank my advisor, Deborah Bell-Pedersen for all of her guidance and support during these long years of study. Lastly, my cohorts in the lab, Dr. Teresa Lamb and Dr. Chuck Goldsmith: you were the reason I maintained any sanity during this world-wind ride.

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INTRODUCTION

Overview

Biological organisms are influenced, both positively and negatively, by their external environment; an environment that is ever changing. The daily rotation of the earth, as well as the annual revolution of the earth around the sun, leads to daily and seasonal variations in many environmental factors, including the amount and intensity of light, temperature, humidity, barometric pressure, as well as nutrients available to the organism. While it is critical for organisms to respond to these changes, it is all the more beneficial for them to be able to anticipate and prepare their cellular processes that are necessary for surviving these reoccurring conditions. To do so, organisms have evolved circadian clocks, internal timekeeping systems that provide a mechanism to coordinate internal biological processes with the time of day. The inner mechanisms of the circadian clock, as well as how the environment influences the clock, have been well studied. However, there is still much to learn regarding the way in which the clock directs organisms to prepare for daily changes. The work in this dissertation is focused on determining how the clock helps organisms to prepare for environmental cycles. I showed that the circadian clock regulates the activity of Mitogen Activated Protein Kinase (MAPK) pathways in *Neurospora crassa* as a method of signaling time of day information that prepares the cells for anticipated changes.

Background

The idea that organisms are not only responsive to daily changes in light and temperature, but that they have an endogenous timing mechanism, was first documented in 1729 by Jean Jacques de Mairan (35). de Mairan noted that mimosa plants showed daily rhythms in leaf movement even when placed in constant darkness (DD). He therefore concluded that the folding was not merely a response to the light to dark transition, but appeared to be regulated internally (35). It wasn't until the 20th century that scientists provided evidence that circadian rhythms were generated endogenously, a property of single cells, and found in light responsive organisms ranging in complexity from cyanobacteria to fungi, insects and mammals. Work with the fruit fly, *Drosophila melanogaster*, identified the first clock deficient mutant, laying the groundwork for a heritable molecular clock (80).

There are three defining characteristics a rhythm must possess in order for it, as well as the oscillator driving the rhythm, to be classified as circadian (126). The first is that the rhythm is sustained, with a period close to 24 hours, when the organism is placed in constant growth conditions. The second dictates that the rhythm can be entrained by external cues, including light or temperature, such that the organism can stay synchronized with its environment. The third property requires that the rhythm is temperature compensated; *i.e.* it persists with a relatively constant period within the biologically relevant temperature range of the organism. A very simple model of a typical circadian system is shown in Figure 1-1. External cues are perceived by the organism through input signals, or zeitgebers, which feed into a central oscillator, synchronizing the oscillator with

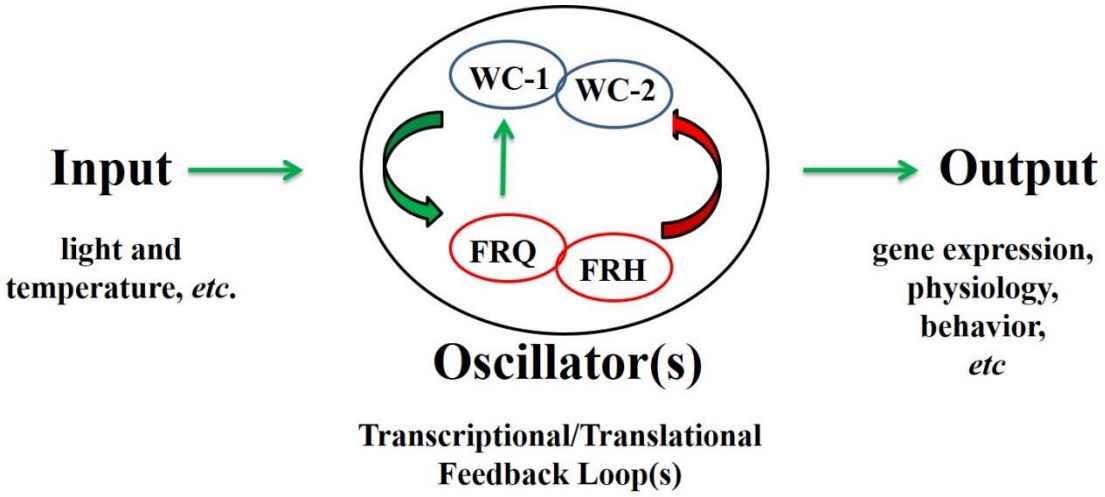

Figure 1-1. A schematic representation of the classic circadian system. Environmental signals, such as light and temperature, are perceived by the the molecular oscillator through input pathways. The oscillator consists of proteins that serve as positive elements that induce the expression of negative element proteins. The negative elements feedback to control the activity of the positive elements in the negative feedback loop. The negative component also positively control the levels of the positive elements. The oscillator then transduces temporal signals through output pathways to control rhythms in gene expression, physiology and behavior. Adapted from Baker *et al.*, 2012 (5).

the external environment. The oscillator relays time of day information, through output pathways, to drive the rhythms in gene expression, behavior and physiology, allowing the organism to better respond to environmental cycles.

Within most eukaryotic organisms, some of the molecular mechanisms of circadian clocks are conserved. The core molecular oscillators that have currently been characterized in eukaryotic organisms including, fungi, flies and mammals, all display a typical transcriptional/translational feedback loop. Briefly, this loop involves the regulated expression of one or more negative component(s) that then feeds back to repress its own transcription (auto-repression) by inhibiting its positive regulators (defined as the negative feedback loop). The negative feedback loop takes about a day to complete cycle. Positive regulation is also observed whereby the negative component(s) plays a role in controlling the levels of the activators, such that they are always present in sufficient amounts to restart the cycle. (10). While it is clear that each level of regulation is important, the significance of each step varies with each organism. For instance, in the circadian oscillator found in *Synechococcus elongatus*, the only prokaryotic clock described to date, a similar transcriptional/translational feedback loop is present, though studies indicate the cycling of post-translational modification of the oscillator components is sufficient to drive rhythmic gene expression (63).

To synchronize the molecular clock with the external environment, namely the 24 hour rotation of the earth around the sun, the oscillator perceives a variety of input signals. While many input signals have been described, depending upon the organism, the primary signals include light and temperature perception. Photoreception ranges with the

complexity of the organism. For instance, in mammals, light is perceived by the photoreceptor melanopsin in the eyes, which transduce light signals through the retinal hypothalamus tract (RHT) directly to the SCN (113). In lower eukaryotes, such as fungi, which have no eyes, photoreceptors in cells allow the organism to recognize and respond to light. After the components of the oscillator receive light input, the positive elements are quickly induced; therefore, independent of the stage in the feedback loop of the molecular feedback loop, the loop is reset as the positive elements induce expression of the negative element(s). This allows organisms, which have endogenous rhythms that are close to, but not exactly 24 hours, to resynchronize to the external light/dark cycles every day (94). Temperature perception is more complicated as temperature elicits two effects on the circadian oscillator. First, the oscillator must be buffered from temperatures within the physiological range of the organism, such that the period of the internal rhythms do not increase, as one would predict to happen with enzymatic activity. Second, temperature changes must also serve as an input to the oscillator, allowing for the synchronization of the oscillator with major changes in the environment.

Additionally, eukaryotic circadian systems have been shown to consist of multiple oscillators, which are hypothesized to be coupled to a central oscillator, or pacemaker. The oscillators are thought to communicate with one another, and thus, a single oscillator would not be required to regulate all circadian outputs or even have all of the canonical properties of a circadian oscillator (33). For example, in mammals, the suprachiasmatic nucleus (SCN), which resides in the hypothalamus of the brain, receives zeitgebers, or external signals from the environment detected by the clock, and, as the pacemaker for the

body, transduces those signals to multiple organs and tissues systems, including the liver, heart, kidneys, and the immune system, which have their own functional oscillators (95, 113). The organs within the body are insensitive to photic cues; therefore, they must be synchronized with the external environment, as well as with other organs, by signals from the SCN (50, 81). The mechanisms of signaling between the SCN and peripheral oscillators are only just beginning to be uncovered, but work thus far indicates signaling through the autonomic nervous system and the neuroendocrine system allows communication between the clocks (8, 23, 70). A similar scenario is hypothesized for microbial organisms, in which the circadian system must be able to regulate all circadian outputs in an individual cell (10). Just as in animals, there may be a central pacemaker that regulates downstream oscillators within a single cell, with each oscillator, at the very least, coupled to the central pacemaker, and possibly to other oscillators (113).

Defects in the molecular oscillator, as well as those that are desynchronized from the environment, of mammalian organisms have been linked to a variety of diseases and psychological disorders, including tumorogenesis, cardiovascular disease, seasonal defective and bipolar disorder (31, 50). Humans are diurnal, or active during the daytime, and the clock in the SCN is strongly synchronized by light and with the environmental light/dark cycles. Because of this, the clock has evolved to regulate proteins necessary for daily activity to be more abundant during the day. For instance, enzymes, proteins and molecules involved in metabolism and immunity peak during the day, a time when humans are most likely to consume food and encounter pathogens (43, 74). Therefore, if one is active during the night, the body is unprepared for that behavior and the challenges that

come with it. Humans (and mice) can also respond to social cues and feeding schedules as inputs to synchronize different clocks within the body; the liver is synchronized by consistent feeding cycles (2, 146). If an organism is kept in a light/dark cycle, but fed on an opposing schedule, the SCN will be reset by the light, while the liver synchronizes with the feeding cycles, creating a situation in which the clocks in the body are desynchronized from each other and/or the environment (25). It is known that shift workers and people who suffer from chronic jet lag show a statistically significant increase in the development of health problems; for instance nurses that work chronic shift work are more likely to develop breast cancer (72). As the shift worker continues activity during the night, normally an inactive time for humans, their internal clocks become desynchronized from one another as the pacemaker is reset by light, while peripheral oscillators synchronize with behavior. Additionally, several genes involved in the cell cycle have been shown to be under the control of the clock, including c-Myc, Wee1 and cyclinD1 (17, 88, 122, 151). Clock regulation of the cell cycle indicates that cellular growth of healthy cells only occurs at specific times a day when the cells are less likely to encounter stress that would affect DNA replication and other biological processes that are sensitive to genotoxic stress (140). Physicians can therefore take advantage of this phenomena and targeted cancer therapy can be applied as chronotherapy, *i.e*. treating cancers with chemotherapy, which inhibits DNA replication, at a time when normal cells are not dividing. This provides a specificity to the cancer cells that are always dividing, and decreases damage done to healthy cells that divide under the control of the clock (150). In order to treat circadian clock-associated

diseases, a thorough understanding of the mechanism of the internal clock, as well as identifying biological processes that are regulated by the clock, has become imperative.

Neurospora as a Model Organism for Circadian Clocks

The filamentous fungus, *Neurospora crassa*, has been a genetic model organism dating back to the early work of Beadle and Tatum, who used the organism to demonstrate a link between one gene and its encoded protein that displays enzymatic activity (9). Use of Neurospora has been further promoted by the sequencing of the genome and community efforts generating knockouts of each of the predicted open reading frames, which can be obtained from the Fungal Genetics Stock Center (32, 51) (www.fgsc.net). *N. crassa* was first identified as a useful model system for the study of circadian clocks when it was shown to have an easily observable circadian rhythm in asexual spore (conidia) formation (143). Strains containing mutations that alter the period of the conidiation rhythm were later identified and most alleles were mapped to the same gene, the *frequency* (*frq*) gene (45). The cloning and molecular characterization of *frq*, and the subsequent identification of WHITE COLLAR-1 (WC-1) and WC-2 as components that form a circadian oscillator feedback loop led to a model in which the FRQ-WC-based oscillator (FWO) forms the core pacemaker of the Neurospora clock (5, 11, 41). In this model (Figure 1-2), the WC-1 and WC-2 proteins dimerize through Per-Arnt-Sim (PAS) domains, forming a heterodimer called the White Collar Complex (WCC) (6). The WCC, acting as a transcription factor complex, then binds to the clock box (C-Box) in the *frq* promoter, and acts as a positive transcriptional regulator (47, 48). *frq* is transcribed, with mRNA

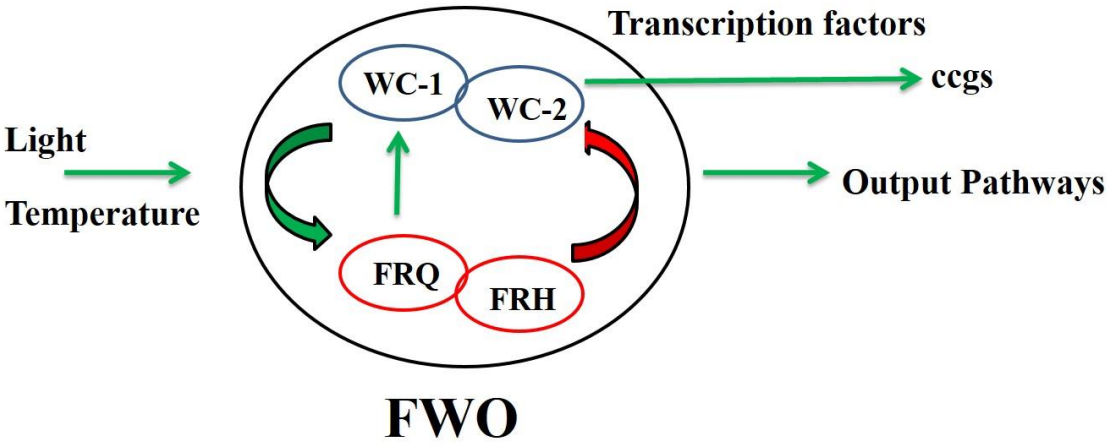

Figure 1-2. A schematic representation of the Neurospora FRQ/WCC (FWO). Input signals are sensed by the FWO via the blue light photoreceptor WC-1. The WCC activates the transcription of *frq*, and then FRQ protein, which interacts with the FRQ interacting helicase (FRH), inhibits the activity of the WCC. Additionally, FRQ acts to positively affect the stability of the WCC. This temporal information is then transduced through output pathways to regulate transcription factors, which, in turn, control rhythms in terminal ccgs.

accumulating to high levels in the morning, and subsequently translated, with FRQ protein levels peaking 4-6 hours later (1). FRQ protein negatively regulates its own transcription by modulating post-translational modification of the WCC; through indirect mechanisms, FRQ, along with its partner, FRQ-interacting helicase (FRH), promote the phosphorylation of the WCC. Phosphorylated WCC is unable to bind to the *frq* promoter, thereby inhibiting the positive transcriptional activity of the complex (145). Throughout the day, FRQ protein is progressively phosphorylated by protein kinases including CKI and CKII (154) and, once fully phosphorylated, is targeted for degradation through the proteosome pathway (61). FRQ protein levels are reduced, which removes the negative regulation of its activators, and renews the cycle. In an opposing role, FRQ positively influence the levels of the WCC by enhancing the transcription of *wc-2* (possibly *wc-1* as well), and stabilizing/increasing levels of WC-1 (29, 142). This activity is likely mediated by the interaction of WC-1 with WC-2, which is necessary for the increased stability of WC-1 (29, 85, 144). While *frq* mRNA, protein and WC-1 protein levels accumulate rhythmically in wild-type Neurospora, *wc-1* and *wc-2* mRNA, as well as WC-2 protein levels do not cycle in constant darkness (29).

The 2 most characterized environmental signals that input to the FWO are light and temperature. The positive element in the FWO is responsible for detecting light, as WC-1 is the major blue light photoreceptor in Neurospora, binding to the flavin cofactor FAD (60). After a light treatment, the WCC induces the expression of *frq*, independent of the time of day, which restarts the FRQ-WCC cycle anew, thereby resetting the clock (34). In addition, in response to light, the WCC directly activates the transcription of hundreds

of light-induced genes that are responsible for biological processes that aid in the health of the cell, including genes involved in carotenoid synthesis (58). Temperature perception is necessary for both temperature compensation, as well as maintenance of a robust oscillation through the range of physiologically relevant temperatures. While very complex, this perception in imparted to the Neurospora clock via transcriptional and translational mechanisms by which several forms of both *frq* and FRQ are produced (45).

Life Cycle and Outputs of the Neurospora Clock

In order to understand the physiological relevance of a circadian clock in *N. crassa*, I must first describe the life cycle of the fungus. Neurospora is a filamentous fungus most commonly found on burned wood after forest fires and was a problematic bread mold in the past before preservatives were invented (99). Briefly, the fungus, under normal conditions, grows as a hyphal structure that is a syncytium, with cytoplasmic streaming of organelles, including multiple nuclei in the hyphae. The hyphae display a polarized growth pattern, with the cell wall of the hyphal tip constantly being remodeled. Neurospora also undergoes various developmental stages, including asexual and sexual structures (20). Under conditions in which nitrogen is limited, sexual development is induced as a mechanism of continuing the species. Mating occurs between strains of 2 different mating types, designated A or a, with one strain acting as the "female" by developing a fruiting body called a perithecia (53, 111, 155). Strains of different mating types can differentiate themselves by detecting mating type dependent pheromones secreted by the cell (18, 77). Once the female strain senses the presence of the opposite mating type, or the "male", it

produces a structure called the trichogyne, which extends from the female structure to fuse with the nucleus of the male to be brought back to fertilize the female (76). Meiosis occurs within the perithecia and homokaryotic nuclear ascospores (sexual spores) are produced and projected away from the perithecia into the air (129). The processes of sexual development and progeny generation are highly sensitive to light (118, 119). Female development occurs in the dark, while the projection of ascospores is directed by light; therefore it was a reasonable assumption that this process might be regulated by the clock. Indeed, investigations by Bobrowicz *et al*., demonstrated that the expression of genes that encode for pheromone precursors are under the control of the clock, with a peak in expression occurring during the day (18). Additionally, when starved for nutrients, exposed to light, encountering a liquid-air interface, or signaled by the circadian clock (see below), hyphae begin to grow away from the medium surface, generating structures called aerial hyphae. Aerial hyphae further undergo sporulation, with the formation of microconidia and macroconidia that can be dispersed to a new location, allowing for the survival of the organism (13, 135).

One of the first circadian outputs observed in Neurospora was the rhythmic production of conidia. This output can be easily monitored when Neurospora is grown on solid media in special tubes called racetubes (Figure 1-3) (143). Neurospora conidia are inoculated on the medium at one end of the racetube and grown in constant light (LL) for 24 hours. The growth front is marked and then the racetube is shifted to DD, which sets the clock to dusk. The growth front is marked every 24 hours using a safe red light until the cultures have grown the end of the tube (about 7 days). The period and phase of the

conidiation rhythm can then be calculated. In *ras-1*^{bd}, the standard "wild-type strain" used due to increased clarity of the banding pattern, the period of conidiation is about 22 hours, with a peak in phase occurring during the subjective day in DD at 25°C (143). In addition to the conidiation rhythm, many early studies identified genes that are rhythmically expressed. Initial studies identified 2 ccgs that peaked in the morning, using subtractive hybridization (14). Further microarray analysis revealed circadian transcripts that peaked at all times of day, indicating a more complex level of clock regulation (14, 33, 168). The last 10 years has revealed a growing body of evidence that the clock is regulating genes that encode for proteins involved in multiple biological processes, including growth, asexual and sexual development and metabolism. With molecular assays predicting that at least 20% of the genes in Neurospora are regulated by the clock at the mRNA level (Renato M. dePaula, Richard Gomer, PB, TLT, and DBP, unpublished data; (33, 39, 117, 166)), studies are ongoing to determine the various mechanisms by which the clock exerts its effects. Thus far, there is no evidence that FRQ can bind DNA; however, as FRQ affects the posttranslational modification and activity of the WCC, it is unclear if FRQ plays a role in the direct regulation of ccgs. As it is known that the WCC is a transcription factor, Chromatin Immunoprecipitation-Sequencing (ChIP-Seq) experiments were carried out to identify direct target sequences. It was shown that there are at least 109 genes with regions of their promoters bound by the WCC after a light treatment, including both light-induced genes and ccgs, and an enrichment in transcription factors (152). The direct targets that also display rhythmic mRNA levels, peak in the morning, consistent with the phase of the

A.

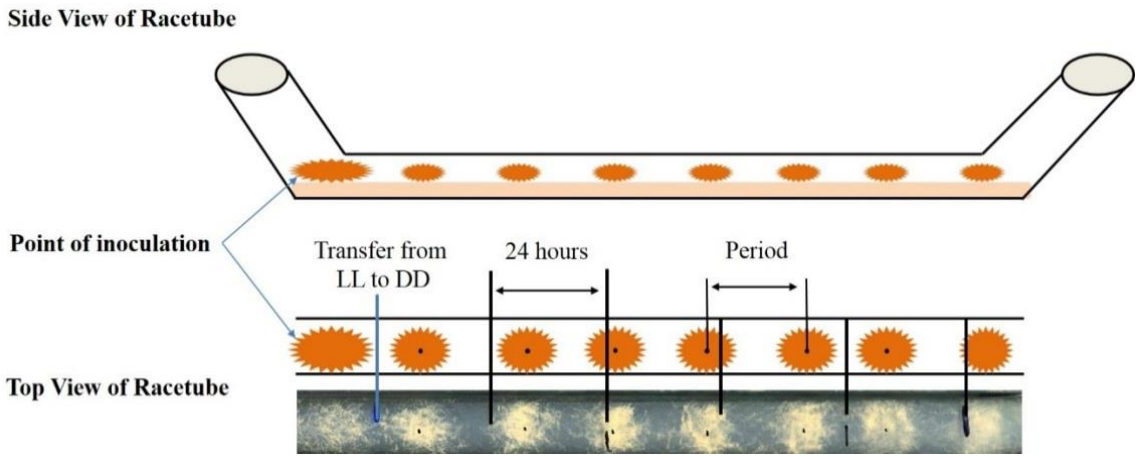

Figure 1-3. Representation of a racetube and a diagram illustrating the period and phase of circadian oscillations. (A). A cartoon representation of a racetube aligned with a racetube with *ras-1*^{bd}, which was grown in LL and moved to DD. The racetube was marked at the time of transfer (blue line) and then marked every 24 hours (black lines). The period of the rhythm of the *ras-1*^{bd} strain is the time that passes between one conidiation peak to the next peak in conidiation, represented by the black dot in the center of the band of conidia. **(B).** Graphical representation of 2 rhythms that peak at different times of day; the black line represents a peak in the dark, while the yellow represents a peak during the day. The period between 2 peaks is indicated, while the phase, or the time in which the peak in oscillation occurs relative to the time of day, is represented by the dashed black line.

B.

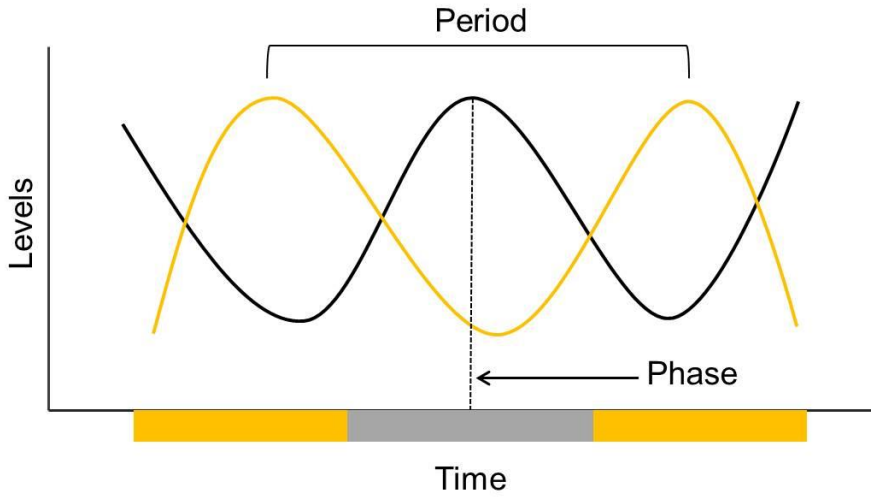

Figure 1-3 Continued.

WCC rhythms. These data suggested that the WCC directly regulates a subset of ccgs, but mostly those that that peak in the morning. This will be discussed in further detail below.

To understand how the clock is regulating genes that peak in the evening, which is anti-phase to WC-1, the rhythmic transcription factor targets of WCC were further characterized. The activity of one such transcription factor, conidial separation-1 (CSP-1) was shown to be a negative regulator of gene expression; the peak of *csp-1* mRNA and protein levels occurs in the morning, allowing it to repress the expression of its target ccgs. As the levels of CSP-1 decrease over the course of the day, the repression of its target genes is relieved, and increased expression is seen of the gene occurring in the evening (141). Taken together, these data support the hypothesis that the Neurospora clock not only directly regulates ccgs, but functions hierarchically by controlling transcription factors that control rhythms of downstream ccgs.

Simultaneously with the ChIP-Seq experiments to identify the direct targets, our lab set out to identify potential pathways that the clock may impinge upon to regulate large numbers of genes. In that effort, a genetic screen performed by Vitalini *et al.* identified mutants that were deficient in the clock regulation of *ccg-1* (160). Two mutants identified in this screen mapped to a single pathway, the OS-2 Mitogen Activated Protein Kinase (MAPK) pathway, involved in osmolarity sensing in Neurospora. Further analysis showed that the activation of the OS-2 pathway was under the control of the clock, and necessary for rhythms in *ccg-1* mRNA accumulation (158). This provided evidence that the molecular oscillator temporally signals through conserved pathways to regulate genes necessary for specific biological processes.

Circadian Regulation of MAPK pathways

MAPK signaling pathways consist of a 3 tier kinase cascade beginning with the MAP Kinase Kinase Kinase (MAPKKK), which, upon phosphorylation, becomes activated and activates the phosphorylation of its substrate, the MAP Kinase Kinase (MAPKK) (Figure1-5). The MAPKK goes on to phosphorylate the terminal step in the cascade, the MAP Kinase (MAPK), which can then signal downstream effector molecules, or directly act as a transcriptional regulator (Figure 1-3). These pathways are highly conserved among eukaryotic organisms ranging from fungi to mammals and have been shown to be important for signal transduction received from both outside, and inside, the cell to control genes necessary for the response to that signal (103). Signals that activate MAPK pathways include molecules that induce proliferation, changes in the environment that lead to stress to the cell, as well as initiating cell death, or apoptosis, in the event that the cell cannot survive the stress. There are 3 major families of MAPK in mammals. The extracellular signal-regulated kinases (ERKs), which are activated in response to growth factors and cytokines, and are responsible for cell growth, survival and differentiation (136), as well as 2 stress response MAPK families, the c-JUN N-terminal kinases (JNKs) and the p38 family of kinases. Homologues of these pathways are found ubiquitously in eukaryotic organisms.

In Neurospora, OS-4 is the MAPKKK in the Osmosensing MAPK pathway, which was first characterized as being responsible for detecting and allowing the cell to adapt to changes in external/ internal osmolarity (167) (Figure 1-4). When the cell detects a change in osmotic conditions, OS-4 is phosphorylated, leading to the subsequential

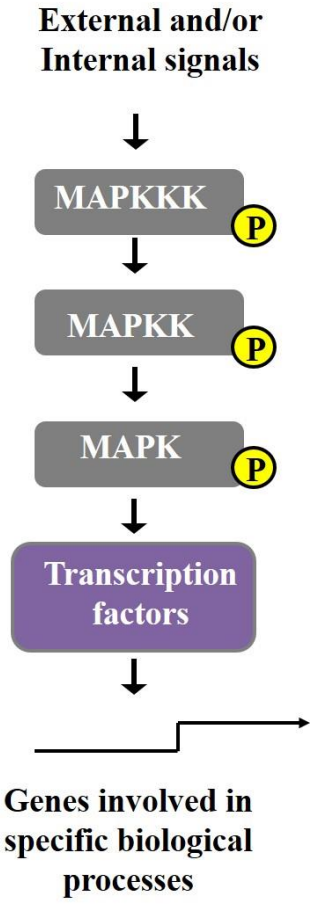

Figure 1-4. Diagram of a MAPK pathway. External and internal stimuli signal to activate the MAPKKK via phosphorylation, which induces the phosphorylation and activation of the MAPKK. Activated MAPKK then induces the activation of the terminal step in the cascade. The MAPK can then go on to activate transcription factors or act as a transcriptional regulator that enhances or represses the expression of genes, depending upon the needs of the cell.

phosphorylation of the MAPKK, OS-5 and the MAPK, OS-2. We have previously shown that the clock regulates the OS-2 pathway, leading to the rhythmic accumulation P-OS-2 (158). The demonstration that OS-4 was a direct target of the WCC led us to determine that OS-4 was also a ccg, and necessary for the rhythmicity of the phosphorylation of OS-2 (P-OS-2) (84). The phosphorylation of P-OS-2 culminates in the activation of genes that will assist in the physiological changes necessary for the cell to adapt to the new osmotic conditions and potentially repress any that are expendable (116). We hypothesized that the clock is impinging upon signaling pathways in eukaryotic cells to coordinately regulate large subsets of genes involved in various biological processes, such that the clock can prime the cell by activating the pathway at a time in which specific stressful conditions are likely to occur. In the case of OS-2, decreasing moisture content in the environment occurs in the late morning, as the sun is rising, drying up the morning dew, thus, P-OS-2 levels peak in the late morning.

In addition to the OS-2 MAPK pathway, there are 2 other MAPK pathways in Neurospora, the MAK-1 and the MAK-2 pathways (Figure 1-5). The MAK-2 pathway is homologous to the Fus-3 MAPK pathway in *Saccharomyces cerevisiae,* which is involved in the mating process (20, 52). Consistent with this, MAK-2 has been shown to be involved in pheromone production and formation of the female sexual structure, the perithecia (89, 123). While MAK-2 has been more thoroughly investigated in Neurospora, the MAK-1 pathway has only recently been characterized. The components of the MAK-1 pathway; the MAPKKK, MIK-1 (NCU02234), the MAPKK, MEK-1 (NCU06419), and the MAPK, MAK-1 (NCU09842), were initially identified by homology to the kinases within the Slt-

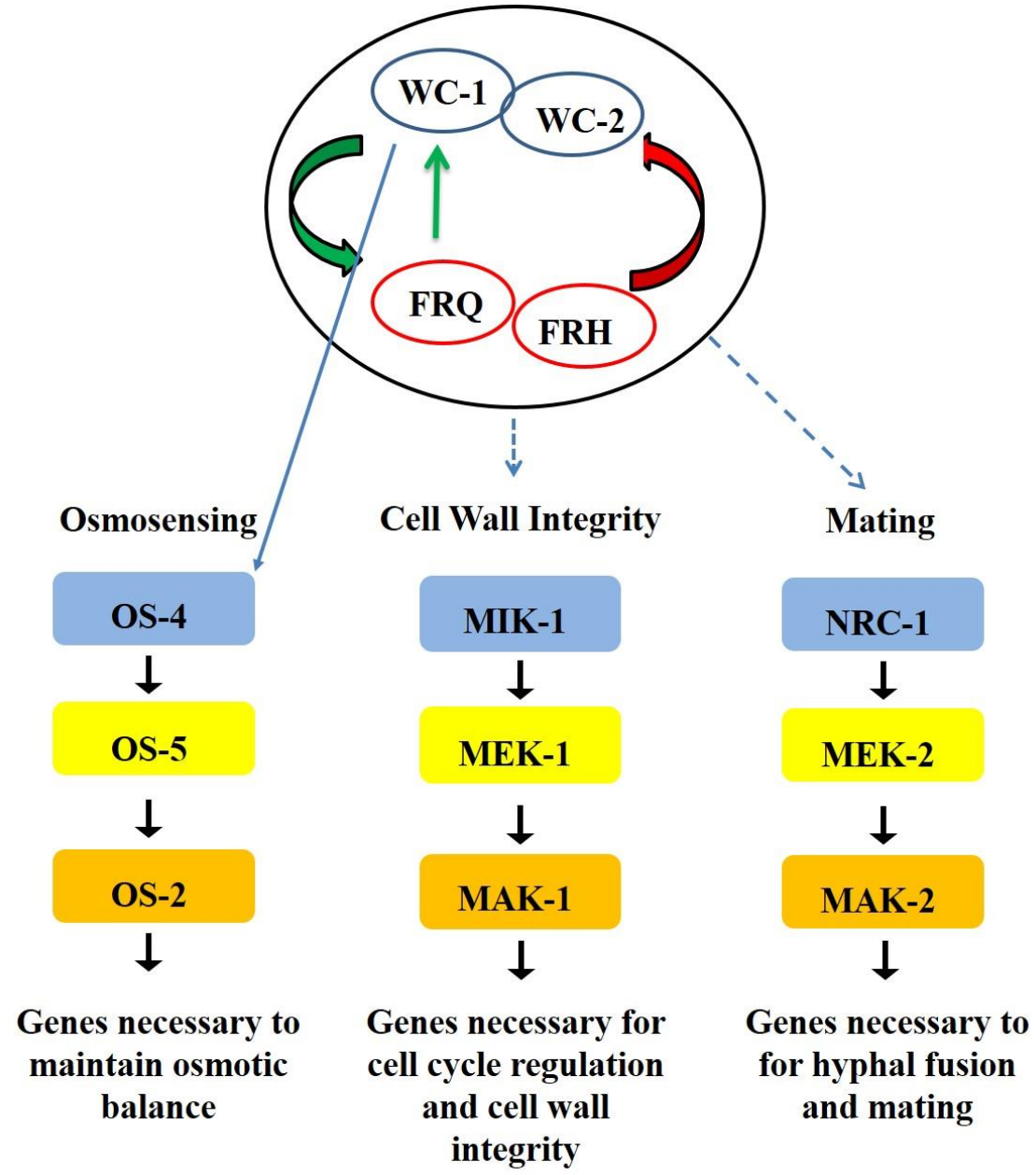

Figure 1-5. A scheme demonstrating the hierarchy of the clock regulating the MAPK pathways in Neurospora. The clock impinges upon the 3 conserved signaling pathways to rhythmically control downstream target genes of the pathway. Direct regulation of OS-4 by the WCC is indicated by the solid blue line. Indirect clock-regulation of the MAK-1 and MAK-2 pathways is indicated by dashed blue lines.

2 pathway in *S. cerevisiae*, which is necessary for the formation and integrity of the cell wall and cell cycle regulation (3, 20) (Figure 1-4). Indeed, phenotypic analysis of strains deleted for the MAK-1 component genes show poor growth, increased branching of hyphae and hyphal lysis, as well as defects in asexual and sexual reproduction (90, 124). Additionally, MAK-1 and MAK-2 are homologous to the ERK1 and ERK2 MAP Kinases, present in mammalian cells, which, consistent with a similar role in fungi, have been shown to be involved in cellular proliferation, morphological changes, and stress responses (136). Numerous studies have identified that changes in the regulation of ERK1/2 contribute to many adverse health effects including, heart disease, cancer, psychological disorders, inflammation and autoimmune diseases (40, 65, 96, 110, 120). The commonality of the diseases associated with defects in MAPK pathways and the circadian clock further underlie the importance of defining and characterizing the relationship between these major signaling pathways.

Our data in Neurospora demonstrates direct regulation of the OS-2 MAPK pathway, which is homologous to the mammalian p38 pathway, by the circadian clock. Studies in our lab have shown that phospho-p38 is rhythmic in constant conditions in several mammalian cell lines, establishing a link between the mammalian clock and the regulation of the p38 pathway (Goldsmith et al., in prep). Ongoing efforts are underway to establish that this link provides beneficial anticipatory effects for mammalian cells (Goldsmith et al., in prep). Taken together, it is likely that the linkage between the circadian clock and MAPK pathways observed in Neurospora also extends to higher eukaryotic organisms, specifically mammals, such that deciphering the mechanism of

clock regulation of MAPK pathways as output pathways, and identification of the downstream targets of the pathways, will lead to a greater understanding of mammalian circadian disease control.

While there have been many recent advances in defining outputs of the circadian oscillator in Neurospora, further characterization of this complex web of transcriptional, translational and post-translation regulation is ongoing. We hypothesized that the clock is utilizing conserved signaling pathways, *i.e.* regulating the activity of all MAPK pathways in *N. crassa* to prime the organism to predict and prepare for daily changes in the environment. To test this hypothesis, I set out to determine if the activity of the MAK-1 (ERK1) and MAK-2 (ERK2) pathways are clock controlled. In Chapter II, I demonstrate that the phosphorylation state, and thus activity, of both MAPK pathways are rhythmic and dependent on the core oscillator. Second, to better understand the function of the MAK-1 pathway, I identified direct target genes of the MAK-1 pathway; specifically genes that are mis-regulated in the ΔMAK-1 strain relative to the wild-type strain. I then cross-referenced these genes with lists of previously identified ccgs to demonstrate a potential connection between the circadian regulation of phospho-MAK-1 (P-MAK-1) and output ccgs. More than 25% of the genes mis-regulated in the ΔMAK-1 strain have also been predicted to be ccgs. To that end, I determined that MAK-1 is required for the rhythmic accumulation of mRNA for 3 of its downstream genes: *ccg-1*, and 2 additional genes that are newly designated ccgs, NCU04352, *chitin synthase-5* (*cs-5*) and NCU07465, a predicted mitochondrial phosphate transport protein. Chapter III documents the study of potential mechanisms by which the clock regulates P-MAK-1. I found that

the mRNA of *mek-1* and *mak-1*, as well as MEK-1 protein levels, accumulate rhythmically, and showed that the rhythms are dependent upon the FWO. Finally, I investigated the role of a transcription factor, ADV-1, in the light and circadian regulation of *mek-1*, *mak-1* and Phospho-MAK-1 (P-MAK-1), revealing that, although ADV-1 affects the regulation of the MAK-1 pathway, it is not essential for light induction or circadian regulation of the MAK-1 pathway components.

CHAPTER II

CIRCADIAN ACTIVATION OF THE MITGEN-ACTIVATED PROTEIN KINASE MAK-1 FACILITATES RHYTHMS IN CLOCK-CONTROLLED GENES IN *NEUROSPORA CRASSA**

Overview

The circadian clock regulates the expression of many genes involved in a wide range of biological functions through output pathways, such as Mitogen Activated Protein Kinase (MAPK) pathways. We demonstrate that the clock regulates the phosphorylation, and thus activation, of the MAPKs, MAK-1 and MAK-2, in the filamentous fungus *Neurospora crassa*. In this study, we identify genetic targets of the MAK-1 pathway, which is homologous to the cell wall integrity pathway in yeast and the ERK1/2 pathway in mammals. When MAK-1 is deleted in Neurospora cells, vegetative growth is reduced and the transcript levels for over 500 genes are affected, with significant enrichment for genes involved in protein synthesis, biogenesis of cellular components, metabolism, energy production and transcription. Additionally, of the ~500 genes affected by the disruption of MAK-1, more than 25% were previously identified as putative clock-controlled genes. We show that MAK-1 is necessary for robust rhythms in two morning-

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specific genes, *ccg-1*, and the mitochondrial phosphate carrier protein gene, NCU07465. Additionally, we show clock regulation of a predicted chitin synthase gene, NCU04352, whose rhythmic accumulation is also dependent upon MAK-1. Together, these data establish a role for the MAK-1 pathway as an output pathway of the circadian clock, and suggest a link between rhythmic MAK-1 activity and circadian control of cellular growth.

Introduction

As a result of the daily rotation of the Earth on its axis, organisms regularly face changes in their environment, including daily exposure to potentially harmful factors, such as ultraviolet light, high temperature, osmotic stress, and oxidative stress. Internal circadian clocks have evolved that allow organisms to coordinate internal biological processes with the cyclic environment, and thus, provide the ability to anticipate and prepare for potential damage. External cues, which serve to reset and synchronize the clock with the environment, are perceived by a molecular circadian oscillator. Time of day information is transduced through output pathways to temporally regulate molecular rhythms, including transcription initiation, transcript stability, translation, post-translational processes, as well as physiology and behavior (10, 22). Significant progress has been made in understanding input pathways to the oscillator, and the molecular mechanisms of the circadian oscillator; however, the mechanisms by which the oscillator regulates rhythmic output processes are just beginning to be unraveled (71, 108, 152, 159).

In *Neurospora crassa*, the FREQUENCY (FRQ)/WHITE COLLAR COMPLEX (WCC) oscillator (FWO) has been well characterized at the molecular level (5, 82). The

FWO consists of a molecular feedback loop, where the WCC, composed of WHITE COLLAR-1 (WC-1) and WC-2, acts as the positive element by directly activating transcription of *frq* mRNA (47). After translation of *frq* mRNA, FRQ protein, in association with the FRQ-interacting RNA helicase (FRH), functions as a negative component by indirectly inhibiting the activity of the WCC (47, 48, 56, 145, 149). Over time, FRQ protein is progressively phosphorylated and degraded, thereby relieving inhibition of WCC, allowing for the cycle to begin again (61). The WC-1 protein is also a blue light photoreceptor and, with its partner WC-2, functions as a transcription factor to induce both light-responsive genes, and clock-controlled genes (ccgs) (27, 60, 152). Studies in Neurospora have suggested that at least 20% of the genome is under control of the circadian clock at the level of transcript abundance (33, 39). The WCC directly controls the expression of about 200 genes, many of which are ccgs that peak in expression in the subjective morning, coincident with the peak of WCC activity (27, 152). However, there are many ccgs that peak in expression at other times of the day, suggesting a complex output regulatory network. Consistent with this idea, several of the direct targets of the WCC are themselves transcription factors, including repressors that control evening-specific gene expression (141, 152). Additionally, clock regulation of major signaling pathways, such as Mitogen Activated Protein Kinase (MAPK) pathways, provides mechanisms for circadian regulation of large sets of functionally related downstream target genes of the pathway. For example, we previously found that the Neurospora FWO controls rhythmic activity of the osmolarity sensitive (OS) MAPK pathway, and downstream ccg targets of the pathway (83, 84, 158).

The evolutionarily conserved signaling MAPK pathways are composed of 3 kinases, the MAP kinase kinase kinase (MAPKKK), the MAP kinase kinase (MAPKK), and finally the MAP kinase (MAPK). The pathway becomes sequentially phosphorylated in response to extracellular signals, and the phosphorylated MAPK then activates downstream effector molecules, including transcription factors that turn on target genes needed for the cell to respond, survive, and adapt to acute environmental signals (163). In Neurospora, the OS-2 MAPK is homologous to HOG1 in yeast, and is responsible for survival during acute hyperosmotic conditions (66, 167). In studies to identify output pathways from the clock, we discovered that the WCC rhythmically binds to the promoter of *os-4*, encoding the OS-4 MAPKKK, and drives rhythms in the accumulation of *os-4* mRNA and OS-4 protein. Rhythmic accumulation of OS-4 protein is necessary for rhythmic phosphorylation and activation of the terminal MAPK, OS-2 and downstream ccgs (83, 84). Taken together, these data demonstrated that clock control of the OS pathway provides a mechanism for Neurospora to prepare for predictable daily hyperosmotic stresses.

In addition to OS-2, there are two other MAPKs present in Neurospora; MAK-1, the homologue of Slt2 in *Saccharomyces cervisiae* and ERK1/2 in mammals, is predicted to be involved in cell wall integrity and maintenance, while MAK-2, the homologue of Fus3 in *S. cerevisiae*, and also ERK1/2 in mammals, has been implicated in the sexual cycle, conidiation, hyphal fusion, and vegetative growth (49, 89, 98, 124, 134). In this study, we show that the phosphorylation state of MAK-1 and MAK-2 is rhythmically controlled by the circadian clock; however, we focus on characterizing the circadian

regulation of the MAK-1 pathway. The MAK-1 pathway is the least characterized MAPK pathway in Neurospora. In *S. cerevisiae*, Slt2 modulates cell wall biogenesis, responses to cell wall perturbations, stress and the cell cycle, while in mammals, ERK1/2 promotes cell growth and cell cycle regulation (26, 28). Although predictions can be made based on homology, there have been no extensive studies to identify the genetic targets of the MAK-1 pathway in Neurospora. Phenotypic analyses of deletion mutants of the MAK-1 pathway showed varying levels of perturbation in the cell wall, increased melanin biosynthesis, and decreased growth rate, polarity, hyphal fusion, conidiation and sexual reproduction (98, 124).

Here, we show that while MAK-1 protein levels are constitutive over the course of the day in constant conditions, the phosphorylation state, and thus activity, of MAK-1 is under control of the clock. We also establish that MAK-1 is phosphorylated after an increase in temperature. Using microarray analysis, we identified potential target genes of the MAK-1 pathway, and compared those to putative ccgs to determine the overlap between these processes. We verified expression levels of several of the MAK-1 downstream target genes, and show that MAK-1 is necessary for regulating mRNA levels in an unstressed environment. Furthermore, we demonstrate that 3 of the targets of the MAK-1 pathway are ccgs, with rhythms that are dependent on a functional MAK-1 pathway, establishing that the circadian clock signal is propagated through the MAK-1 pathway.

Materials and Methods

Strains and culture conditions

N. crassa wild-type strain 74OR23-1 (FGSC 2489), and Δ*mak-1* (FGSC 11320) were obtained from the Fungal Genetics Stock Center (http://www.fgsc.net). The Δ*mak-1* strain was created by replacing the *mak-1* coding region with hygromycin (32), and the knockout was verified using PCR (data not shown). The Δ*frq* and Δ*wc-1* strains were generated by replacing the coding region with the *bar* gene conferring resistance to Basta in 74OR23-1 (Teresa M. Lamb and DBP, unpublished data; (121)). Correct integration of *bar* was verified using PCR (data not shown). The Δfrq^{BAR} and $\Delta wc\text{-}1^{BAR}$ (hereafter named Δ*frq* and Δ*wc-1,* respectively) strains were back-crossed to 74OR23-1 to obtain homokaryons. The strain expressing the MAK-1:LUC translation fusion was created in a 3-way PCR using a fully codon optimized luciferase gene (Renato M. dePaula and DBP, unpublished data; (55)) and transformed into 74OR23-1, where the *mak-1* gene was replaced with the MAK-1:LUC fusion by homologous recombination. Transformants displaying luciferase activity were chosen and crossed to wild-type to obtain homokaryons. Integration at the *mak-1* locus was verified by PCR (data not shown). Circadian time-courses were performed in liquid culture as previously described (84), with the following modifications; mycelial mats from the Δ*mak-1* strain required 4 additional days of growth in constant light (LL), as compared to wild-type strains, because of poor growth. For RNA isolation, liquid cultures were grown in LL 25°C for 24 h and then transferred to constant darkness (DD) at 25°C for 24 h and then harvested at the indicated times. For the heat shock experiments, cells were shifted after growth in LL 25°C for 24

h to DD at 42°C and harvested at the indicated times. To measure the linear growth rate of the wild-type and Δ*mak-1* strains, race tube analysis was done as previously described (143).

Protein extraction and western blot assays

Total protein extracts, protein quantification, and western blot analyses were done as previously described (84). To detect the phosphorylated state of MAK-1 (P-MAK-1), membranes were blocked with 5% non-fat milk in phosphate buffered saline (PBST) with 0.1% Tween-20 overnight at 4ºC, and then washed 3 times for 5 min in PBST before being incubated with rabbit anti-phospho p44/42 (#9101, Cell Signaling, Danvers, MA) at a 1:200 dilution in 5% BSA PBST. After an overnight incubation at 4ºC, membranes were washed 6 times for 10 min in PBST, and incubated with a 1:10,000 dilution of secondary antibody (Goat Anti-Rabbit IgG (H + L)-HRP Conjugate -170-6515, BioRad, Hercules, CA) in 5% non-fat milk PBST at 4ºC overnight. Membranes were washed 6 times for 10 min in PBST. Immuno-reactivity was then visualized with Super Signal West Pico Chemi-luminescence Detection (Thermo Scientific, Waltham, MA). To detect MAK-1::LUC protein, western blots were performed as described above, except membranes were probed with primary goat α-luciferase (#G7451, Promega, Madison, WI) at 1:10,000 dilution, and secondary donkey α-goat (#2020, Santa Cruz Biotechnology, Santa Cruz, CA) at 1:5,000 dilution. To verify phosphate-specific signal, protein extracts were treated with λ-protein phosphatase (#P0753S, New England Biolabs, Ipswich, MA) as indicated. After exposure to X-ray film, images were scanned at 600 dpi in tiff format. Densitometry was performed

using Image J software (UA National Institute of Health, Bethesda, Maryland) and signal levels were normalized to the amido black stained membrane.

Luciferase assays

Mycelia mats of the MAK-1::LUC strain were grown in 1X Vogel's salts, 2% glucose, 0.5% Arginine-HCl, pH 6.0 in LL at 25°C for 3 days. Mycelia mats were cut with a cork borer, generating disks with a 0.5 cm diameter. The disks were washed in sterile water for 30 min before transfer to 96 well plates. Each well was inoculated with 170 µl media containing 1X Vogel's salts with 25 µM luciferin (#LUCNA-300, Gold Biotechnology, St. Louis, MO), and no carbon source. The plates were incubated in LL at 30°C for 4 h, and then transferred to a Packard Topcount Microplate Scintillation and Luminescence Counter (PerkinElmer Life Sciences, Boston, MA). The plates were kept in DD at 25°C and light production was assayed every 90 min. Data was collected and analyzed using the Import and Analysis (I&A) program designed by the S.A. Kay laboratory (127).

RNA extraction and northern blot assay

Total RNA was extracted from tissue harvested and ground in liquid N_2 as described previously (12). All transcripts were detected by northern blot using [α-^{32}P]-UTP-labeled RNA probes as described (152). Primer sequences used to make gene-specific templates are listed in Table 2.1. Images were processed as described for western blots, and normalized to rRNA levels. To determine significant differences in expression

of selected genes between the wild-type and Δ*mak-1* strains, a student's t-test was performed and p-values were required to be equal to or less than 0.05.

Microarray analysis

Wild-type and Δ*mak-1* strains were grown in LL for 24 h, and transferred to DD for 24 h before being harvested and total RNA extracted. RNA samples were treated with DNA-free (Ambion, Grand Island, NY) to remove residual genomic DNA, and then amplified using SenseAmp (Genisphere, Hatfield, PA). The high quality of the original and amplified RNA was confirmed with an Agilent Bioanalyzer (Santa Clara, CA) using RNA 6000 nano chips. The amplified RNA was used to prepare Cy-dye-labeled aminoallyl cDNA targets using the Pronto Indirect Labeling system (Promega, Madison, WI). Targets obtained from mutant and wild-type cells at designated time points were labeled either with Cy5 or Cy3. The 70-mer oligonucleotide probes were synthesized by Illumina (San Diego, CA) and represented 10036 ORFs identified by the Broad Institute (http://www.broadinstitute.org/annotation/genome/neurospora). The printed arrays were cross-linked to the gamma amino propyl silane coating of UltraGAPS slides (Corning Life Sciences, Tewksbury, MA) with 600 mJ of UV energy in a Stratagene Stratalinker (Agilent Technologies, Santa Clara, CA). The arrays were pre-soaked and pre-hybridized to lower background fluorescence and then hybridized with the labeled cDNAs using reagents supplied in the Corning Pronto Universal Hybridization kit according to the manufacturer's specifications. As an internal control, Cy5 and Cy3-labeled wild-type derived cDNAs were hybridized together in duplicate. Wild-type to Δ*mak-1* sample

Table 2.1: Primer sequences used to make gene-specific probes for northern blot experiments.

Gene	Forward Primer Sequence	Reverse Primer Sequence [1]
NCU06871	TTTCCTGTCCTTCTACTACGCCCA	ACAACCAAGAGACGGATCAAGGAG
NCU04352	ACTGCTCTGACCAACGCCAAG	AACATGTCGGAGTTGGCGAAAG
NCU02044	AACTTCCCCTATGCCACCATTGAC	ACCTCCTTGCCGTCCTTGA
NCU08923	ACTCCGGCTCCGGTGAGAA	TCGATGCATTACGCTCGCAT
acw-5	TCCCTGCCACCGAGAAGTTCT	CAATGTAACCGGCAGAGGCG
hsp88	AAACGAAGTCTCCAACCGGGCTA	TCATCCTTGACCTCCTCCTCGA
hsp70	CCAAGAAGGATCTCTCCGGTGAT	TTAGCGGGAAGACATGGCCTT
NCU03980	GAAGAAGAGGCAATACGGAAACGAA	TGTTGCTACCCCTGACAAAGACAG
crp-4	TTCCAGACCAAGTACAAGCGCAG	AGAGCGGCAATGCGCTCCT
NCU07465	ATACCCAGGACTCGATATCCTCCA	AAGAGTGCTGCGCGGGCTT
acw-2	CGCCATCTCTCTTCTGTCAACAGT	TGACGGTGCTGCTAGCATCAGT

[1] The following T7 Primer binding sequence was added to the 5' end of ALL reverse primers: TAATACGACTCACTATAGGG

hybridization was performed in triplicate. Two of the replicates were carried out with Cy5-labeled mutant derived cDNAs and Cy3-labeled wild-type derived cDNAs. In the third replicate the dyes used to label the cDNAs were swapped. Following hybridization, the arrays were washed with the buffers provided in the Pronto Universal Hybridization kit (Promega, Madison, WI) as recommended. The washed arrays were quickly dried under a stream of nitrogen and then scanned using an Affymetrix (Santa Clara, CA) 428 array scanner. Image and data analysis were carried out with GenePix 6.1 and GeneSpring 7.3 software as previously described (4, 73). Data were filtered using the following criteria: the wild-type to wild-type average value was required to be between 0.5 and 2.0. Genes that met these conditions were then subjected to the following restrictions: when comparing RNA from Δ*mak-1* to wild-type in triplicate, values for each of the three experiments were required to be less than 0.67 or greater than 1.5 of wild-type, or the average values of the 3 replicates were required to be statistically different from wild-type using the student's t-test with a p-value cutoff of 0.05.

The raw data are available at the Filamentous Fungal Gene Expression Database (http://bioinfo.townsend.yale.edu/), experiment ID number 225, and the Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/), accession number.

Statistical data

Nonlinear regression was used to fit the rhythmic data to a sine wave (fitting period, phase, and amplitude) or a line (fitting slope and intercept). Akaike's information criteria tests (24) to compare the fit of each data set to the 2 equations were carried out

using the Prism Software package (GraphPad Software, San Diego, CA). The p-values reflect the probability that the sine wave fits the data better than a straight line.

Results

The levels of phosphorylated MAK-1 and MAK-2 are regulated by the circadian clock

In previous studies in Neurospora, the phosphorylation state of MAK-1 and MAK-2 has been detected using available phospho-specific antibodies against the mammalian homologs p44/p42 (123, 124). As expected, using this antibody in western blots, bands were observed corresponding to the predicted size for MAK-1 (46.8 kD) and MAK-2 (40.8 kD) that were absent in the Δ*mak-1* and the Δ*mak-2* strains, respectively (Figure 2-1A). Under these growth conditions, the levels of phosphorylated-MAK-2 (P-MAK-2) were lower relative to phosphorylated-MAK-1 (P-MAK-1) in the wild-type strain, and the levels of P-MAK-2 were increased in the Δ*mak-1* strain as compared to the wild-type strain. To determine if the phosphorylation state of the MAPK's are under clock control, the levels of P-MAK-1 and P-MAK-2 were examined from cultures grown in a circadian time-course, in which the cells were maintained in DD and constant temperature, and harvested every 4 h over two days. Under these conditions, the only time cue is from the endogenous circadian clock. A daily rhythm in both P-MAK-1 and P-MAK-2 was observed in wild-type strains, peaking in the subjective afternoon (about DD16 and DD40/DD44) (Figure 2-2A). Consistent with control of MAK-1 and MAK-2 phosphorylation by the circadian clock, the rhythms were disrupted in cells that lacked the core oscillator component FRQ (Figure 2-2A and B). Additionally, unlike with P-OS-2,

where the levels of P-OS-2 were significantly reduced in Δ*wc-1* and increased in Δ*frq* cells (158), we did not observe a significant change in overall levels of P-MAK-1 or P-MAK-2 in Δ*frq* and Δ*wc-1* strains as compared to wild-type (Figure 2-2C), suggesting a different mechanism of clock regulation.

The MAK-1 pathway is the least characterized MAP kinase pathway in Neurospora. Therefore, we focused further efforts on defining the MAK-1 pathway. MAK-1 was previously shown to be phosphorylated after treatment with a β-1,3 glucan inhibitor, aculeacin-A (124), suggesting that the MAK-1 pathway functions in the cell wall stress-response pathway in Neurospora, similar to Slt2 in yeast (62). Slt2 is also activated in response to increased temperatures, which can destabilize the integrity of the cell wall (102). Under constant conditions, the peak in phosphorylation of MAK-1 occurs in the subjective afternoon, a time of day when environmental temperatures are likely to be higher; therefore, MAK-1 may be activated under similar conditions. Indeed, Vogt and Seiler observed an increase in P-MAK-1 after a shift in temperature from 25ºC to 37ºC (161), and we determined that, after a temperature shift from 25ºC to 42ºC, wild-type cells consistently showed a large increase in the levels of P-MAK-1 after 30 and 60 min at the elevated temperature (Figure 2-2D).

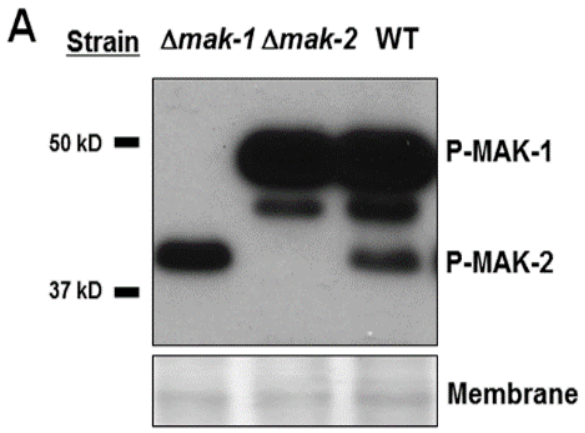


Figure 2-1. Detection of MAK-1 and MAK-2. (A) Western blot showing P-MAK-1 and P-MAK-2 were detectable using α-phospho-p44/42 antibody. Protein was isolated from the indicated strains, and the stained membrane is shown as a loading control. **(B)** Western blot showing total MAK-2 protein, but not total MAK-1 protein, is detectable using α-p44/42 antibody. Protein was isolated from the indicated strains, and the stained membrane is shown as a loading control. No specific bands were observed in the wild type (WT) or Δ*mak-2* that were absent in the Δ*mak-1* strain, indicating that the antibody does not recognize MAK-1. Lanes 2, 4 and 6 were treated with λ-phosphatase in attempts to increase resolution of the bands, while lanes 1, 3, and 5 were untreated.

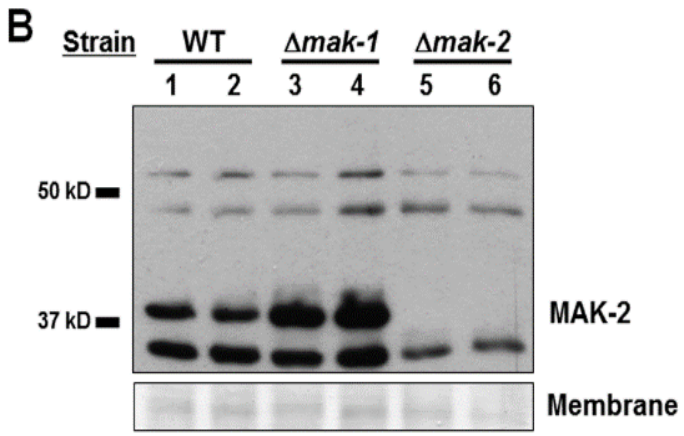


Figure 2-1 Continued.

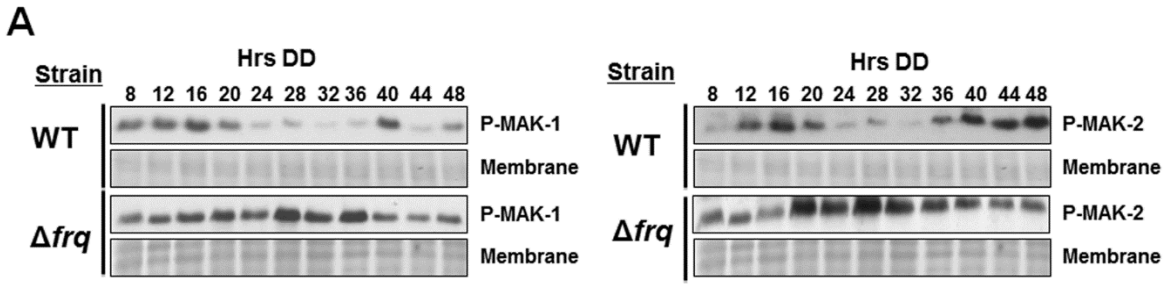


Figure 2-2 Circadian regulation of MAK-1 and MAK-2 phosphorylation. (A) The accumulation of P-MAK-1 (left) and P-MAK-2 (right) are shown in representative western blots of total protein from the indicated strains grown in a standard circadian time-course, in constant dark (DD) and harvested every 4 h. The blots were probed with α–phospho-p44/42 antibody. The stained membranes were used as loading controls. **(B)** blots of normalized P-MAK-1 and P-MAK-2 from **(A)** are shown. WT (solid black line) was better fit to a sine wave (dotted black line; P-MAK-1: $p < 0.001$; P-MAK-2: $p < 0.0001$), whereas Δ*frq* (solid grey line) was better fit to a line (dotted grey line) (values are ± SEM, n=3). **(C)** The levels of P-MAK-1 and P-MAK-2 are not altered in clock mutant strains. Western blot of total protein harvested from the specified strains grown in DD for 16 h, and probed with α–phospho-p44/42 antibody. The stained membrane is shown as a loading control. The data are plotted on the right. The level of P-MAK-1 and P-MAK-2 in WT cells were set to 1 (values are ± SEM, n=3). **(D)** Representative western blot showing the levels of P-MAK-1 at 25ºC, and after 10, 30 or 60 min at 42ºC. Stained membranes are shown as a loading control. This experiment was performed 3 times with similar results.

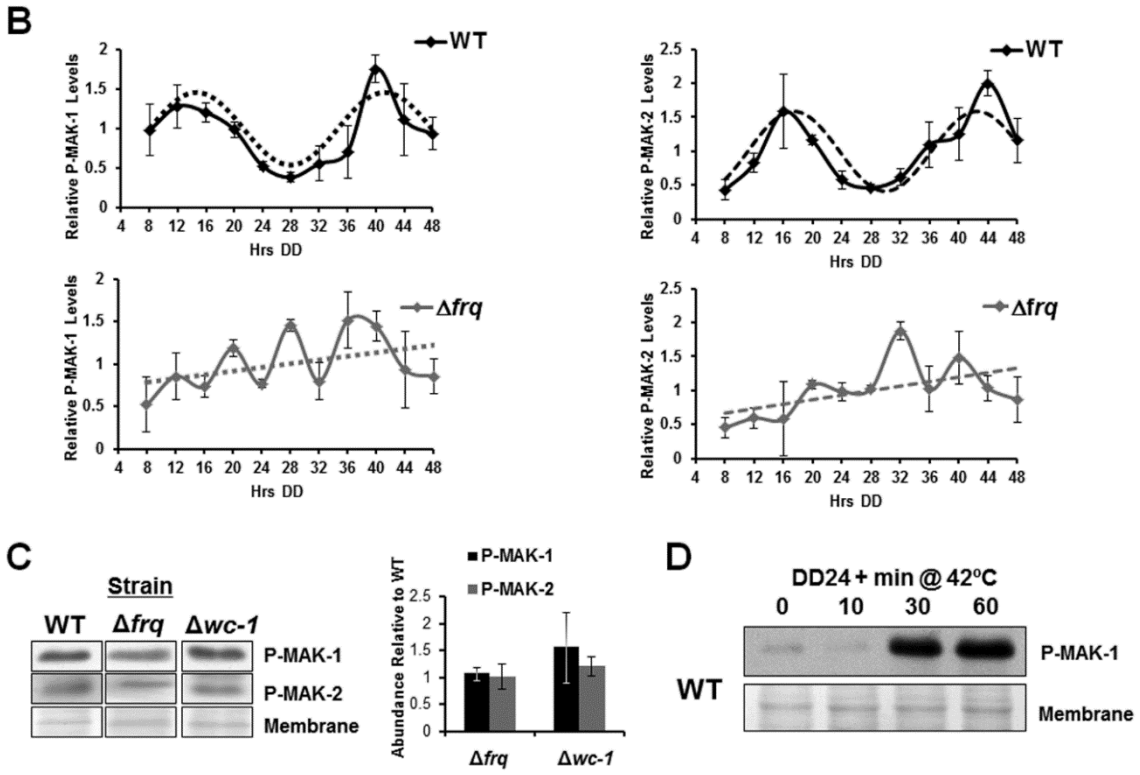


Figure 2-2 continued.

To resolve whether the rhythm in P-MAK-1 is due to rhythms in the accumulation of total MAK-1 protein, we first examined if available antibodies against total mammalian p44/42 would recognize MAK-1 (123); however, this antibody detected MAK-2, and not MAK-1 (Figure 2-1B). Therefore, to detect MAK-1, a translational fusion between MAK-1 and luciferase was generated. No circadian rhythms in MAK-1:LUC were observed by western blot using anti-Luc antibodies or assays to measure luciferase activity (Figure 2-3). While it is possible that the tag interferes with the stability of the protein, the levels of P-MAK-1 cycle normally and no obvious phenotype is associated with this strain (Figure 2-3, and data not shown). Furthermore, using a different tag (V5), no circadian rhythm in MAK-1:V5 was observed (data not shown).

A Δ*mak-1* strain, generated by replacing the *mak-1* locus with the gene conferring resistance to hygromycin (32), has a severe defect in growth, hyphal fusion, protoperithecia formation, and asexual conidiation, indicating that MAK-1 is important for the overall growth and health of the cell (124). Using the race tube assay, the circadian phenotype and linear growth rate of the Δ*mak-1* strain grown in DD 25°C were assayed. The Δ*mak-1* strain grew at 0.27±0.01 cm/day, which was significantly reduced relative to the wild-type growth rate of 6.6±0.57 cm/day. As expected, the Δ*mak-1* strain did not conidiate, and therefore, a circadian rhythm in development could not be observed (data not shown). To ensure that the FWO was functional in the Δ*mak-1* strain, FRQ protein levels were assayed in wild-type and Δ*mak-1* over a circadian time-course and no major difference was observed between the two strains (Figure 2-4A). We also tested rhythmicity of the anchored cell wall protein-1 (*acw-1*) gene, also known as *ccg-15*, a ccg

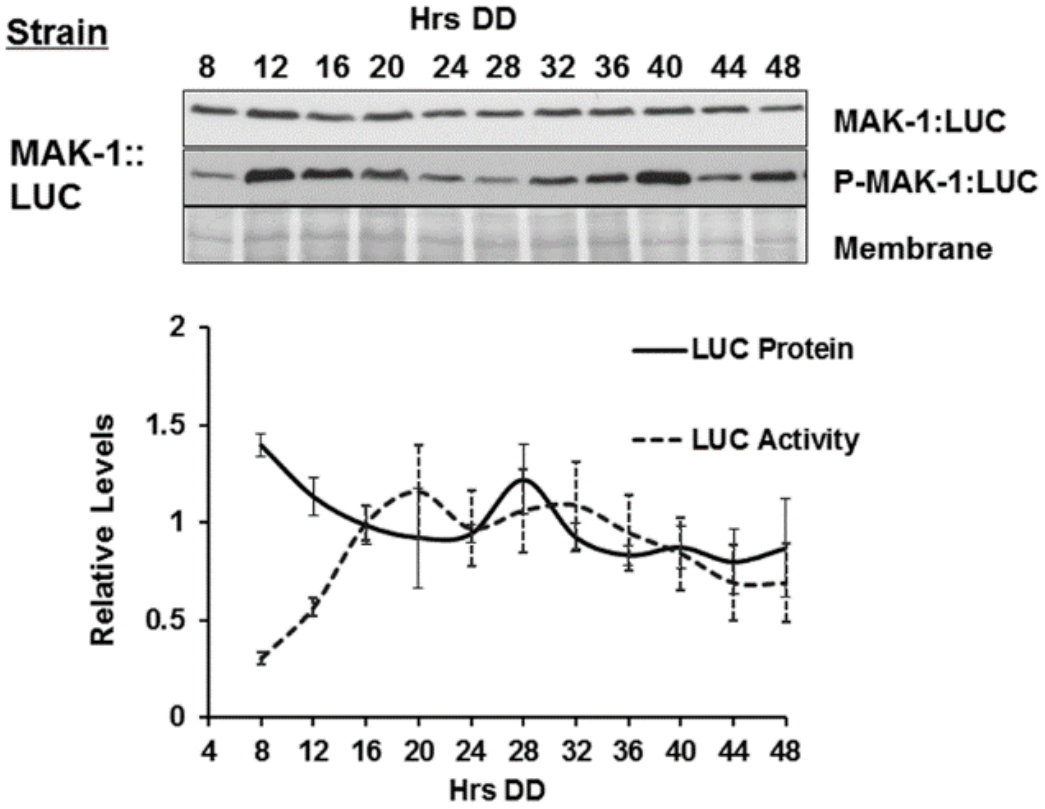


Figure 2-3. The total levels of MAK-1 are not clock-controlled. Western blot of total protein from MAK-1::LUC cells grown in a circadian time-course (see Figure 2-2). The blots were probed with α–LUC or α-phospho-p44/42. The stained membrane is used as a loading control. Plots of MAK-1:LUC protein levels and luciferase activity are shown below (values are ± SEM, n=3).

rhythmicity of regulated by the FWO (168), which encodes a protein homologous to the extracellular mutant (Ecm33) regulated by the Slt2 pathway in yeast (69). No major differences in expression levels or rhythms were observed between wild-type and Δ*mak-1* cells (Figure 2-4B). Based on these results, we concluded that, although the Δ*mak-1* strain grows poorly, the FWO remains functional, and any effects observed on ccg rhythms in the mutant strain can be attributed to a defect in output from the clock.

Identification of downstream targets of P-MAK-1

Phosphorylation of MAPK is associated with activation of target transcription factors, kinases, and other regulatory networks (101). The demonstration that P-MAK-1 is clock-regulated suggested that at least some of the downstream targets of P-MAK-1 would likely be clock-controlled. To test this idea, we first needed to identify downstream genes of the MAK-1 signaling pathway. To accomplish this, microarray analyses were carried out to identify genes with altered expression in Δ*mak-1* as compared to wild-type strains grown in DD and harvested at 24 h. At this time of day, the levels of P-MAK-1 are decreasing. Thus, any observed changes in mRNA levels may reflect clock-dependent (such as a change in period or phase of the rhythms) or clock–independent events. The microarray analyses identified 517 genes that were mis-regulated in the Δ*mak-1* strain (Appendix A), including 111 that were down-regulated and 406 that were up-regulated (Appendix A), suggesting a role for MAK-1 in both activation and repression of gene expression. The genes were sorted into functional categories using the Munich Information Center for Protein Sequences Functional Catalogue (MIPS FunCat), and

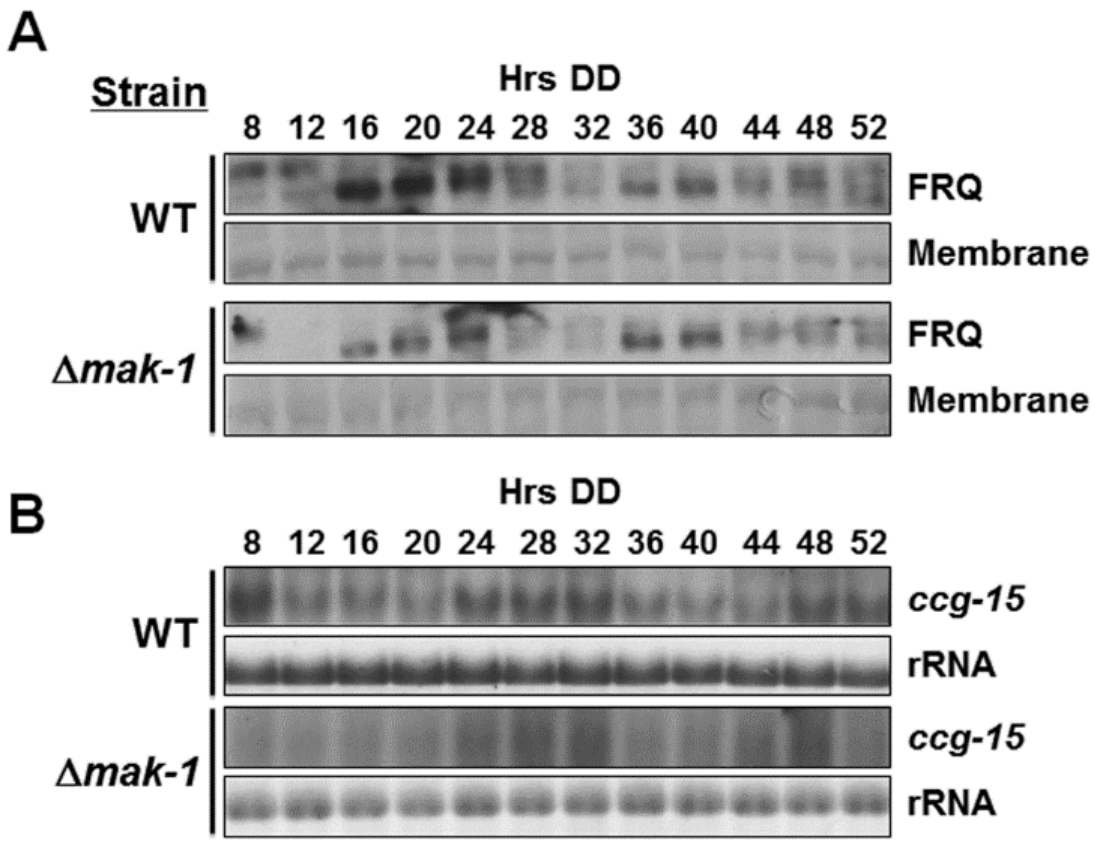


Figure 2-4. The FWO remains functional in the absence of MAK-1. (A) Western blots probed with α-FRQ, showing both the levels and phosphorylation state of FRQ protein in WT and Δ*mak-1* cells grown in a circadian time-course (see Figure 2). The stained membrane is used as a loading control. **(B)** Northern blots probed with *ccg-15* in WT and Δ*mak-1* strains grown in DD and harvested every 4h. Ribosomal RNA (rRNA) was used as a loading control. Consistent results were obtained from two independent experiments.

included genes in 15 of the 20 major functional classifications represented in the Neurospora genome (Appendix A; (138)). Significant enrichment of genes under the control of MAK-1 included genes involved in metabolism, energy production, biogenesis of cellular components, transcription, and protein synthesis, suggesting a role for MAK-1 in these processes. Interestingly, of the 111 genes with decreased levels of mRNA at DD24, 38 (34%) were classified as being involved in protein synthesis, indicating a positive role for MAK-1 in protein synthesis; specifically, in the absence of MAK-1, genes encoding many ribosomal proteins were down-regulated. Furthermore, mutations in the MAK-1 homolog Slt-2 in yeast result in defects in the cell wall and growth (86), and loss of MAK-1 MAPK pathway components also show weak cell walls and a significantly reduced growth rate (124). Although few of the MAK-1 candidate downstream target genes overlapped with those known to be regulated by Slt2 in *S. cerevisiae*, the positive regulation of genes necessary for protein synthesis by the MAK-1 pathway correlates with the decreased growth rate observed when the pathway is disrupted. Using northern blot analysis, mRNA levels for 14 candidate genes identified as mis-regulated in Δ*mak-1* cells, and which represented 8 of the 15 functional categories, were analyzed (Figure 2-5A). We primarily focused on those genes with decreased expression in Δ*mak-1* at DD24 and are involved in cellular processes predicted to be regulated by MAK-1, including biosynthesis of cellular components, metabolism, cellular communication/signal transduction, and stress response/interaction with the environment, as well as the most highly enriched category, protein synthesis (Appendix A). mRNA levels of the candidate genes were examined in wild-type and Δ*mak-1* cells, from cultures harvested under the same growth

conditions used for the microarray analyses. In accordance with the microarray data, NCU06871, NCU04352, *acw-5*, NCU02044, NCU08923, *hsp88*, *hsp70* (NCU02075), NCU03980, *crp-4,* and *ccg-1* mRNA levels were reduced, while the levels of NCU07465 and *acw-2* were elevated, in the Δ*mak-1* strain as compared to wild-type. The expression of 2 predicted MAK-1 downstream target genes, NCU05429 and NCU07472, were inconsistent with the microarray results (Figure 2-5A and B). To differentiate between genes that are mis-regulated in the Δ*mak-1* strain at all times of the day, or that show mis-regulation due to variation in period or phase of ccgs in Δ*mak-1*, mRNA levels of 2 of the downstream target genes, *acw-2* and NCU06871, were assayed at 3 different times of the day, including subjective afternoon (DD16), subjective late afternoon (DD20) and subjective evening (DD24). Consistent with the microarray results, *acw-2* and NCU06871, neither of which is predicted to be a ccg, were mis-regulated in Δ*mak-1* cells at all 3 time-points (Figure 2-5C). Three additional genes were assayed every 4 hours during a circadian time-course. *ccg-1* and NCU04352 showed decreased expression in Δ*mak-1* over the course of 2 days in DD, while NCU07465 mRNA levels were increased in Δ*mak-1* at all times of the day (Figure 2-6). These data indicated that the MAK-1 pathway is necessary for normal mRNA accumulation of at least a subset of its target genes, including known and predicted ccgs. To identify rhythmic targets of the P-MAK-1 pathway, we compared the Δ*mak-1* microarray data to 3818 unique genes that were previously classified using microarrays as putative ccgs (Renato M. dePaula, Richard Gomer, PB, TLT, and DBP, unpublished data; (33, 39, 117, 166)) and found that, of the 517 genes

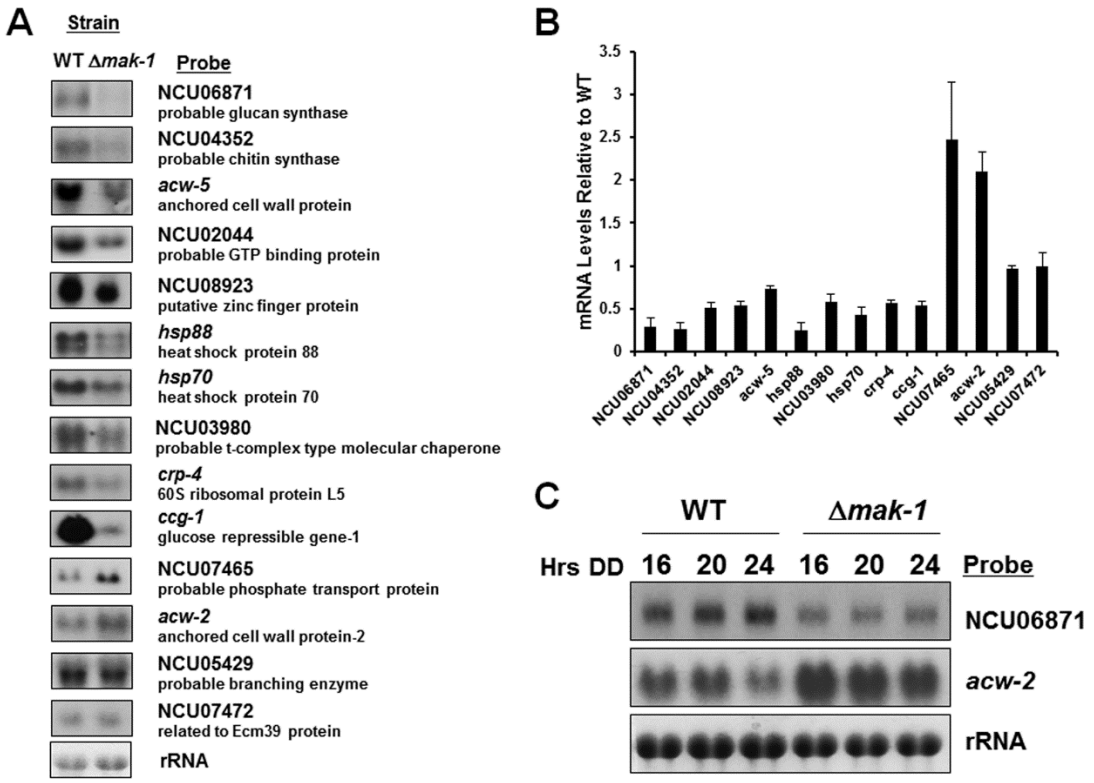


Figure 2-5. MAK-1 is required for the regulation of target genes. (A) Representative northern blots of total RNA from the indicated strains validating the MAK-1-dependent regulation of 12 of 14 genes identified by microarray analysis. The membranes were probed with the indicated genes. The known or predicted functions or domains of the genes are shown in parenthesis below. rRNA was used as a loading control. (**B)** Plots of mRNA levels of the indicated genes in Δ*mak-1* cells normalized to rRNA from **(A)** (values are ± SEM, n=3) relative to the levels in WT cells. The levels of mRNA in WT cells in each blot were set to 1. Twelve out of 14 genes tested showed significantly different expression levels between WT and Δ*mak-1* strains (NCU06871 $p \leq 0.02$, NCU04352 $p \leq 0.01$, NCU02044 $p \leq 0.05$, NCU08923 $p \leq 0.04$, *acw-5* $p \leq 0.02$, *hsp88* $p \leq 0.02$, NCU03980 $p \leq 0.05$, NCU02075 $p \leq 0.03$, *crp-4* $p \leq 0.004$, *ccg-1* $p \leq 0.01$, NCU07465 $p \leq 0.02$, and *acw-2* $p \leq 0.04$, NCU05429 $p \leq 0.39$, NCU07242 $p \leq 0.21$). **(C)** Northern blots probed with NCU06871 and *acw-2* in WT and Δ*mak-1* strains grown in DD for the indicated times. rRNA is shown as a loading control.

predicted to be differentially expressed in Δ*mak-1* at DD24, at least 145 genes were also predicted to be ccgs (Appendix A). Similar to the total number of MAK-1 responsive genes, an enrichment of ccgs involved in metabolism, energy and protein synthesis was observed. Taken together, these data support the idea that the clock regulates rhythms in the activity of MAK-1, which, in turn, controls rhythmic expression of many target genes of the pathway.

MAK-1 is required for rhythmic expression of some pathway target genes

To determine if ccgs that are targets of the MAK-1 pathway require MAK-1 for rhythmic mRNA accumulation, a subset of MAK-1 target ccgs was examined for circadian rhythms in mRNA accumulation in the Δ*mak-1* strain. As shown in Figure 2-3, levels of *ccg-1* transcript were reduced in the Δ*mak-1* strain under non-stress inducing conditions, suggesting that MAK-1 is important for *ccg-1* mRNA accumulation. This led us to investigate if MAK-1 is necessary for rhythmic *ccg*-1 mRNA accumulation. The amplitude of the observed rhythms in *ccg-1* accumulation was reduced in Δ*mak-1* versus wild-type cells (Figure 2-6A), and a rhythm was not reliably reproducible; low amplitude rhythms were detected in half of the experiments. We previously showed that the OS-2 MAPK pathway regulates rhythms in *ccg-1*; thus, it is not unexpected that when MAK-1 was deleted in cells, some elements of rhythmicity were maintained under control of the OS pathway, which peaks in phase with *ccg-1*. Regulation of *ccg-1* by at least 2 MAPK pathways may also underlie the observation that the peak in *ccg-1* expression precedes the peak in MAK-1 phosphorylation rhythms (Figures 2-2 and 2-6A).

We next focused on genes known, or predicted, to be a part of the biogenesis of cellular components. The structural elements of the cell wall in Neurospora primarily consist of polymers made of glucan and chitin (100). There are at least 2 proteins required for glucan synthesis in Neurospora; the enzyme GLUCAN SYNTHASE-1 is encoded by the gene *gs-1* (NCU06871), and the regulatory subunit RHO-1, encoded by the gene NCU01484 (44, 147). Transcripts for both genes were mis-regulated in Δ*mak-1* cells; transcript levels of *gs-1* were reduced, while *rho-1* levels were elevated in Δ*mak-1* when compared to wild-type cells at DD24 (Figure 2-5, and data not shown). Although there are 10 genes predicted to be involved in chitin synthesis in Neurospora, only the gene, NCU04352, encoding a predicted chitin synthase, was found to be mis-regulated in Δ*mak-1* in the microarray experiments. As with *gs-1*, NCU04352 mRNA levels were reduced by about half in the Δ*mak-1* strain under non-stress inducing conditions. While neither of these cell wall biogenesis genes had been previously reported to be clock-controlled, based on their regulation by the rhythmic MAK-1 pathway, we assayed mRNA levels of *gs-1* and NCU04352 in wild-type and Δ*mak-1* strains over a circadian time-course. No rhythm was observed for *gs-1* mRNA levels in wild-type cells (data not shown); however, a rhythm in mRNA accumulation was observed for NCU04352 in wild-type cells, with peaks occurring around DD16 and DD40 (Figure 2-6B), a phase similar to that of P-MAK-1 (Figure 2-2). Consistent with clock regulation of the MAK-1 pathway, the rhythm in NCU04352 was abolished in Δ*frq* and Δ*mak-1* strains (Figure 2-6B). The levels of NCU04352 mRNA were low in the Δ*mak-1* strain at all times of day as compared to wild-type cells, supporting the idea that P-MAK-1 positively regulates NCU04352 expression.

Although the levels were decreased, after a 24 hour exposure to film, transcript levels of NCU04352 were detectable in Δ*mak-1*, but with no obvious circadian rhythm in mRNA levels (Figure 2-6B).

Finally, we tested the necessity for MAK-1 in the rhythmic accumulation of an additional putative ccg, NCU07465, encoding a predicted mitochondrial phosphate carrier protein. As shown in Figure 2-3, the levels of NCU07465 mRNA were increased when *mak-1* was deleted, which indicated that MAK-1 contributes to the repression of NCU07465. In wild-type cells, accumulation of NCU07465 mRNA was rhythmic; however, the rhythm was abolished in the Δ*frq* and the Δ*mak-1* strains (Figure 2-6C). Similar to *ccg-1*, the peak phase of NCU07465 mRNA was in the subjective early morning (DD12 and DD36), occurring earlier than the peak of P-MAK-1 levels. Taken together, these data support the hypothesis that the clock signals through the MAK-1 MAPK pathway to regulate rhythmic gene expression via positive and negative regulation, and with peak phases at different circadian times.

Discussion

Endogenous circadian clocks provide a mechanism for organisms to anticipate environmental stress and prepare cellular response pathways needed for survival. Here, we show that in Neurospora, the circadian clock regulates the activity of the MAK-1 and MAK-2 MAPK pathways. In constant conditions, and in the absence of stress, the levels of active P-MAK-1 and P-MAK-2 peak during the subjective afternoon. At this time of

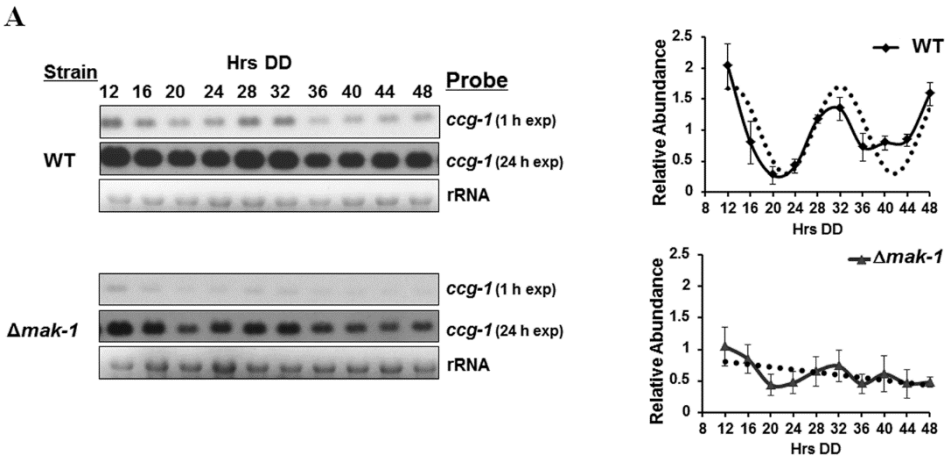


Figure 2-6. MAK-1 is necessary for robust rhythmic accumulation of downstream target ccgs. (A) Representative northern blots of *ccg-1* mRNA from WT and Δ*mak-1* strains grown in a circadian time-course (see Figure 2). rRNA is shown as a loading control. A 24 h exposure is shown in order to detect *ccg-1* mRNA in Δ*mak-1* cells. The data are plotted on the right (values are ±SEM, n=3). WT is represented by a solid black line, and Δ*mak-1* by a solid gray line. Plots of *ccg-1* from WT cells were fit to a sine wave (dotted black line) ($p < 0.02$), whereas *ccg-1* from Δ*mak-1* was better fit to a line (dotted black line). **(B).** Representative northern blots of NCU04352 mRNA from the indicated strains, and labeled and plotted as in **(A)**. The Δ*frq* strain is represented as a dotted gray line. Plots of NCU04352 in WT cells were fit to a sine wave (dotted black line) ($p < 0.0001$), whereas NCU04352 from Δ*mak-1* and Δ*frq* were better fit to a line (dotted black line). **(C)** Representative northern blots of NCU07465 mRNA from the indicated strains, and labeled and plotted as in (**A** and **B**). Plots of NCU07465 from WT cells were fit to a sine wave (dotted black line) ($p < 0.0001$), whereas in Δ*mak-1* and Δ*frq* cells, NCU07465 was better fit to a line (dotted black line).

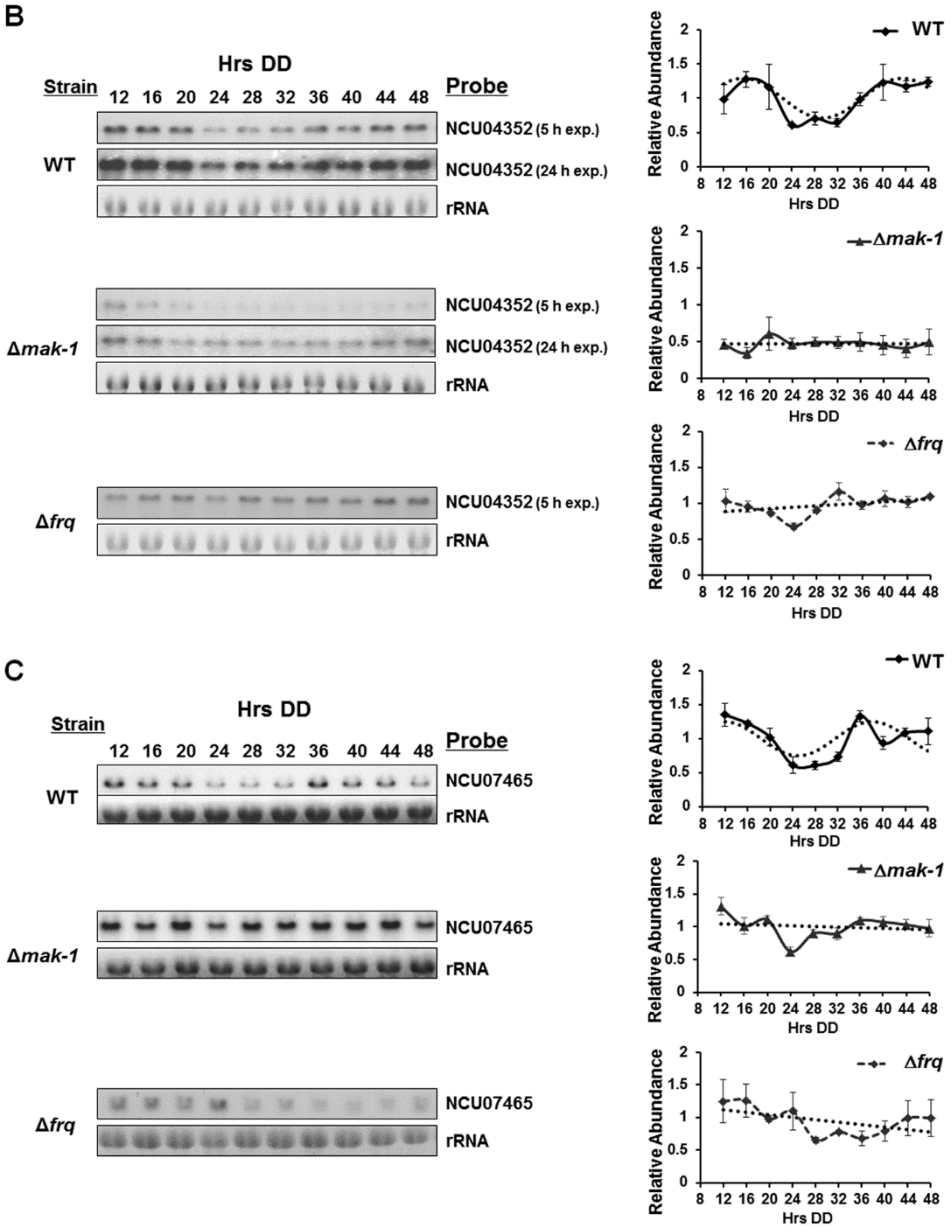


Figure 2-6 Continued.

day, in the naturally changing environment, the ambient temperatures would generally be higher; thus, activation of P-MAK-1 at this time may provide a mechanism for the cell to anticipate stress on the cell wall induced by increased temperatures. Consistent with this idea, increased levels of P-MAK-1 were observed when wild-type cells were subjected to an increase of temperature. A role for P-MAK-2 in the regulation of vegetative growth, conidiation and mating was previously established (89, 123), processes that are under the control of the circadian clock in Neurospora (18, 54, 143). Thus, the clock may signal through the MAK-2 pathway to control rhythms in genes involved in these biological processes. However, the biological functions of the MAK-1 pathway were not known. In *S. cerevisiae*, not only is Slt2, the MAK-1 homolog in yeast, activated in response to stress that affects the cell wall, it is activated during cell cycle transitions, leading to budding and polarized pseudohyphal growth (106). The ERK1/2 pathways in mammals are activated in response to stress, as well as extracellular signals leading to the regulation of cellular growth and polarity (137). To better understand the role of the MAK-1 pathway in Neurospora, we performed microarray analysis to identify genes that are mis-regulated in the Δ*mak-1* under normal growth conditions. About 500 genes were mis-regulated in Δ*mak-1* cells, with independent validation of the downstream targets suggesting an accuracy of 85% (12/14 tested were significant). We did not observe considerable overlap between genes regulated by MAK-1 in our arrays and those regulated by Slt2 in *S. cerevisiae* (3, 69, 105). These results may be explained by differences in morphogenesis of these organisms; Neurospora hyphae grow as a syncytium (133), while budding is observed in Saccharomyces (92). However, the slow growth of Δ*mak-1* cells, as well as

the decreased expression of a significant number of genes encoding ribosomal proteins, suggests that, similar to ERK1/2 in mammals, MAK-1 may be needed for cells to proceed through the G_1 phase of the cell cycle, a process requiring an increase in protein synthesis and the biogenesis of structural components of the cell membrane or wall (26). Although the microarray results yielded fewer MAK-1 pathway-regulated genes with characterized roles in cell wall biogenesis than might be expected, some genes with predicted roles in cell wall biogenesis are classified by FunCat as metabolic or energy-related genes. Furthermore, some of the MAK-1 target genes that are uncharacterized may turn out to be important in cell wall biogenesis and maintenance. The MAK-1 downstream target gene and ccg, NCU07465, encodes a mitochondrial phosphate carrier protein homologous to yeast Pic2, which is induced in response to stress (57). This gene is not known to be regulated by the Slt2 pathway in yeast; however, this family of carrier proteins is necessary for transport of inorganic phosphate into the mitochondria in order to generate ATP, supporting a role for the MAK-1 pathway in rhythmic energy production (37) and cellular growth control.

Of the genes affected in the Δ*mak-1* strain, 28% were previously characterized in microarray experiments as putative ccgs, supporting the idea that by controlling rhythmic activation of MAPK pathways, the clock can coordinately control a subset of functionally related genes. However, similar to the OS-pathway (83), not all downstream targets of MAK-1 are ccgs. For example, no circadian rhythm in mRNA accumulation was observed for the two heat shock genes, *hsp88* and *hsp70* (data not shown), despite these two genes showing different levels of mRNA in Δ*mak-1* relative to wild-type cells. In agreement

with this idea, all 3 MAPK pathways in Neurospora are clock-controlled, albeit with varying phases. These pathways are likely responsible for controlling both unique, as well as overlapping, cellular processes and stress responses that occur at various times in the day, consistent with our findings that not all ccg targets of the MAK-1 pathway behave arrhythmically in Δ*mak-1* cells; circadian rhythms in mRNA accumulation of 2 downstream targets of MAK-1, *ccg-7* and NCU08949, were maintained in the Δ*mak-1* strain (data not shown). P-OS-2 peaks in the morning at a time when the cell is beginning to encounter potential osmotic changes in the environment (158), while P-MAK-1 and P-MAK-2 peak later in the afternoon at a time when the growth rate of the cells is increasing and conidiation is repressed (54). Interestingly, when MAK-1 was deleted, the levels of *ccg-1* mRNA were greatly reduced. Despite this, low amplitude *ccg-1* mRNA rhythms were observed in the deletion strain, albeit with less reproducibility than in wild-type cells. We have previously reported that *ccg-1* rhythmicity depends upon a functional OS-2 pathway (158), and *ccg-1* is induced in response to a variety of stresses (42, 93, 162, 165). Thus, it appears that both MAPK pathways contribute to robust rhythmicity in the expression of some target genes, including *ccg-1*. This may not be surprising as previous studies have demonstrated crosstalk between MAPK pathways (107, 148), and more specifically in yeast, have indicated that dual activation of the Hog1 and Slt2 pathways are required for cell survival during cell wall stress (16). In contrast to MAK-1 pathway regulation of *ccg-1*, the levels of target gene NCU07465 mRNA were increased in the Δ*mak-1* cells at all times of the day, and circadian rhythms in mRNA accumulation were abolished. Taken together, these data illustrate the complexity of clock output

mechanisms; MAK-1 is necessary to both promote and repress gene expression, and through these activities, control rhythmic gene expression. Additionally, both *ccg-1* and NCU07465 mRNA levels peak in the morning, prior to the peak of P-MAK-1, supporting the existence of dual MAPK module inputs to some of the MAK-1 downstream targets, and/or a complex, hierarchical regulation of downstream effector molecules that transduce the rhythmic signal from MAK-1 to the downstream target genes. Alternatively, the correspondence of peak levels of NCU04352 mRNA and P-MAK-1 suggests a short regulatory pathway from P-MAK1 to NCU04352.

In summary, we have shown the activity of the ERK1/2 like MAP kinases, MAK-1 and MAK-2, to be under the control of the circadian clock in Neurospora. The conservation of the clock and these signaling cascades underlies their relevance in the health of eukaryotic organisms. Both the clock and MAP kinase pathways have been linked to diseases, including cancer; defects in either can lead to uncontrolled growth and tumor formation (64, 128). The circadian activation of MAK-1 suggests that clock regulation of rhythmic growth and development in Neurospora may be, in part, acting through the MAK-1 MAPK pathway. Therefore, in both Neurospora and mammalian cells, these ERK like kinases may provide a mechanism to temporally limit cellular growth, such that progression through the cell cycle occurs at times of the day when cells are the least susceptible to genotoxic stress.

CHAPTER III

CLOCK INPUT TO THE MAK-1 PATHWAY

Introduction

After demonstrating the circadian regulation of the activation of all three of the Mitogen Activated Protein Kinases (MAPKs) in *Neurospora crassa* (15, 84, 158), our effort focused on elucidating the mechanisms by which the clock controls these MAPK pathways at the molecular level. We previously showed that the WHITE COLLAR Complex (WCC) transcription factor rhythmically binds to the *os-4* promoter. Furthermore, we showed that WCC binding to the *os-4* promoter is necessary for the circadian accumulation of *os-4* mRNA and OS-4 protein (84). Additionally, when the WCC binding sequences are deleted from the *os-4* promoter, rhythms in phospho-OS-2 (P-OS-2) are abolished, indicating a direct link between the core FRQ-WCC oscillator (FWO) and the circadian input to the OS MAPK pathway (84). While Chromatin-Immunoprecipitation sequencing (ChIP-seq) experiments performed to find direct targets of the WCC identified the *os-4* promoter, no direct targets were found in the promoters of genes that reside in the MAK-1 and the MAK-2 pathways (152). As the peak in activation of both MAK-1 and MAK-2 is anti-phase to the peak in WCC activity and P-OS-2, these data were not surprising, and suggested indirect control of the MAK-1 and MAK-2 pathways by the clock. The WCC ChIP-seq experiments revealed that the WCC bound at least 24 known, or predicted, transcription factors, which included proteins that either activate or repress the activity of gene expression (152). One of these transcription factors,

arrested development-1 (ADV-1), is homologous to a zinc finger transcription regulatory protein required for fruiting body formation, Pro-1, in the fungus *Sordara macrospora* (104). To identify ADV-1 targets, ChIP-seq experiments were performed with ADV-1 translationally fused to the V5 epitope. The results of these experiments identified ADV-1 binding sites in the promoters of 2 of the MAK-1 pathway genes, the MAPKK, *mek-1*, and the MAPK, *mak-1* (Dekhang and Bell-Pedersen, in prep). Consistent with these data, ADV-1 deleted strains have similar phenotypes to strains deleted for the MAK-1 pathway components, including female sterility, decreased aerial hyphae, and loss of circadian regulation of conidiation (15, 124, 152). Both the mRNA and the protein levels of ADV-1 are light-induced and accumulate with a circadian rhythm (152), providing further evidence that ADV-1 may mediate the regulation of the output pathway from the FWO to the MAK-1 pathway.

In this study, I show that the clock regulates mRNA levels of the MAPKK, and the MAPK, *mek-1* and *mak-1*, while the levels of the MAPKKK, *mik-1*, remain constant throughout the day. I also confirmed ADV-1 binds to the promoter regions of both *mek-1* and *mak-1*. Light induction experiments suggested that *mek-1* was mildly induced by light, and while this induction is not completely abolished in the ΔADV-1 strain, it is less robust, indicating a role for ADV-1 in the regulation of *mek-1*. Additionally, I show that the *mak-1* transcript was not light-induced in wild-type or ΔADV-1 cells, but *mak-1* mRNA levels were consistently lower in the ΔADV-1 strain when compared to wild-type. Moreover, *mek-1*, *mak-1* and P-MAK-1 rhythms were maintained in ΔADV-1. Taken together, these

data indicated that while ADV-1 likely plays a role in regulating the MAK-1 pathway under certain conditions, it is not required for the circadian activation of MAK-1.

Materials and Methods

Strains and culture conditions

N. crassa wild-type strain 74OR23, mating type a, and ΔADV-1 mutant strain, mating type a (FGSC11041) were obtained from the Fungal Genetics Stock Center. The ΔADV-1 strain was created by replacing the *adv-1* (NCU07932) coding region with a hygromycin resistance gene (152), and the knockout was verified using PCR (data not shown). The ΔFRQ strain was generated by replacing the coding region with the *bar* gene, conferring resistance to Basta (glufosinate-ammonium) in 74OR23 (15). A strain expressing a MEK-1:V5 translation fusion was created by 3-way PCR, and then transformed into 74OR23, where the endogenous *mek-1* gene was replaced with the MEK-1:V5 fusion by homologous recombination. Transformants were screened by western blot analysis to identify the correct size protein present, and crossed with the wild-type and ΔFRQ strains to obtain homokaryons. Circadian time course experiments were performed in liquid culture as previously described (15). For RNA isolation, liquid cultures were grown in constant light (LL) at 25°C for 24 h, transferred to constant darkness (DD) at 25°C for 24 h, and then harvested at the indicated times. For light pulse experiments, cells were grown in LL at 25°C, shifted to DD at 25°C for 24 h, before being shifted back to LL, and harvested at the indicated times.

RNA extraction and northern blot assay.

Total RNA was extracted from tissue harvested and ground in liquid N_2 as described previously (12). All transcripts were detected by northern blots using [α-32P]UTP-labeled RNA probes as described previously (152). Primer sequences used to make gene-specific templates are *mek-1* forward primer: 5' CAGCAAGACCCACCAGTGTA 3', *mek-1* reverse primer: 5' CAAACCAACGGTTTCTTCGT 3'; *mak-1* forward primer: 5' CAAGCAACGATCCGTAAACA 3', *mak-1* reverse primer: 5' CTGTGATTTGCAACCCTCCT 3'. After exposure to X-ray film, images were scanned at 600 dpi in TIFF format. Densitometry was performed using ImageJ software, and signal levels were normalized to the rRNA.

Protein extraction and western blot assays

Total protein extracts, protein quantification, and western blot analyses were done as previously described (83). The phosphorylated state of MAK-1 (P-MAK-1) was detected as previously described (15). To detect the MEK-1:V5 protein, western blots were performed as described above, except that membranes were probed with a primary rabbit anti-V5 tag antibody (ab9116; Abcam, Cambridge, MA) at a 1:10,000 dilution, and a secondary donkey anti-rabbit antibody (2020; Santa Cruz Biotechnology, Santa Cruz, CA) at a 1:5,000 dilution. Immunoreactivity was then visualized with a Super Signal West Pico chemiluminescence detection kit (Thermo Scientific, Waltham, MA). Images were processed, as described for northern blots, and normalized to amido black-stained

membrane. To verify the phosphate-specific signal, protein extracts were treated with λ-phosphatase (New England BioLabs, Ipswich, MA) as directed by the supplier. After exposure to X-ray film, images were scanned at 600 dpi in TIFF format. Densitometry was performed using ImageJ software (National Institute of Health, Bethesda, MD), and signal levels were normalized to the amido black-stained membrane. To detect phosphorylated MEK-1:V5, western blots were performed using the Phos-tag™ system according to manufacturer's specifications (http://www.wako-chem.co.jp/english/labchem/product/life/Phos-tag/pdf/AAL107_v8.pdf), (Wako Pure Chemical Industries, Ltd Osaka, Japan).

Results

Clock regulation of MAK-1 pathway components

To determine how the clock is impinging upon the MAK-1 pathway, I first investigated if the transcript levels of each of the kinases within the cascade, the MAPKKK, *mik-1*, the MAPKK, *mek-1*, and the MAPK, *mak-1* accumulated with a circadian rhythm. RNA from wild-type and ΔFRQ strains grown in DD and harvested every 4 hours was isolated and probed for the each of the transcripts. As shown in Figure 3-1, the mRNA levels of *mik-1* were constitutive throughout the time course in the wild-type strain. The mRNA levels of *mek-1* and *mak-1* showed a circadian oscillation with a period of about 24 hours in the wild-type strain, peaking around DD20 and DD44, and the rhythm was abolished in the ΔFRQ strain, indicating the rhythms are dependent upon the circadian clock mechanism.

As we have previously reported, the protein levels of the MAPK, MAK-1, show no detectable rhythm when analyzed using antibody to the epitope tagged strains in western blots (3). I next asked if the levels of MEK-1 protein, similar to OS-4, are rhythmic and lead to rhythms in activation of the MAK-1 pathway. No antibodies known to detect the MEK-1 protein in Neurospora are available; therefore, I generated a translation fusion, adding the V5 epitope tag to the C-terminus of MEK-1. Four out of eight transformants screened expressed the MEK-1:V5 protein, which is predicted to be about 65 kDa (Figure 3-2A). Transformant number 4 was crossed to a wild-type strain, and to a ΔFRQ strain, to obtain homokaryons with tagged MEK-1 (MEK-1:V5 and MEK-1:V5, ΔFRQ, respectively). The tagged strains displayed no phenotype, suggesting the tag did not interfere with the function of MEK-1 (data not shown). To determine if MEK-1 protein levels followed the rhythms in the *mek-1* mRNA levels, the MEK-1:V5 and ΔFRQ, MEK-1:V5 strains were grown in a circadian time course, and MEK-1:V5 levels were assayed by western blot analysis. In Figure 3-2B, I show that the levels of MEK-1:V5 oscillated over the time course of the day in the wild-type strain, with a period of about 16 hours. While the levels also fluctuated in the ΔFRQ strain, there was no consistent rhythm with a measurable period (Figure 3-2B). Using Prism software, I show that, although the 16 h period of MEF-1:V5 is shorter than would be predicted for a circadian rhythm, it is within the error range of ± 8 hours using 4 hour time points, and the average levels of MEK-1:V5 in wild-type were better fit to a sine wave ($p \leq 0.003$), than a straight line. The ΔFRQ strain, the average levels of MEK-1:V5 were better fit to a line, consistent with circadian control

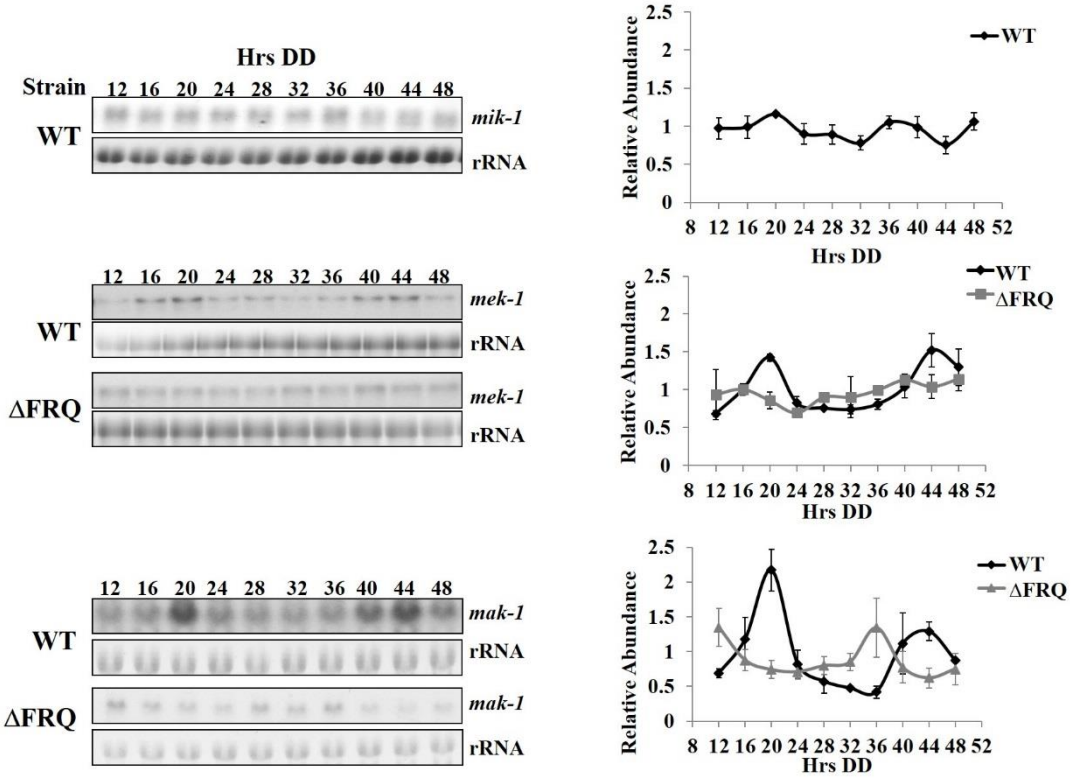


Figure 3-1: Clock regulation of the MAK-1 transcripts. Representative northern blots probed with *mik-1* (top), *mek-1* (middle), and *mak-1* (bottom) in WT and Δ*frq* strains grown in DD and harvested every 4 hrs are shown on the left. The data are plotted on the right (values are ±SEM, n=3). Ribosomal RNA (rRNA) was used as a loading control.

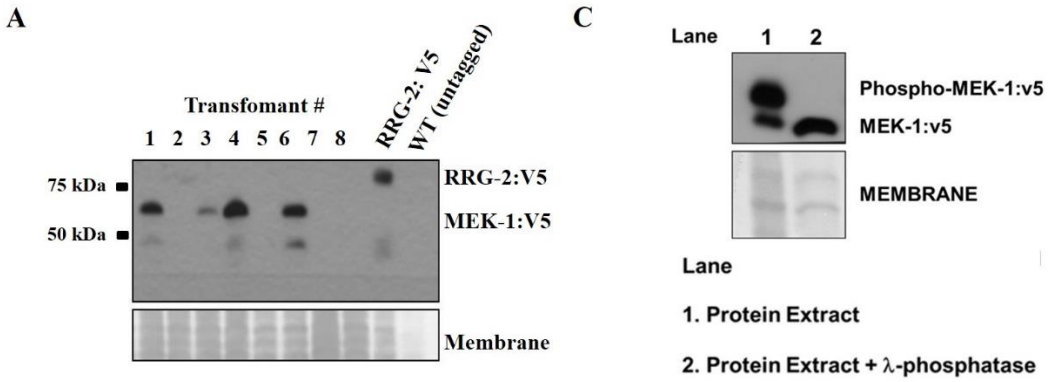


Figure 3-2. MEK-1 detection and regulation. (A) Western blot of total protein from MEK-1:V5 transformants 1-8. RRG-2:V5 was used as a positive control, and WT as a negative control. The blots were probed with α-V5, and the stained membrane is shown as a loading control. **(B)** Representative western blots of total protein from MEK-1:V5 and MEK-1:V5, DFRQ cells over a circadian time course and harvested every 4 hrs. Plots of MEK-1:V5 protein levels are shown below. The MEK-1:V5 strain is represented by a solid black line, while the MEK-1:V5, ΔFRQ is represented by a dashed grey line (values are ± SEM, n=3). **(C)** Western blot of total protein from the MEK-1:V5 strain (lane 1 is untreated; lane 2 is treated with α-phosphase) using the Phos-tag method (see materials and methods) and probed with α-V5.

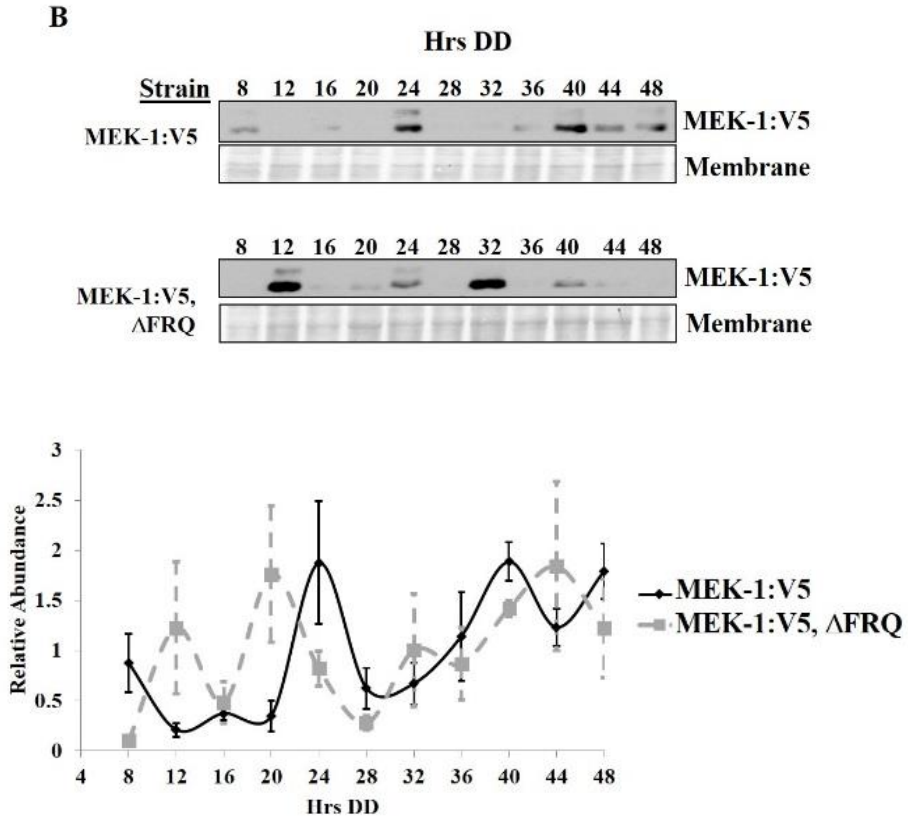


Figure 3-2 Continued.

of MEK-1:V5. When analyzing the western blot signal for MEK-1:V5, were specific to the MEK-1-tagged strain. I considered that these bands may be different forms of MEK-1, and set out to determine if the bands were post-translationally modified forms of MEK-1:V5, using the phos-tag system that allows the separation of the phosphorylated and unphosphorylated forms of a protein. MEK-1:V5 protein samples treated with λ-phosphatase, and control untreated, samples were separated on phos-tag gels, transferred to a membrane and probed with anti-V5 antibody. In the untreated sample, 2 bands were present, whereas in the phosphatase treated sample, the slower migrating band is missing, suggesting this larger band is the phosphorylated, or activated form, of MEK-1 (Figure 3-3C). Experiments are currently in progress to determine whether the phosphorylation in total MEK-1 protein is rhythmic, and if the rhythm is dependent upon the FWO. However, both forms of the protein display similar accumulation profiles in the blot, suggesting that the clock controls accumulation of MEK-1 protein, and does not specifically control phosphorylation of MEK-1.

The discovery of an enrichment of transcription factors bound by the WCC in response to light led to studies to determine the subsets of genes that are regulated by the transcription factors. Specifically, all of the 27 transcription factors controlled by the WCC were epitope-tagged, and ChIP-seq experiments have been done on about ½ of these so far, to identify the direct targets of the transcription factors before and after various timed light treatments. The results of the ADV-1 ChIP-seq experiments revealed the promoters of *mek-1* and *mak-1* of the MAK-1 pathway were bound by ADV-1 in DD and a small increase in binding observed following a light treatment (Figure 3-3.A). To investigate a

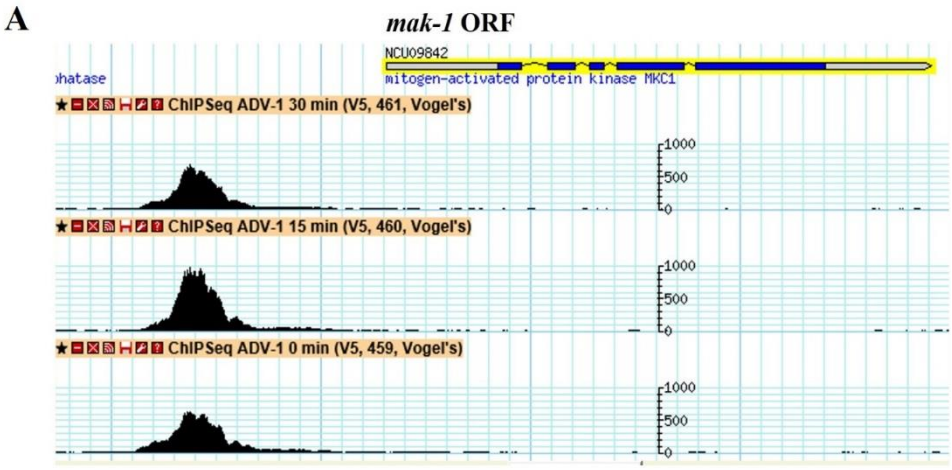
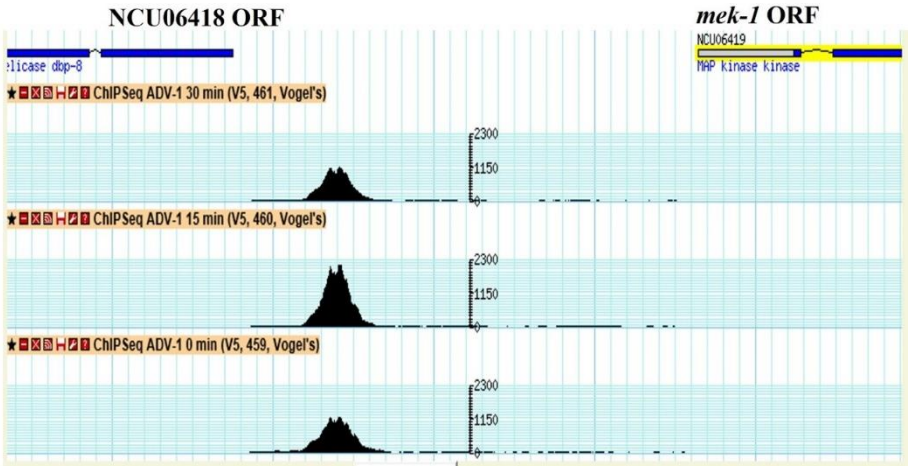
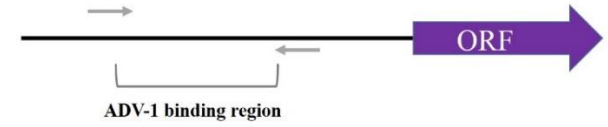


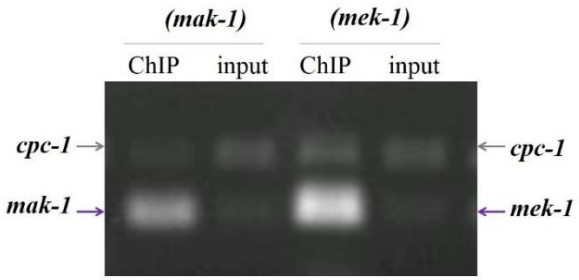
Figure 3-3. ADV-1 binds to the promoters of *mek-1* and *mak-1*. (A) The graphical representation of the relative intensity of ADV-1:V5 epitope tagged protein binding to the promoters of mak-1 and mek-1 as determined by ChIP from cultures grown in DD (0 min), or after 15 or 30 min of light exposure. **(B)** The cartoon representation of the promoter region of an open reading frame (ORF). After a 15 minute light treatment, ChIP experiments were performed and PCR was performed using the DNA pulled down in the ChIP experiment. Primers that are specific to the promoter regions (grey arrows) of the ORFs, *cpc-1*, *mek-1* and *mak-1* (top). The amplified DNA fragments were separated in an agarose gel and stained with ethidium bromide. Amplified sequences from DNA of the input to the ChIP experiment were used as a positive control (middle). The signal of *mek-1* and *mak-1* promoters amplified from the ChIP-PCR was normalized to the *mek-1* and *mak-1* promoter signal amplified from the input, and graphed (bottom).

A



B





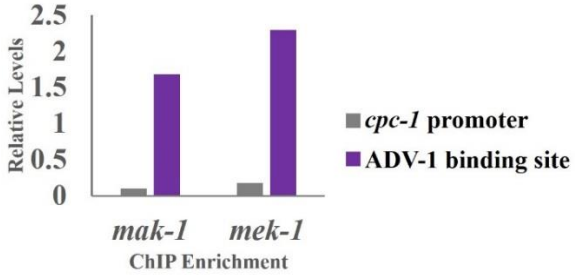


Figure 3-3 Continued.

possible role of ADV-1 in *mek-1* and *mak-1* transcriptional regulation, I independently validated that ADV-1 was binding to these regions by ChIP-PCR. ΔADV-1 cultures were grown DD for 24 hours, before being treated with light for 15 minutes. PCR was carried out using primers flanking the sequence predicted to be bound by ADV-1 in the promoters of *mek-1* and *mak-1*. The *cpc-1* gene, which is not bound by the by ADV-1, served as a negative control.

After confirming that ADV-1 binds upstream of *mek-1* and *mak-1*, I then investigated the role ADV-1 may play in their expression. ADV-1 is induced by the WCC after a light induction, therefore, I first tested if the *mek-1* and *mak-1* mRNA levels are induced by light, and if the induction was ADV-1-dependent. Wild-type and ΔADV-1 cultures were transferred from DD to LL for 0, 15, 30, and 60 minutes before being harvested. As shown in Figure 3-4A, *mek-1* mRNA levels were induced by light between 15 to 30 minutes in the wild-type strain. However, a similar induction was also observed in the ΔADV-1 strain, but the induction varied widely in different experiments, as reflected in the large error. Contrary to the results obtained for *mek-1* and despite ChIP-Seq data showing increased binding of ADV-1:V5 to the *mak-1* promoter following light treatment, *mak-1* mRNA levels remained constant throughout the light induction experiment in the wild-type and ΔADV-1 strains. The *mak-1* transcript levels were consistently lower in ΔADV-1 cells, suggesting ADV-1 is a positive regulator in its expression.

To determine if rhythms in *mek-1* and *mak-1* mRNA levels are a consequence of the circadian regulation of ADV-1, wild-type and ΔADV-1 strains were grown in DD and

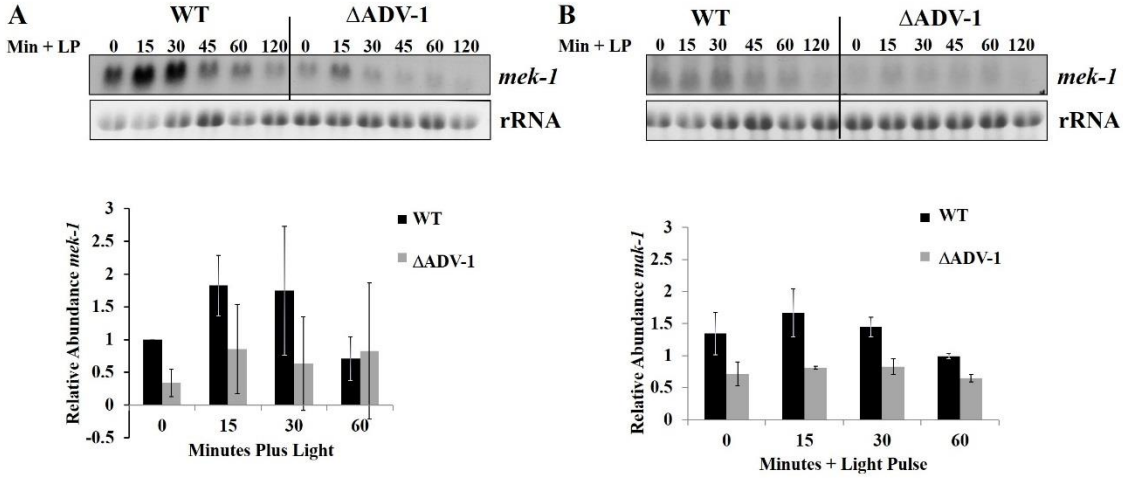


Figure 3-4. Light regulation of *mek-1* and *mak-1*. Representative northern blots probed with *mek-1* (**A**), and *mak-1* (**B**) in WT and ΔADV-1 strains grown in DD and harvested after 0, 15, 30, 45, 60 and 120 minutes of light treatment. The data are plotted on the bottom. For *mek-1*, values plotted are the average of n=2 and error bars represent standard deviation. For *mak-1*, values plotted are the average of n=3, ±SEM. Ribosomal RNA (rRNA) was used as a loading control.

harvested every 4 hours. While the levels of *mak-1* were reduced in the ΔADV-1 strain, the mRNA accumulated with a circadian rhythm that was similar in phase and amplitude to wild-type strains (3-5). For *mek-1*, no difference in levels or rhythmicity of the mRNA was observed in the ΔADV-1 strain when compared to wild-type cells.

Currently, it is unclear how the circadian clock activates the MAK-1 pathway, but we predict that similar to the OS-2 pathway, the clock regulates multiple components in the pathway. To test this idea, experiments were initiated to determine if ADV-1 functions in the rhythmic activation of MAK-1. In 2 experiments, P-MAK-1 was assayed over a circadian time course in wild-type and ΔADV-1 strains. As shown in Figure 3-5A, rhythmic P-MAK-1 was observed in ΔADV-1; however, the overall levels were decreased and the amplitude of the rhythm was reduced when compared to wild-type (Figure 3-5C), further suggesting that, while ADV-1 is not required for the rhythmic activity of the MAK-1 pathway, it is involved in maintaining wild-type levels of components of the pathway.

Discussion

The circadian clock regulates the activity of signal transduction pathways of *N. crassa* in order to coordinate the expression of subsets of genes necessary for various environmental stresses the cell may encounter throughout the day (36). In this study, I investigated the manner by which the circadian oscillator mediates the input to one of these pathways, the MAK-1 pathway. I show that transcript accumulation of the MAK-1 pathway components *mek-1* and *mak-1* are regulated by the clock, and that the rhythm is abolished in strains deleted for the clock component FRQ. Interestingly, I showed that

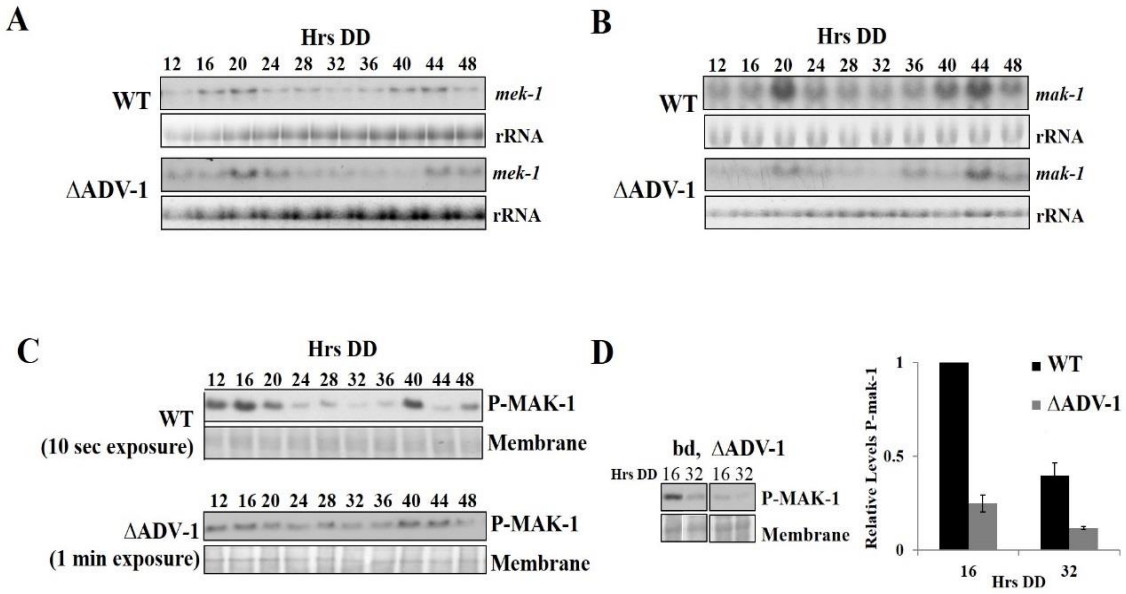


Figure 3-5 MAK-1 Pathway rhythms are maintained in the ΔADV-1 strain. Representative northern blots probed with mek-1 **(A)** and mak-1 **(B)** in WT and ΔADV-1 strains grown in DD and harvested every 4 hrs are shown. rRNA is shown as a loading control. Each experiment was performed at least 2 times, with similar results obtained. **(C)** The accumulation of P-MAK-1 is shown in representative western blots of total protein from the WT and ΔADV-1 strains grown in DD and harvested every 4 hrs. The blots were probed with α-phospho-p44/42 antibody. The stained membranes are shown as loading controls. The experiment was performed at least 2 times, with similar results. Exposure times for the blots are indicated under the strain name. **(D)** Western blot of total protein harvested from the WT and ΔADV-1 strains grown in DD for 16 and 32 hrs, and probed with α-phospho-p44/42 antibody. The stained membrane is shown as a loading control. The data are plotted on the right, with error bars representating the standard deviation of n=2.

MAK-1 protein levels are constitutive, despite rhythmic mRNA accumulation. These data suggest that while there might be a rhythm in MAK-1 translation, the stability of MAK-1 protein may mask this rhythm. This hypothesis is similar to what has been observed at the mRNA level for some genes targeted by the clock. For many genes, rhythms in protein levels generated from stable mRNA have been observed (91)**.** To test this idea, it would be necessary to study translation initiation of MAK-1 or generate an unstable version of MAK-1 protein.

To detect MEK-1 protein, I generated a translational fusion with V5, in both wild-type and ΔFRQ cells. The levels of MEK-1:V5 were assayed over a circadian time course in both strains. In the wild-type strain, I identified a reproducible rhythm, albeit with a short period of 16 hours. This rhythm was abolished in the ΔFRQ strain; the levels of MEK-1:V5 fluctuated over the course of the day, but displayed no consistency in the time of the peaks or troughs. To determine if the circadian regulation of the *mek-1* transcript is necessary for the circadian activation of MAK-1, it will be important to test if altering *mek-1* mRNA rhythms using a constitutive promoter abolishes rhythms in P-MAK-1. Finally, while our data suggests that the phosphorylation rhythm in MEK-1 is dependent upon the levels of total MEK-1 protein, this needs to be verified.

Although we have shown that ADV-1 binds regions upstream of the *mek-1* and *mak-1* ORFs, I was unable to identify a specific role for ADV-1 in light or circadian regulation of these genes. Our data suggest that *mek-1* was light-induced, but that light induction is independent of ADV-1, while *mak-1* was unaffected by light in our experiments. It is interesting that *mek-1* is induced in response to light, as the activity of

the MAK-1 pathway peaks in the early to late subjective afternoon. While it is currently unknown if P-MAK-1 is induced by light treatment, studies in both *S. cerevisiae* and mammals demonstrated that the MAK-1 homologous pathways, Slt-2 and ERK1/2 respectively, are involved in responding to genotoxic stress generated by ultraviolet (UV) light (114, 153). As a natural protection from UV stress, Neurospora produces spores with carotenoids during the daytime, a time in which the cells are most likely to be exposed to this stress (59, 130, 143). However, if hyphal cells, which do not produce carotenoids, are exposed to light, induction of the MAK-1 pathway and its downstream target genes, would provide an additional mechanism for cell survival.

Further analysis of ChIP-Seq experiments of the other WCC direct target transcription factors revealed that the developmental regulator, VOS-1, is also bound to similar regions of DNA in the *mek-1* and *mak-1* promoters. VOS-1 (NCU05964) is a predicted transcription factor homologous to the VosA gene in *Aspergillus nidulans*, which is involved in sporogenesis, trehelose biogenesis, heat tolerance and oxidative stress responses, biological processes similar to those regulated by the MAK-1 pathway (15, 115, 124). It would be interesting to explore the role of ADV-1 and VOS-1 in the transcriptional regulation of *mek-1* and *mak-1* under conditions in which the MAK-1 pathway is known to be activated, such as heat or oxidative stress.

It is becoming increasingly evident that the circadian regulation of MAP Kinase pathways is quite complex. In addition to the circadian accumulation of *os-4* in the OS-2 pathway, there is evidence that the clock also regulates proteins upstream of OS-4; specifically, the clock controls the circadian accumulation of the transcript and protein of

HPT, the histidine phospho-transferase in the 2-component regulatory system involved in the OS-2 response to acute osmotic stress (67, 84). The regulatory components of the MAK-1 pathway are only recently being identified and characterized. Based on homology to the Slt-2 pathway in *S. cerevisiae*, the activation of MIK-1, the MAPKKK in the MAK-1 pathway, is predicted to be through Protein Kinase C (PKC) (86). In Neurospora, PKC is light-induced and its activity is also regulated by the FWO (46, 75); therefore, PKC may be an additional mechanism by which the clock is regulating P-MAK-1. PKC is an essential protein in Neurospora so it cannot be deleted; however, functional mutants that produce either an overactive, or dominant negative function of the protein are available (46). It will be interesting to determine if the circadian accumulation of P-MAK-1 is affected in the PKC mutant strains. Further studies will help to elucidate the complete clock regulation of the MAK-1 pathway in Neurospora. Because MAPK pathways are highly conserved, I expect my studies in *N. crassa* will shed light on the connection between the circadian clock and MAPK pathways in mammalian cells.

CHAPTER IV

CONCLUSIONS

Summary

The research conducted in this study provides further insight into the output pathways of the molecular oscillator in *Neurospora crassa* by demonstrating the circadian regulation of 2 highly conserved MAP kinase signaling pathways. The ubiquity of both the circadian clock and MAPK pathways in eukaryotic organisms illustrates the significance of these findings; it is likely that this mechanism of clock output extends to higher eukaryotic organisms, including humans. Because of the wide range of human disorders that are associated with dysfunctions of both the circadian clock and MAPK signaling cascades conserved systems, defining the link between the clock regulations may be key to identifying potential therapeutic treatments.

There were 3 primary outcomes of my research; I first demonstrated that the activity of the Mitogen Activated Protein Kinases, MAK-1 and MAK-2 are regulated by the circadian clock in Neurospora. Secondly, I further characterized the MAK-1 pathway by identifying genetic targets; the results of both are described in chapter II. Lastly, I sought to unravel the mechanism(s) by which the clock impinges upon the MAK-1 pathway. The results of these experiments are presented in Chapter III, where I show that the components of the MAK-1 pathway are regulated by the clock at the mRNA level. In addition, evidence suggests that the transcription factor, ADV-1 is involved in the

regulation of transcripts of 2 of the pathway components, yet it is not required for their rhythmicity, nor the activity of the pathway's rhythmicity.

What the Output Pathways of the Circadian Clock in Neurospora Tell Us

Data presented in this dissertation show that the activity of 2 of the MAPK pathways are regulated by the clock in Neurospora. These data, in combination with previous data reported by our lab, indicate that all 3 MAPK pathways are clock regulated (158). MAPK signaling pathways are known, or predicted, to be involved in most biological processes occurring in the cell including, growth, proliferation, polarity, development, and stress responses. The circadian regulation of OS-2 was found to be important for anticipation of daily changes in osmolarity. Cells with a functional FWO showed an increased response time when treated with salt in the subjective morning when compared to cells in a clock deficient strain, as well as to a strain that was deleted for the WCC binding sequence in the *os-4* promoter. In this assay, we found that after 3 minutes of osmotic stress, the DD12 wild-type cells produced more glycerol, a molecule necessary to balance osmotic conditions in the cell, than cells from either of the deficient strains at DD12 or DD24, or the wild-type at DD24. These data indicate that the cell is primed for a change in osmotic conditions in the morning, a time when the sun is rising and temperatures are increasing, causing a decrease in environmental moisture content (84).

The MAK-2 MAPK pathway has been shown to modulate several biological processes, including hyphal fusion, conidia production, and sexual reproduction. The ΔMAK-2 strain displays short aerial hypha, de-repressed conidiation, and the mis-

regulation of many genes including, the pheromone precursor gene, *ccg-4*, which is necessary for communication mating type between 2 strains, (18, 89, 123). Previous studies indicate that the pheromone precursor genes, *ccg-4* and *mfa-1*, are rhythmic and dependent upon the clock (18). Furthermore, when MAK-2 is absent, *ccg-4* levels are increased, suggesting that MAK-2 plays an inhibitory role in the regulation of *ccg-4* (89). *ccg-4* levels peak in the subjective morning, at a time when conidia are likely to be carried to new locations by aerial winds, which are dryer and more conducive to the dispersal of conidia during the day. Additionally, P-MAK-2 levels peak in the subjective afternoon, around DD16, the time of day in which *ccg-4* levels are low, further supporting the idea that MAK-2 rhythmically represses *ccg-4* expression during the late day, and that repression is relieved in the morning, when P-MAK-2 levels decrease. Clock regulation of pheromone precursor genes, as well as hyphal fusion, both of which are necessary for mating establishes a link between the clock and sexual reproduction, such that this process, which is influenced by and sensitive to environmental conditions, including light and nutrient availability, occurs at a time that is most advantageous for the organism. Identification of the ccgs regulated by MAK-2 is necessary to decipher the mechanism of clock regulation in mating in Neurospora.

Because the MAK-1 pathway is the least characterized in Neurospora, I focused on unraveling details of this this pathway that include genes and biological processes under the control of MAK-1. Using microarray analysis, I established that over 500 genes were misregulated in a strain lacking MAK-1; the results, in combination with the phenotypes displayed by the MAK-1 component deleted strains, establish the significance

of this pathway in metabolism, hyphal growth, asexual and sexual reproduction, and the overall health of Neurospora cells. Of the 517 genes affected by MAK-1 deletion, greater than 25% of these genes were previously identified as ccgs; this large overlap suggests the clock may be signaling through MAK-1 to generate rhythms in a subset of ccgs (15). It has been shown that, while Neurospora hyphae continue to grow throughout the day and night, the overall growth rate is under the control of the clock (54). I suggest that this output of the circadian clock may allow the cells to limit processes that can be negatively affected by light, such as DNA replication, to a time of day in which the cell is most protected. We propose that by regulating the activity of MAK-1, the clock is able to propagate a rhythm in growth rate by generating rhythms in genes involved in cell wall biosynthesis. The genes necessary for the biogenesis of structural components are those that synthesize glucans and chitin. In Neurospora, there is only one gene encoding for glucan synthase; therefore, it is not surprising that this gene is not clock controlled, as Neurospora is constantly growing, and glucan synthesis is necessary for growth. However, there are 8 characterized or predicted chitin synthase genes, and these proteins have been shown to localize to difference regions of the growing hypha (133). I showed that *chitin synthase-5* (*cs-5*; NCU04352), which localizes to the growing hyphal tip, is regulated by the clock, suggesting a role for this chitin synthase in the circadian regulation of growth in Neurospora (15, 133).

Microscopic analysis of Neurospora hyphae has revealed that mitochondria are one of the few organelles uniformly distributed, and also the most abundant organelle present, implicating the importance of energy production in hyphal growth (133). I found

that the transcript of the predicted gene encoding a mitochondrial phosphate transporter (NCU07465) accumulates rhythmically, and that rhythm is dependent upon the clock, as well as MAK-1. The data suggest the clock may be exerting its control on hyphal growth, in part by regulating mitochondrial phosphate transport. In addition to a possible contribution to rhythmic growth, the clock may be regulating phosphate transport in the mitochondria to control overall metabolism, a process that generates oxidative stress, which is genotoxic (157). My data suggests the clock may temporally regulate this stress through MAK-1, such that it occurs at a time when DNA replication is minimal. Consistent with this, MAK-1 has been shown to be induced by a hydrogen peroxide treatment, suggesting a function in the response to oxidative stress (97). Additionally, our microarray data showed an enrichment of genes involved in protein synthesis, including ribosomal proteins and translation elongation factors, to be down regulated in the ΔMAK-1 strain. This data implicates a positive role for the MAK-1 pathway in protein production and, similar to ERK1/2 in mammals, likely for the cell to progress through G1 phase of the cell cycle (109). There is increasing evidence to support the circadian regulation of translation; there are many examples of rhythmic proteins generated from stable mRNA (79, 131). Studies in our lab indicate that the circadian activation of RCK-2 and eEF-2, both involved in translational regulation, is through the OS-2 pathway (Caster and Bell-Pedersen, in prep). Furthermore, recent mammalian studies provide evidence that support the idea that ribosome biosynthesis is under the control of the clock (68). Taken together, it is reasonable to hypothesize that the clock may signal through the MAK-1 pathway as one

mechanism to temporally regulate protein synthesis, a process that is also necessary for the growth a health of the cell.

Interestingly, I discovered that MAK-1 is necessary for robust rhythms in accumulation of the stress response gene, *ccg-1*. We previously showed that rhythmic accumulation of *ccg-1* also requires the OS-2 pathway (158), indicating a more complex regulation of ccgs. We propose that there is an overlap of ccgs regulated by multiple MAPK pathways, potentially providing increased amplitude in the rhythm, and/or continued circadian regulation in the absence or malfunction of one MAPK pathway. It is also possible that multiple MAPKs regulate ccgs so that in a situation of acute stress that activates one of the MAPKs, circadian regulation of any ccgs that are not involved in the stress are maintained. In the MAK-1 strain, the levels and the amplitude of *ccg-1* are significantly reduced, suggesting a positive role for MAK-1 in *ccg-1* regulation. We showed that MAK-1 peaks anti-phase to *ccg-1*; therefore, MAK-1 regulation of *ccg-1* is probably indirect. However, additional studies are necessary to test this hypothesis.

From Neurospora to Humans

The conservation of MAPK pathways from yeast to mammalian cells implicate the necessity of these pathways in intra and intercellular signaling, cellular growth and survival, as well as stress responses. We have now shown that all MAPK pathways are clock controlled in Neurospora, supporting the idea that the clock impinges upon these pathways to regulate subsets of genes involved in various cellular processes such that the

cell can prepare for predictable extracellular conditions. Taken together, this strongly suggests that the clock regulation of signaling pathways extends to higher eukaryotes.

The mammalian homologues of MAK-1 and MAK-2 are ERK1 and ERK2, which share overlapping functions, and are therefore often referred to as interchangeable kinases, (ERK1/2) (21, 125). ERK1/2 pathways are activated by extracellular signals, such as growth factors and cytokines, to regulate cellular proliferation, the cell cycle, and polarity of the cell (137). Progression through the cell cycle relies on external growth factors that activate ERK1/2, which in turn, regulate the kinase complexes that either block or activate movement through the cycles of growth and DNA replication (136). Studies in mammals have shown that deregulation of ERK1/2 pathways leads to a loss of cell cycle regulation; depending upon the cell type and cell cycle stage, the cell either arrests its progression and senesces to prevent the cell from abnormal growth, or the cell may continue to grow, without regulation, leading to tumor oogenesis (30, 120). Additionally, once cellular growth in no longer regulated, the cell can potentially secrete growth signals that affect the cells around them, increasing growth and tumor formation (38). It was recently shown that the clock regulates ERK1/2 activation in the mouse liver, consistent with a conserved link between the clock, MAPK activation and rhythmic control of the cell cycle (156).

The JNK and p38 MAPK pathways are activated more specifically by signaling molecules that indicate stress to cells within an multicellular organism, such as tumor necrosis factor (TNF), interleukin-1, ionizing and UV radiation, oxidative, and hyperosmotic stress (7, 139, 164). Once activated, these MAPKs direct the cell to either repair the damage or induce apoptosis, depending upon the extent of damage and cell type

(87, 112). Mis-regulation of these pathways are also associated with cancer; if a cell encounters the previously mentioned stresses and the JNK or p38 pathways are dysfunctional, damage that occurs in the cell, such as mutations in DNA, is passed on to daughter cells. Circadian activation of these pathways in a time of day manner would allow the cell to anticipate these daily stresses, stimulating a quicker response. Our lab has demonstrated that the activity of the p38 MAPK, which is homologous to OS-2 in Neurospora, is rhythmic in 2 mammalian cell lines (Goldsmith et al., in prep). Together, these data support a conserved role for clock control of MAPK activation. Importantly, disruption of the clock, and mis-regulation of MAPK pathways share many of the same disease symptoms in humans; therefore, understanding this link is likely to have therapeutic value.

Future Directions: Continuing the Investigation Using the Model Organism *Neurospora crassa*

Understanding how the clock signals through MAPK pathways in Neurospora will help to further our knowledge of circadian outputs in eukaryotic organisms. The proteins that activate the MAK-1 pathway are currently being identified and characterized (Figure 4-1). Extracellular signals are detected by receptors in the cell wall; recent studies have identified a cell wall protein, WSC-1, involved in the activation of MAK-1 in response to oxidative stress (97). Cells in which WSC-1 is deleted showed a significant decrease in P-MAK-1 after treated with hydrogen peroxide. Additionally, ΔWSC-1 cells show a similar defect in growth, hyphal fusion, as well as asexual and sexual development. WSC-1

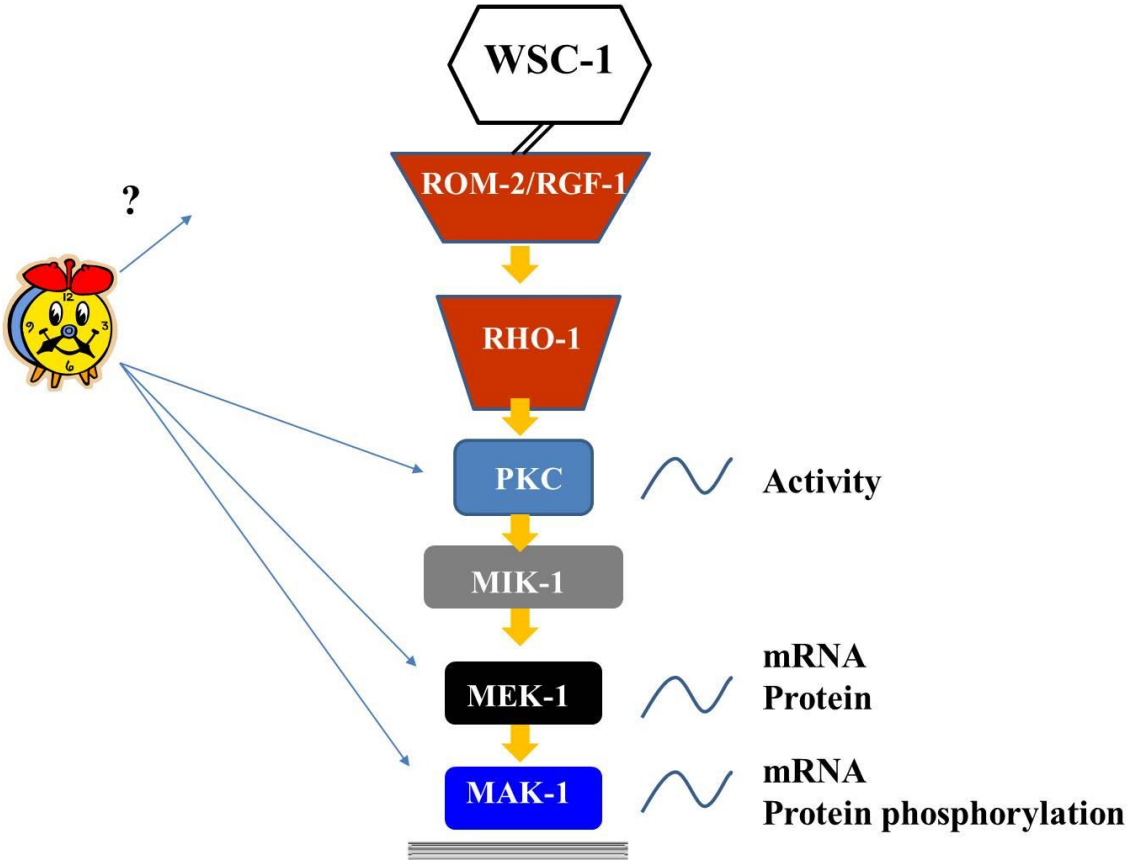


Figure 4-1. Schematic representation of the regulatory components upstream of MAK-1 and the clock inputs into the pathway. WSC-1 senses external signals and activates the small GTPase RHO-1, through the guanine exchange factor (GEF) ROM-2/RGF-1. Once activated, RHO is predicted to activate the phosphorylation of PKC, which is thought to activate the MAK-1 MAPK cascade. Previous studies show that PKC activity is under the control of the clock. Data presented in this disseration showed that the clock regulates the mRNA and protein accumulation of MEK-1, and mRNA and phosphorylation of MAK-1.

interacts with a plasma membrane-associated small GTPase, RHO-1, which becomes activated through the GTP exchange factor, ROM-2 (132). It is not yet known whether any of these regulatory proteins are regulated by the clock. Deletion mutants can be examined for P-MAK-1 rhythms to determine their role in the circadian control of P-MAK-1. Once activated, RHO-1 is predicted to activate protein kinase C (PKC), the kinase that is predicted to activate the phosphorylation of MIK-1, and thereby activating the MAK-1 pathway (Figure 4-1). Thus far, no studies have reported an interaction between PKC and MIK-1, or on the necessity of PKC in the activation of the MAK-1 pathway. As mentioned in Chapter III, PKC activity is regulated by the clock, suggesting that PKC may provide a rhythmic signal to activate MAK-1 (75). Studies are currently being conducted to investigate the role of PKC in circadian regulation of the MAK-1 pathway. Independent of any clock input to the regulatory proteins upstream of the MAK-1 pathway, I have demonstrated the circadian accumulation of the mRNA of *mek-1* and *mak-1*. While I was unable to demonstrate a rhythm in protein levels of MAK-1, my studies revealed an oscillation in MEK-1 protein levels with a detectable period of about 16 hours; a rhythm that becomes deregulated in a clock mutant strain. Further studies are necessary to determine the role the rhythm of MEK-1 plays in the circadian activation of P-MAK-1. To test this, the *mek-1* gene should be placed under the control of either an inducible, or constitutive, promoter and levels of P-MAK-1 assayed over a circadian time course. Evidence from the circadian regulation of the OS-2 pathway indicate that the direct activation of transcription of *os-4* by the WCC is necessary for rhythms in P-OS-2 (84). The promoter region of *mek-1* was not identified as a direct target of the WCC in ChIP

experiments, and MEK-1 protein levels peak anti-phase to the peak in WCC, indicating that the clock must be acting indirectly, possibly through transcription factors that are direct targets of the WCC. Consistent with this idea, 2 transcription factors, ADV-1 and VOS-1, were shown to directly bind promoter regions of both *mek-1* and *mak-1* (Dekhang and Bell-Pedersen, in prep). I investigated the role of ADV-1 in the light and circadian regulation of *mek-1* and *mak-1*, finding that while ADV-1 plays a positive role in the activation of this pathway, it is not required for either light or circadian control of *mek-1*, *mak-1* or P-MAK-1. Similar studies using a ΔVOS-1 strain will reveal what role, if any VOS-1 plays in the circadian regulation of the MAK-1 pathway. It is possible that multiple transcription factors can regulate these genes, either as a mechanism of compensation, or in response to different signals or cellular stress.

Little is known about the transcriptional regulatory proteins downstream of MAK-1; however, information can be gained by identifying homologous transcription factors regulated by the MAK-1 homologue, Slt-2 in *S. cerevisiae*. Three transcription factors are activated by Slt-2, the proteins Swi-4 and Swi-6, which form a complex and activate the transcription of genes involved in the cell cycle, and Rlm1, which is responsible for the regulation of cell wall integrity (19, 78). These yeast transcription factors have homologues in Neurospora, NCU07246 (Swi4), NCU07587 (Swi6), and NCU02558 (Rlm1) (Figure 4-2) and strains deleted for each of these genes are available. Experiments should be performed to determine if MAK-1 acts through these transcription factors to temporally regulate ccgs, starting with *cs-5*, NCU07465 and *ccg-1*, all of which were shown to require MAK-1 for rhythmic accumulation. Finally, phosphorylation is

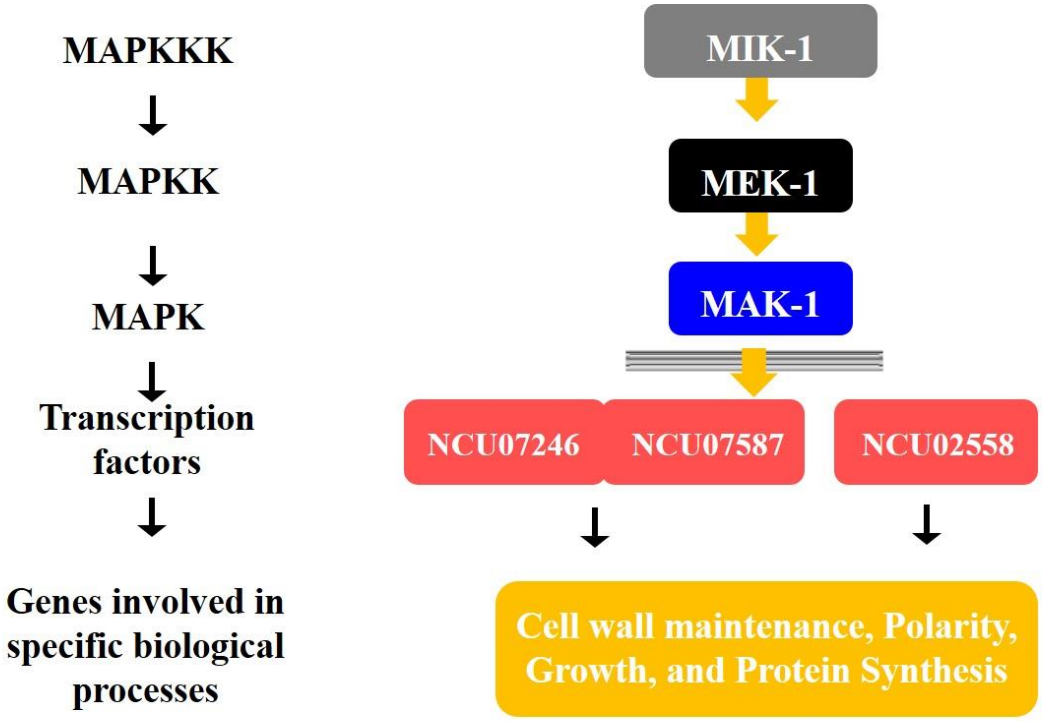


Figure 4-2. Diagram of the predicted downstream transcription factors of MAK-1. Based on homology to the yeast Slt2 pathway, predicted transcription factors were identified. See text for additional details.

necessary for the activation of MAPKs, and dephosphorylation to inactivate them. In order for these pathways to be inactivated in *S. cerevisaie*, tyrosine, serine/threonine and dual-specificity phosphatases act on MAPKs (103). While these phosphatase homologues have been identified in Neurospora, their role in the de-phosphorylation of any of the MAPKs has not been established. Strains with the phosphatases genes deleted were obtained from the Fungal Genetics Stock Center. Circadian time-courses were carried out using these strains to analyze P-MAK-1 and P-MAK-2; however, these phosphatases are extremely important to the cell, so the knockout strains grew very poorly, making it difficult to determine a direct effect on circadian regulation of the MAPKS (data not shown). Strains with either conditional mutations in the phosphatase genes, or with the genes under the control of an inducible promoter may provide further insight into their role in the dephosphorylation of MAPKs.

Clock Regulation of Mammalian MAPKs

To further characterize the circadian regulation of mammalian MAPKs, investigations need to be carried out to test for rhythms in the activation of ERK1/2, JNKs mammalian cells, and describe the role the circadian regulation of these pathways plays in cellular physiology. Studies are ongoing using immortalized cell lines; however, by definition, the cell cycle in these lines has been modified, and MAPK signaling may be compromised, undermining any effect of circadian regulation of MAPK pathways linked to the cell cycle, as is expected of all mammalian MAPK families. It will be of more interest to determine if the MAPKs are rhythmic in different organs and tissue types in

whole organisms, because it will provide a more realistic picture of what is occurring within the cell and organism and possibly reveal mechanisms of communication between clocks in different tissue. It is our assertion that the clock is using these conserved signaling pathways to transduce temporal signals between cells and within the cell to temporally regulate sensitive biological processes.

Final Thoughts

The data reported in this dissertation provide a greater understanding of the circadian output pathways in the model organism, *Neurospora crassa*. We have shown that the 3 conserved MAPK pathways present in Neurospora are activated in a circadian manner; specifically, I present data showing that the clock facilitates the rhythms of ccgs involved in, metabolism, cellular growth and development, as well stress responses, through the MAK-1 pathway. Additionally, microarray analysis revealed that MAK-1 is necessary in the positive regulation of a large percent of genes encoding proteins involved in protein synthesis; we hypothesize that the clock may be mediating translational regulation through the MAK-1 pathway, though additional experiments are necessary to test this hypothesis.

As the MAPK pathways are conserved among eukaryotic organisms, we predict that the clock is also regulating MAPKs in mammals. In support of this, recent evidence has shown that the ERK1/2 phosphorylation is clock controlled in the mouse liver (156). It is still unclear what role the circadian activation of MAPKs play in mammalian cells; therefore, more studies are needed to investigate the potential physiological effects. We

have also demonstrated circadian regulation of the transcripts of several MAPK components, which is a novel finding as most studies involving MAPK signaling have focused on the posttranslational modification of the kinases upstream, within, and downstream of the cascade. It is likely that transcriptional regulation of MAPK components is conserved in mammals, but has not been thoroughly explored. Therefore, it represents an additional avenue for targeted treatments for mammalian diseases associated with the mis-regulation of MAPK pathways.

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APPENDIX A

Supplemental Table 2: Microarray data showing genes with altered expression levels in Δ*mak-1*

NCU # or Gene I.D.	Known or Predicted Function[1]	FunCat Classification	Predicted ccg[2]
NCU05136	related to endo alpha-1,4 polygalactosaminidase precursor	01 METABOLISM	
NCU08535	probable acetyl-CoA carboxylase	01 METABOLISM	
NCU05429	probable branching enzyme (be1)	01 METABOLISM	YES
NCU07307	probable fatty-acyl-CoA synthase, beta subunit	01 METABOLISM__70 SUBCELLULAR LOCALIZATION	
NCU08287	probable carbamoyl-phosphate synthetase, pyrimidine-specific / aspartate transcarbamylase	01 METABOLISM	YES
NCU07308	probable fatty acid synthase, alpha subunit	01 METABOLISM__70 SUBCELLULAR LOCALIZATION	
NCU03117	probable IMP dehydrogenase	01 METABOLISM	
NCU06687	glycogen synthase	01 METABOLISM	
NCU06727	spermidine synthase SPE-3	01 METABOLISM	YES
NCU09326	related to beta-glucosidase	01 METABOLISM__42 BIOGENESIS OF CELLULAR COMPONENTS	YES

NCU # or Gene I.D.	Known or Predicted Function[1]	FunCat Classification	Predicted ccg[2]
NCU01258	probable cyanate lyase	01 METABOLISM	
NCU06783	probable ATP citrate lyase subunit 2	01 METABOLISM	YES
NCU04292	probable branched-chain amino acids aminotransferase	01 METABOLISM	
NCU06785	probable ATP citrate lyase subunit 1	01 METABOLISM	
NCU02333	arginase AGA	01 METABOLISM__70 SUBCELLULAR LOCALIZATION	
NCU07659	dihydrolipoamide acetyltransferase component of pyruvate dehydrogenase complex, mitochondrial precursor MRP-3	01 METABOLISM__02 ENERGY__70 SUBCELLULAR LOCALIZATION	
NCU06871	probable glucan synthase	01 METABOLISM__43 CELL TYPE DIFFERENTIATION	
NCU03813	formate dehydrogenase	01 METABOLISM__02 ENERGY	YES
NCU03191	related to rab geranylgeranyl transferase component A	01 METABOLISM__14 PROTEIN FATE (folding, modification, destination)	
NCU05810	CPC2 protein	01 METABOLISM	YES
NCU06549	Sno-type pyridoxine vitamin B6 biosynthetic protein SNO1	01 METABOLISM	
NCU09911	probable formyltetrahydrofolate deformylase	01 METABOLISM	

NCU # or Gene I.D.	Known or Predicted Function[1]	FunCat Classification	Predicted ccg[2]
NCU03748	probable saccharopine reductase	01 METABOLISM	
NCU06346	probable acyl-coenzyme A-binding protein (diazepam binding inhibitor)	01 METABOLISM__20 CELLULAR TRANSPORT, TRANSPORT FACILITATION AND TRANSPORT ROUTES	
NCU07930	probable adenosylhomocysteinase (S-adenosyl-L-homocysteine hydrolase)	01 METABOLISM	YES
NCU04923	probable reductase	01 METABOLISM	YES
NCU02252	probable phosphoglyceromutase	01 METABOLISM__02 ENERGY	YES
NCU08615	related to tyrosinase precursor (monophenol monooxygenase)	01 METABOLISM__16 PROTEIN WITH BINDING FUNCTION OR COFACTOR REQUIREMENT (structural or catalytic)	
NCU08117	related to O-succinylhomoserine (thiol)-lyase	01 METABOLISM	
NCU07042	related to alcohol dehydrogenase	01 METABOLISM__02 ENERGY	YES
NCU03118	probable saccharopine dehydrogenase (NAD, L-lysine-forming)	01 METABOLISM__16 PROTEIN WITH BINDING FUNCTION OR COFACTOR REQUIREMENT (structural or catalytic)__70 SUBCELLULAR LOCALIZATION	
NCU00552	phytoene dehydrogenase AL-1 (carotenoid biosynthesis protein al-1)	01 METABOLISM	YES

NCU # or Gene I.D.	Known or Predicted Function[1]	FunCat Classification	Predicted ccg[2]
NCU07589	related to acetyltransferase	01 METABOLISM	YES
NCU06386	related to dolichyl-phosphate beta-glucosyltransferase	01 METABOLISM__14 PROTEIN FATE (folding, modification, destination)__70 SUBCELLULAR LOCALIZATION	YES
NCU02542	probable hexokinase	01 METABOLISM	
NCU05762	related to monophenol monooxygenase (tyrosinase)	01 METABOLISM	YES
NCU10507	related to triacylglycerol lipase	01 METABOLISM	
NCU07318	probable mannitol-1-phosphate dehydrogenase	01 METABOLISM__20 CELLULAR TRANSPORT, TRANSPORT FACILITATION AND TRANSPORT ROUTES	YES
NCU03755	related to flavin-containing monooxygenase	01 METABOLISM	
NCU04865	probable type I polyketide synthase	01 METABOLISM__32 CELL RESCUE, DEFENSE AND VIRULENCE	YES
NCU08404	related to coenzyme a synthetase	01 METABOLISM	
NCU03693	probable NADPH-dependent beta-ketoacyl reductase (rhlG)	01 METABOLISM	YES
NCU07931	related to D-mandelate dehydrogenase	01 METABOLISM	

NCU # or Gene I.D.	Known or Predicted Function[1]	FunCat Classification	Predicted ccg[2]
NCU05078	related to protein-L-isoaspartate(D-aspartate) O-methyltransferase	01 METABOLISM	
NCU02786	aldose 1-epimerase	01 METABOLISM	
NCU01753	related to gamma-glutamyltransferase	01 METABOLISM	YES
NCU04039	GNAT family N-acetyltransferase	01 METABOLISM	
NCU09810	related to succinate-CoA ligase alpha and beta chain	01 METABOLISM__02 ENERGY	
NCU03525	3-oxoacyl-[acyl-carrier-protein]-reductase (oar-1) OAR-1	01 METABOLISM__02 ENERGY	YES
NCU05038	related to gibberellin 20-oxidase	01 METABOLISM	
NCU02097	related to D-arabinitol 2-dehydrogenase	01 METABOLISM	
NCU03282	probable 3-hydroxyanthranilic acid dioxygenase	01 METABOLISM	
NCU07761	related to SUR1 protein	01 METABOLISM__43 CELL TYPE DIFFERENTIATION	
NCU07608	related to D-ribose-5-phosphate ketol-isomerase	01 METABOLISM__02 ENERGY	
NCU06348	related to quinic acid utilisation protein QUTG (inositol-1(or 4)-monophosphatase)	01 METABOLISM	

NCU # or Gene I.D.	Known or Predicted Function[1]	FunCat Classification	Predicted ccg[2]
NCU02083	related to phosphatidylinositol 3-phosphate 5-kinase	01 METABOLISM__14 PROTEIN FATE (folding, modification, destination)__20 CELLULAR TRANSPORT, TRANSPORT FACILITATION AND TRANSPORT ROUTES__42 BIOGENESIS OF CELLULAR COMPONENTS	YES
NCU09456	related to flavin-containing monooxygenase	01 METABOLISM	
NCU04349	related to proteinase inhibitor PBI2	01 METABOLISM	
NCU06402	probable C-4 methyl sterol oxidase	01 METABOLISM	
NCU06071	2-dehydropantoate 2-reductase	01 METABOLISM	
NCU09327	RNA-splicing regulatory protein phosphatase CYT-4, mitochondrial CYT-4	01 METABOLISM__10 CELL CYCLE AND DNA PROCESSING__11 TRANSCRIPTION__14 PROTEIN FATE (folding, modification, destination)__70 SUBCELLULAR LOCALIZATION	
NCU07965	probable dolichyl-phosphate beta-D-mannosyltransferase	01 METABOLISM__70 SUBCELLULAR LOCALIZATION	
NCU03492	related to fatty acid hydroxylase	01 METABOLISM	
NCU05973	related to CobB protein	01 METABOLISM	

NCU # or Gene I.D.	Known or Predicted Function[1]	FunCat Classification	Predicted ccg[2]
NCU04575	related to phthalate 4,5-dioxygenase oxygenase reductase subunit	01 METABOLISM	
NCU00437	related to nitrilase	01 METABOLISM	
NCU08689	related to polyphosphoinositide phosphatase family member	01 METABOLISM	
NCU01080	related to laminaripentaose-producing beta-1,3-glucanase	01 METABOLISM	YES
NCU06471	pyrroline-5-carboxylate reductase PRO-1	01 METABOLISM	
NCU05278	probable cytochrome P450 (involved in C-22 denaturation of the ergosterol side-chain)	01 METABOLISM	
NCU07001	homoserine O-acetyltransferase MET-5	01 METABOLISM	
NCU01494	related to myo-inositol transport protein ITR1	01 METABOLISM__20 CELLULAR TRANSPORT, TRANSPORT FACILITATION AND TRANSPORT ROUTES__70 SUBCELLULAR LOCALIZATION	
NCU01904	related to 2-deoxy-D-gluconate 3-dehydrogenase	01 METABOLISM	YES
NCU03404	probable acid phosphatase Pho610	01 METABOLISM__70 SUBCELLULAR LOCALIZATION	YES
NCU03542	enoyl-CoA hydratase/isomerase	01 METABOLISM	

NCU # or Gene I.D.	Known or Predicted Function[1]	FunCat Classification	Predicted ccg[2]
NCU01546	probable coproporphyrinogen oxidase precursor	01 METABOLISM	YES
NCU07737	related to salicylate 1-monooxygenase	01 METABOLISM	YES
NCU05969	probable endoglucanase IV precursor	01 METABOLISM	YES
NCU01744	probable glutamate synthase (NADPH)	01 METABOLISM	
NCU01441	probable inosine triphosphate pyrophosphatase	01 METABOLISM	
NCU01364	oxidoreductase	01 METABOLISM	YES
NCU03139	histidine biosynthesis trifunctional protein HIS-3	01 METABOLISM	
NCU08418	related to tripeptidyl-peptidase I	01 METABOLISM	
NCU01866	probable lactonohydrolase	01 METABOLISM	YES
NCU06416	related to gibberellin 20-oxidase	01 METABOLISM	
NCU06112	related to glutamic acid decarboxylase	01 METABOLISM	
NCU06191	related to glutathione synthase	01 METABOLISM	YES
NCU01562	related to 2,4-dichlorophenoxyacetate dioxygenase	01 METABOLISM	YES

NCU # or Gene I.D.	Known or Predicted Function[1]	FunCat Classification	Predicted ccg[2]
NCU03718	related to CAX4 protein	01 METABOLISM	
NCU08050	related to salicylate 1-monooxygenase	01 METABOLISM	
NCU00944	probable threonine aldolase	01 METABOLISM	YES
NCU06711	possible lysine decarboxylase	01 METABOLISM	YES
NCU02475	probable glycine decarboxylase P subunit	01 METABOLISM	YES
NCU04385	probable 3-isopropylmalate dehydratase	01 METABOLISM	
NCU01420	dienelactone hydrolase family protein	01 METABOLISM	
NCU09637	related to sterigmatocystin 7-O-methyltransferase precursor	01 METABOLISM__32 CELL RESCUE, DEFENSE AND VIRULENCE	
NCU10107	related to triose-phosphate isomerase	01 METABOLISM	
NCU09425	related to cellodextrin-phosphorylase	01 METABOLISM	YES
NCU01571	related to geranylgeranyl transferase alpha chain	01 METABOLISM__14 PROTEIN FATE (folding, modification, destination)	
NCU08643	related to acid phosphatase precursor	01 METABOLISM	
NCU04791	related to sorbitol utilization protein	01 METABOLISM	

NCU # or Gene I.D.	Known or Predicted Function[1]	FunCat Classification	Predicted ccg[2]
NCU05127	related to D-alanine:D-alanine ligase 2	01 METABOLISM	YES
NCU01050	related to cel1 protein precursor	01 METABOLISM	YES
NCU09638	related to type I polyketide synthase	01 METABOLISM__32 CELL RESCUE, DEFENSE AND VIRULENCE	
NCU00797	related to NADPH-dependent aldehyde reductase	01 METABOLISM	
NCU02663	related to L-lysine 2,3-aminomutase	01 METABOLISM	
NCU02220	DSBA oxidoreductase; Thioredoxin-like fold	01 METABOLISM	
NCU00732	related to isotrichodermin C-15 hydroxylase (cytochrome P-450 monooxygenase CYP65A1)	01 METABOLISM	
NCU05133	related to UDP-glucose 4-epimerase	02ENERGY	
NCU07452	related to flavin oxidoreductase	02ENERGY	
NCU01692	mitochondrial citrate synthase	02 ENERGY__70 SUBCELLULAR LOCALIZATION	
NCU05430	H+-transporting ATP synthase beta chain	02 ENERGY__16 PROTEIN WITH BINDING FUNCTION OR COFACTOR REQUIREMENT (structural or catalytic)__34 INTERACTION WITH THE ENVIRONMENT__70 SUBCELLULAR LOCALIZATION	YES

NCU # or Gene I.D.	Known or Predicted Function[1]	FunCat Classification	Predicted ccg[2]
NCU02549	mitochondrial-processing peptidase beta subunit precursor Pep	02ENERGY	YES
NCU03156	NADH2 dehydrogenase (ubiquinone) 10.5K chain NUO-10.5	02 ENERGY__20 CELLULAR TRANSPORT, TRANSPORT FACILITATION AND TRANSPORT ROUTES__70 SUBCELLULAR LOCALIZATION	YES
NCU09228	aminopeptidase 2	02 ENERGY	
NCU06303	alcohol dehydrogenase	02 ENERGY	
NCU01528	glyceraldehyde 3-phosphate dehydrogenase (ccg-7)	02 ENERGY	YES
NCU00087	6-phosphogluconolactonase	02 ENERGY	
mito_060	probable NADH dehydrogenase (ubiquinone) chain 3	02 ENERGY__70 SUBCELLULAR LOCALIZATION	YES
mito_190	NADH dehydrogenase (ubiquinone) chain 1	02 ENERGY__70 SUBCELLULAR LOCALIZATION	YES
NCU02480	related to short-chain alcohol dehydrogenase	02 ENERGY	YES
mito_080	NADH dehydrogenase (ubiquinone) chain 2	02 ENERGY__70 SUBCELLULAR LOCALIZATION	
mito_220	ubiquinol--cytochrome-c reductase cytochrome b	02 ENERGY__70 SUBCELLULAR LOCALIZATION	YES

NCU # or Gene I.D.	Known or Predicted Function[1]	FunCat Classification	Predicted ccg[2]
NCU02734	related to citrate lyase beta subunit	02 ENERGY	
mito_130	cytochrome-c oxidase chain II	02 ENERGY__70 SUBCELLULAR LOCALIZATION	
mito_010	NADH dehydrogenase (ubiquinone) chain 5	02 ENERGY__70 SUBCELLULAR LOCALIZATION	
mito_170	probable NADH dehydrogenase (ubiquinone) chain 4	02 ENERGY__70 SUBCELLULAR LOCALIZATION	
mito_140	H+-transporting ATP synthase protein 9	02 ENERGY__70 SUBCELLULAR LOCALIZATION	
NCU02435	histone H2B H2B	10 CELL CYCLE AND DNA PROCESSING__70 SUBCELLULAR LOCALIZATION	
NCU08290	MUS-51 related to ATP-dependent DNA helicase II, 70 kDa subunit	10 CELL CYCLE AND DNA PROCESSING__70 SUBCELLULAR LOCALIZATION	
NCU04505	cell cycle control protein cwf19	10 CELL CYCLE	
NCU02791	related to telomerase reverse transcriptase	10 CELL CYCLE AND DNA PROCESSING__42 BIOGENESIS OF CELLULAR COMPONENTS__70 SUBCELLULAR LOCALIZATION	
NCU04354	DEAD box family helicase	10 CELL CYCLE AND DNA PROCESSING	
NCU01370	related to origin recognition complex subunit 2	10 CELL CYCLE AND DNA PROCESSING__34 INTERACTION WITH THE	

NCU # or Gene I.D.	Known or Predicted Function[1]	FunCat Classification	Predicted ccg[2]
		ENVIRONMENT__43 CELL TYPE DIFFERENTIATION	
NCU04327	probable DNA replication licensing factor (nimQ)	10 CELL CYCLE AND DNA PROCESSING__70 SUBCELLULAR LOCALIZATION	
NCU08115	related to DNA mismatch repair protein	10 CELL CYCLE AND DNA PROCESSING__16 PROTEIN WITH BINDING FUNCTION OR COFACTOR REQUIREMENT (structural or catalytic)__70 SUBCELLULAR LOCALIZATION	
NCU08484	probable sepB protein	10 CELL CYCLE AND DNA PROCESSING	
NCU01951	catalytic subunit of DNA polymerase zeta UPR-1	10 CELL CYCLE AND DNA PROCESSING	
NCU04650	related to kinetoplast-associated protein KAP	10 CELL CYCLE AND DNA PROCESSING__70 SUBCELLULAR LOCALIZATION	
NCU09240	proliferating cell nuclear antigen [Grosmannia clavigera kw1407]	10 CELL CYCLE AND DNA PROCESSING	YES
NCU01634	histone H4	11 TRANSCRIPTION__70 SUBCELLULAR LOCALIZATION	YES
NCU03702	probable fibrillarin (NOP1)	11 TRANSCRIPTION__70 SUBCELLULAR LOCALIZATION	
NCU01635	histone H3	11 TRANSCRIPTION__70 SUBCELLULAR LOCALIZATION	
NCU08063	(predicted, still listed as hypothetical protein) Fungal transcriptional regulatory	11 TRANSCRIPTION	

NCU # or Gene I.D.	Known or Predicted Function[1]	FunCat Classification	Predicted ccg[2]
	protein, N-terminal; GAL4-like Zn(II)2Cys6 (or C6 zinc) binuclear cluster DNA-binding domain		
NCU07331	related to RNA binding protein LCP5	11 TRANSCRIPTION__16 PROTEIN WITH BINDING FUNCTION OR COFACTOR REQUIREMENT (structural or catalytic)	
NCU04859	related to silencing protein (SIR2 gene family)	11 TRANSCRIPTION	
NCU05637	predicted, still hypothetical protein) BZIP family transcription factor [Glomerella graminicola M1.001]	11 TRANSCRIPTION	
NCU06256	related to transcription initiation factor	11 TRANSCRIPTION__70 SUBCELLULAR LOCALIZATION	YES
NCU04784	related to Nup98-Nup96 precursor	11 TRANSCRIPTION__20 CELLULAR TRANSPORT, TRANSPORT FACILITATION AND TRANSPORT ROUTES__70 SUBCELLULAR LOCALIZATION	
NCU03184	related to putative C2H2 zinc finger protein flbC	11 TRANSCRIPTION__70 SUBCELLULAR LOCALIZATION	
NCU01954	related to pre-mRNA-splicing factor cwc24	11 TRANSCRIPTION__16 PROTEIN WITH BINDING FUNCTION OR COFACTOR REQUIREMENT (structural or catalytic)	
NCU03417	predicted fungal specific transcription factor; GAL4-like Zn2Cys6 binuclear cluster DNA-binding domain	11 TRANSCRIPTION	

NCU # or Gene I.D.	Known or Predicted Function[1]	FunCat Classification	Predicted ccg[2]
NCU01367	related to a component of the yeast U3 snoRNP Rrp9p	11 TRANSCRIPTION	
NCU03266	related to LIM homeobox protein	11 TRANSCRIPTION__70 SUBCELLULAR LOCALIZATION	
NCU04009	related to 3`->5` exoribonuclease	11 TRANSCRIPTION__70 SUBCELLULAR LOCALIZATION	
NCU08000	cutinase transcription factor 1 alpha	11 TRANSCRIPTION	YES
mito_155	predicted ATP6 intron maturase	11 TRANSCRIPTION__38 TRANSPOSABLE ELEMENTS, VIRAL AND PLASMID PROTEINS__70 SUBCELLULAR LOCALIZATION	
NCU03536	regulatory protein Cys-3; transcription	11 TRANSCRIPTION	
NCU02082	related to pre-mRNA 3`-end processing factor CF II	11 TRANSCRIPTION__70 SUBCELLULAR LOCALIZATION	YES
NCU03956	related to Pol II transcription elongation factor	11 TRANSCRIPTION__70 SUBCELLULAR LOCALIZATION	
NCU01321	related to DNA polymerase Tdt-N	11 TRANSCRIPTION	
NCU00730	Fungal transcriptional regulatory protein, N-terminal	11 TRANSCRIPTION	
NCU07409	related to tRNA (guanine-N(7)-)-methyltransferase-associated WD repeat protein TRM82	11 TRANSCRIPTION__16 PROTEIN WITH BINDING FUNCTION OR COFACTOR REQUIREMENT (structural or catalytic)	YES

NCU # or Gene I.D.	Known or Predicted Function[1]	FunCat Classification	Predicted ccg[2]
mito_230	related to cob intron2 maturase/endonuclease	11 TRANSCRIPTION__38 TRANSPOSABLE ELEMENTS, VIRAL AND PLASMID PROTEINS__70 SUBCELLULAR LOCALIZATION	
mito_240	related to cob intron1 maturase	11 TRANSCRIPTION__38 TRANSPOSABLE ELEMENTS, VIRAL AND PLASMID PROTEINS__70 SUBCELLULAR LOCALIZATION	
NCU01235	related to RNA splicing factor PRP9	11 TRANSCRIPTION	YES
NCU04179	related to finger protein AZF1	11 TRANSCRIPTION__70 SUBCELLULAR LOCALIZATION	
NCU08983	probable poly(A) polymerase	11 TRANSCRIPTION	YES
NCU02533	probable DNA-directed RNA polymerase	11 TRANSCRIPTION__16 PROTEIN WITH BINDING FUNCTION OR COFACTOR REQUIREMENT (structural or catalytic)__70 SUBCELLULAR LOCALIZATION	
mito_050	predicted ND4L intron protein	11 TRANSCRIPTION__38 TRANSPOSABLE ELEMENTS, VIRAL AND PLASMID PROTEINS__70 SUBCELLULAR LOCALIZATION	
NCU00282	Fungal transcriptional regulatory protein, N-terminal	11 TRANSCRIPTION	
NCU02208	related to translation initiation factor eIF-3 Prt1 chain	12 PROTEIN SYNTHESIS	

NCU # or Gene I.D.	Known or Predicted Function[1]	FunCat Classification	Predicted ccg[2]
NCU07831	related to translation initiation factor eIF3	12 PROTEIN SYNTHESIS	
NCU06661	probable ribosomal protein L22	12 PROTEIN SYNTHESIS	YES
NCU03038	probable ribosomal protein S13.e	12 PROTEIN SYNTHESIS__70 SUBCELLULAR LOCALIZATION	
NCU00294	probable ribosomal protein L10a.e, cytosolic	12 PROTEIN SYNTHESIS__70 SUBCELLULAR LOCALIZATION	
NCU01331	probable 13 kD U4/U6.U5 snRNP associate protein Snu13	12 PROTEIN SYNTHESIS	
NCU00258	40S ribosomal protein CRPS-7 CRPS-7	12 PROTEIN SYNTHESIS__70 SUBCELLULAR LOCALIZATION	
NCU01317	probable ribosomal protein L12	12 PROTEIN SYNTHESIS	YES
NCU06047	probable 40S ribosomal protein S2	12 PROTEIN SYNTHESIS__70 SUBCELLULAR LOCALIZATION	
NCU07700	elongation factor 2 COT-3	12 PROTEIN SYNTHESIS	YES
NCU08502	probable 40s ribosomal protein S6.e, cytosolic	12 PROTEIN SYNTHESIS__70 SUBCELLULAR LOCALIZATION	
NCU08389	probable 60S large subunit ribosomal protein	12 PROTEIN SYNTHESIS__70 SUBCELLULAR LOCALIZATION	

NCU # or Gene I.D.	Known or Predicted Function[1]	FunCat Classification	Predicted ccg[2]
NCU00464	probable 60S ribosomal protein L32	12 PROTEIN SYNTHESIS	YES
NCU07922	probable translation elongation factor eEF-3	12 PROTEIN SYNTHESIS__70 SUBCELLULAR LOCALIZATION	
NCU04331	60S ribosomal protein L5 crp-4	12 PROTEIN SYNTHESIS	YES
NCU04552	ribosomal protein S26.e CRP-5	12 PROTEIN SYNTHESIS__70 SUBCELLULAR LOCALIZATION	
NCU07014	ribosomal protein L17.e, cytosolic crp-3	12 PROTEIN SYNTHESIS__70 SUBCELLULAR LOCALIZATION	YES
NCU02707	probable ribosomal protein L6.e.B, cytosolic	12 PROTEIN SYNTHESIS__70 SUBCELLULAR LOCALIZATION	
NCU02509	probable ribosomal protein L11.e.A, cytosolic	12 PROTEIN SYNTHESIS__70 SUBCELLULAR LOCALIZATION	YES
NCU09089	probable 40S ribosomal protein S30.e, cytosolic	12 PROTEIN SYNTHESIS__70 SUBCELLULAR LOCALIZATION	YES
NCU01949	probable 40S ribosomal protein S9 (S7)	12 PROTEIN SYNTHESIS__70 SUBCELLULAR LOCALIZATION	YES
NCU01452	probable ribosomal protein 10, cytosolic	12 PROTEIN SYNTHESIS	YES
NCU07829	probable 60s ribosomal protein L7 subunit	12 PROTEIN SYNTHESIS__70	

NCU # or Gene I.D.	Known or Predicted Function[1]	FunCat Classification	Predicted ccg[2]
		SUBCELLULAR LOCALIZATION	
NCU08344	probable ribosomal protein L31.e.A, cytosolic	12 PROTEIN SYNTHESIS__70 SUBCELLULAR LOCALIZATION	
NCU03988	probable ribosomal protein L18, cytosolic	12 PROTEIN SYNTHESIS__70 SUBCELLULAR LOCALIZATION	YES
NCU01001	related to ribosomal protein s25	12 PROTEIN SYNTHESIS	
NCU06035	probable translation elongation factor eEF-1 beta chain	12 PROTEIN SYNTHESIS	YES
NCU03635	probable ribosomal protein L38	12 PROTEIN SYNTHESIS	YES
NCU01452	probable ribosomal protein 10, cytosolic	12 PROTEIN SYNTHESIS	
NCU01827	probable 60S large subunit ribosomal protein	12 PROTEIN SYNTHESIS	YES
NCU02181	probable ribosomal protein S4.e, cytosolic	12 PROTEIN SYNTHESIS	YES
NCU00706	probable 60S ribosomal protein L44	12 PROTEIN SYNTHESIS__70 SUBCELLULAR LOCALIZATION	
NCU08620	probable ribosomal protein S16.e, cytosolic	12 PROTEIN SYNTHESIS__70 SUBCELLULAR LOCALIZATION	YES
NCU01981	probable translation initiation factor SUI1	12 PROTEIN SYNTHESIS	YES

NCU # or Gene I.D.	Known or Predicted Function[1]	FunCat Classification	Predicted ccg[2]
NCU09476	probable ribosomal protein S25.e.c7	12 PROTEIN SYNTHESIS__70 SUBCELLULAR LOCALIZATION	
NCU00971	probable ribosomal protein S12, cytosolic	12 PROTEIN SYNTHESIS__70 SUBCELLULAR LOCALIZATION	
NCU03738	probable ribosomal protein S29.e.A, cytosolic	12 PROTEIN SYNTHESIS	YES
NCU05804	probable 60S ribosomal protein L19	12 PROTEIN SYNTHESIS__70 SUBCELLULAR LOCALIZATION	
NCU09102	related to glu/asp-tRNA amidotransferase subunit A	12 PROTEIN SYNTHESIS__14 PROTEIN FATE (folding, modification, destination)	
NCU03548	related to eukaryotic translation initiation factor EIF-2B subunit 3	12 PROTEIN SYNTHESIS	YES
NCU05890	related to mitochondrial 60s ribosomal protein	12 PROTEIN SYNTHESIS__70 SUBCELLULAR LOCALIZATION	
NCU05233	p60 domain-containing protein	12 PROTEIN SYNTHESIS	YES
NCU05717	probable ribosomal protein MRP17, mitochondrial	12 PROTEIN SYNTHESIS__70 SUBCELLULAR LOCALIZATION	
NCU02075	heat shock protein 70	14 PROTEIN FATE (folding, modification, destination)__32 CELL RESCUE, DEFENSE AND VIRULENCE__70 SUBCELLULAR LOCALIZATION	

NCU # or Gene I.D.	Known or Predicted Function[1]	FunCat Classification	Predicted ccg[2]
NCU04140	FK506-binding protein (FKBP) fkr-2	14 PROTEIN FATE (folding, modification, destination)	
NCU01792	related to Hsp90 associated co-chaperone	14 PROTEIN FATE (folding, modification, destination)__32 CELL RESCUE, DEFENSE AND VIRULENCE	
NCU05773	related to prolyl aminopeptidase A	14 PROTEIN FATE (folding, modification, destination)	
NCU01315	related to udp-n-acetylglucosamine-peptide n-acetylglucosaminyltransferase	14 PROTEIN FATE (folding, modification, destination)	
NCU08733	related to histone-lysine N-methyltransferase	14 PROTEIN FATE (folding, modification, destination)	
NCU06756	related to ubiquitin-protein ligase HUL4	14 PROTEIN FATE (folding, modification, destination)__18 REGULATION OF METABOLISM AND PROTEIN FUNCTION	YES
NCU03309	probable ubiquitin-like protein ubl1	14 PROTEIN FATE (folding, modification, destination)__32 CELL RESCUE, DEFENSE AND VIRULENCE	
NCU06250	related to F-box protein Fbl2	14 PROTEIN FATE (folding, modification, destination)	
NCU09006	related to AOS1 protein	14 PROTEIN FATE (folding, modification, destination)	
NCU02074	related to ERO1 protein, required for protein disulfide bond formation in the ER	14 PROTEIN FATE (folding, modification, destination)__70 SUBCELLULAR LOCALIZATION	
NCU07159	probable endopeptidase K	14 PROTEIN FATE (folding, modification, destination)	

NCU # or Gene I.D.	Known or Predicted Function[1]	FunCat Classification	Predicted ccg[2]
NCU10927	related to signal recognition particle 68 kDa protein	14 PROTEIN FATE (folding, modification, destination)	
NCU02404	related to heterogeneous nuclear ribonucleoprotein	16 PROTEIN WITH BINDING FUNCTION OR COFACTOR REQUIREMENT (structural or catalytic)	
NCU01389	mitogen-activated protein kinase organizer 1	16 PROTEIN WITH BINDING FUNCTION OR COFACTOR REQUIREMENT	
NCU04120	calmodulin CMD-1	16 PROTEIN WITH BINDING FUNCTION OR COFACTOR REQUIREMENT (structural or catalytic)__30 CELLULAR COMMUNICATION/SIGNAL TRANSDUCTION MECHANISM	
NCU07328	related to potassium channel regulator	16 PROTEIN WITH BINDING FUNCTION OR COFACTOR REQUIREMENT (structural or catalytic)	
NCU11050	conserved hypothetical protein	16 PROTEIN WITH BINDING FUNCTION OR COFACTOR REQUIREMENT (structural or catalytic)	
NCU04606	probable pre-mRNA splicing factor prp1	16 PROTEIN WITH BINDING FUNCTION	
NCU01348	agmatinase	16 PROTEIN WITH BINDING FUNCTION OR COFACTOR REQUIREMENT (structural or catalytic)	

NCU # or Gene I.D.	Known or Predicted Function[1]	FunCat Classification	Predicted ccg[2]
NCU04861	ankyrin repeats; ankyrin repeats mediate protein-protein interactions in very diverse	16 PROTEIN WITH BINDING FUNCTION OR COFACTOR REQUIREMENT (structural or catalytic)	
NCU03826	probable translation elongation factor eEF-1, gamma chain	18 PROTEIN SYNTHESIS__70 SUBCELLULAR LOCALIZATION	YES
NCU05276	Yip1 domain family; Integral membrane protein required for the biogenesis of ER-derived COPII transport vesicles	18 REGULATION OF METABOLISM AND PROTEIN FUNCTION__70 SUBCELLULAR LOCALIZATION	YES
NCU06067	related to Scd1 protein	18 REGULATION OF METABOLISM AND PROTEIN FUNCTION__70 SUBCELLULAR LOCALIZATION	
NCU09477	ADP, ATP carrier protein (ADP/ATP translocase) acp	20 CELLULAR TRANSPORT, TRANSPORT FACILITATION AND TRANSPORT ROUTES__70 SUBCELLULAR LOCALIZATION	YES
NCU01791	related to endosomal Vps protein complex subunit	20 CELLULAR TRANSPORT, TRANSPORT FACILITATION AND TRANSPORT ROUTES	
NCU03497	related to high-affinity iron permease	20 CELLULAR TRANSPORT, TRANSPORT FACILITATION AND TRANSPORT ROUTES__34 INTERACTION WITH THE ENVIRONMENT	YES
NCU05902	related to vacuolar assembly protein	20 CELLULAR TRANSPORT, TRANSPORT FACILITATION AND TRANSPORT ROUTES__42 BIOGENESIS OF CELLULAR COMPONENTS	

NCU # or Gene I.D.	Known or Predicted Function[1]	FunCat Classification	Predicted ccg[2]
NCU00648	related to cholin permease	20 CELLULAR TRANSPORT, TRANSPORT FACILITATION AND TRANSPORT ROUTES__70 SUBCELLULAR LOCALIZATION	YES
NCU04065	potassium channel	20 CELLULAR TRANSPORT, TRANSPORT FACILITATION AND TRANSPORT ROUTES	YES
NCU09869	related to SEC3 protein	20 CELLULAR TRANSPORT, TRANSPORT FACILITATION AND TRANSPORT ROUTES__43 CELL TYPE DIFFERENTIATION	
NCU07960	related to transporter protein HOL1	20 CELLULAR TRANSPORT, TRANSPORT FACILITATION AND TRANSPORT ROUTES__70 SUBCELLULAR LOCALIZATION	
NCU05068	related to phospholipid-translocating ATPase	20 CELLULAR TRANSPORT, TRANSPORT FACILITATION AND TRANSPORT ROUTES	
NCU06946	related to ATP-binding cassette transporter	20 CELLULAR TRANSPORT, TRANSPORT FACILITATION AND TRANSPORT ROUTES	
NCU02337	mitochondrial carrier protein	20 CELLULAR TRANSPORT, TRANSPORT FACILITIES AND TRANSPORT ROUTES	
NCU08092	related to NIPSNAP protein	20 CELLULAR TRANSPORT, TRANSPORT FACILITATION AND TRANSPORT ROUTES	
NCU07465	probable phosphate transport protein, mitochondrial	20 CELLULAR TRANSPORT, TRANSPORT FACILITATION AND	YES

NCU # or Gene I.D.	Known or Predicted Function[1]	FunCat Classification	Predicted ccg[2]
NCU05045	related to monocarboxylate transporter 4	TRANSPORT ROUTES__70 SUBCELLULAR LOCALIZATION 20 CELLULAR TRANSPORT, TRANSPORT FACILITATION AND TRANSPORT ROUTES	
NCU01750	conserved hypothetical protein	20 CELLULAR TRANSPORT, TRANSPORT FACILITATION AND TRANSPORT ROUTES	
NCU07343	major facilitator family transporter	20 CELLULAR TRANSPORT, TRANSPORT FACILITIES AND TRANSPORT ROUTES	
NCU03395	H+-transporting ATPase, vacuolar, 41 kDa subunit	20 CELLULAR TRANSPORT, TRANSPORT FACILITATION AND TRANSPORT ROUTES__70 SUBCELLULAR LOCALIZATION	
NCU08147	P-type ATPase (Ca2+-transporting ATPase) PH-7	20 CELLULAR TRANSPORT, TRANSPORT FACILITATION AND TRANSPORT ROUTES__32 CELL RESCUE, DEFENSE AND VIRULENCE__34 INTERACTION WITH THE ENVIRONMENT__70 SUBCELLULAR LOCALIZATION	
NCU01132	probable sugar transporter	20 CELLULAR TRANSPORT, TRANSPORT FACILITATION AND TRANSPORT ROUTES	
NCU09027	related to putative tartrate transporter	20 CELLULAR TRANSPORT, TRANSPORT FACILITATION AND TRANSPORT ROUTES	

NCU # or Gene I.D.	Known or Predicted Function[1]	FunCat Classification	Predicted ccg[2]
NCU06137	probable cytochrome P450 55A2	20 CELLULAR TRANSPORT, TRANSPORT FACILITATION AND TRANSPORT ROUTES	
NCU01198	related to RNA export mediator GLE1	20 CELLULAR TRANSPORT, TRANSPORT FACILITATION AND TRANSPORT ROUTES	
NCU05585	related to quinate transport protein	20 CELLULAR TRANSPORT, TRANSPORT FACILITATION AND TRANSPORT ROUTES__70 SUBCELLULAR LOCALIZATION	
NCU01330	SacI domain-containing protein	20 CELLULAR TRANSPORT, TRANSPORT FACILITIES AND TRANSPORT ROUTES	
NCU05796	probable BET3	20 CELLULAR TRANSPORT, TRANSPORT FACILITATION AND TRANSPORT ROUTES	
NCU04218	RTA1 domain-containing protein	20 CELLULAR TRANSPORT, TRANSPORT FACILITIES AND TRANSPORT ROUTES	
NCU09773	related to peptide transporter	20 CELLULAR TRANSPORT, TRANSPORT FACILITATION AND TRANSPORT ROUTES	
NCU09820	related to protein YBT1 (bile acid transporter)	20 CELLULAR TRANSPORT, TRANSPORT FACILITATION AND TRANSPORT ROUTES	
NCU07820	related to transporter protein	20 CELLULAR TRANSPORT, TRANSPORT FACILITATION AND TRANSPORT ROUTES	
NCU04541	related to VPS60 involved in vacuolar protein sorting	20 CELLULAR TRANSPORT, TRANSPORT	

NCU # or Gene I.D.	Known or Predicted Function[1]	FunCat Classification	Predicted ccg[2]
		FACILITATION AND TRANSPORT ROUTES	
NCU02826	sodium/calcium exchanger protein	20 CELLULAR TRANSPORT	
NCU00043	serine/threonine protein phosphatase PP1 PPP-1	30 CELLULAR COMMUNICATION/SIGNAL TRANSDUCTION MECHANISM	YES
NCU02044	probable GTP-binding protein	30 CELLULAR COMMUNICATION/SIGNAL TRANSDUCTION MECHANISM	
NCU09132	tubulin alpha-A chain TUB-alpha A	30 CELLULAR COMMUNICATION/SIGNAL TRANSDUCTION MECHANISM	
NCU08923	Putative zinc finger protein, proposed to be involved in the RAS/cAMP signaling pathway	30 CELLULAR COMMUNICATION/SIGNAL TRANSDUCTION	
NCU01523	probable GTP-binding protein Drab11	30 CELLULAR COMMUNICATION/SIGNAL TRANSDUCTION MECHANISM	
NCU06202	related to serine/threonine kinase ARK1	30 CELLULAR COMMUNICATION/SIGNAL TRANSDUCTION MECHANISM__42 BIOGENESIS OF CELLULAR COMPONENTS	
NCU00108	probable protein kinase Eg22	30 CELLULAR COMMUNICATION/SIGNAL TRANSDUCTION MECHANISM	
NCU00109	TIP41-like protein	30 CELLULAR COMMUNICATION/SIGNAL TRANSDUCTION MECHANISM__34	

NCU # or Gene I.D.	Known or Predicted Function[1]	FunCat Classification	Predicted ccg[2]
NCU09520	Histidine kinase A	INTERACTION WITH THE ENVIRONMENT__43 CELL TYPE DIFFERENTIATION__70 SUBCELLULAR LOCALIZATION 30 CELLULAR COMMUNICATION/SIGNAL TRANSDUCTION MECHANISM__34	
NCU04201	related to SYG1 protein	INTERACTION WITH THE ENVIRONMENT__43 CELL TYPE DIFFERENTIATION__70 SUBCELLULAR LOCALIZATION 30 CELLULAR COMMUNICATION/SIGNAL TRANSDUCTION MECHANISM__34	
NCU03571	related to 3-phosphoinositide dependent protein kinase-1 (PDK1)	INTERACTION WITH THE ENVIRONMENT__43 CELL TYPE DIFFERENTIATION__70 SUBCELLULAR LOCALIZATION 30 CELLULAR COMMUNICATION/SIGNAL TRANSDUCTION MECHANISM	YES
NCU06419	probable MAP kinase kinase	30 CELLULAR COMMUNICATION/SIGNAL TRANSDUCTION MECHANISM	
NCU05591	probable ABC1 transport protein	30 CELL RESCUE, DEFENSE AND VIRULENCE	
NCU09427	G-protein coupled receptor	30 CELLULAR COMMUNICATION/SIGNAL TRANSDUCTION MECHANISM__34	

NCU # or Gene I.D.	Known or Predicted Function[1]	FunCat Classification	Predicted ccg[2]
NCU04834	related to phytochrome	INTERACTION WITH THE ENVIRONMENT__43 CELL TYPE DIFFERENTIATION__70 SUBCELLULAR LOCALIZATION 30 CELLULAR COMMUNICATION/SIGNAL TRANSDUCTION MECHANISM__34 INTERACTION WITH THE ENVIRONMENT	
NCU06111	related to GTPase Ras2p	30 CELLULAR COMMUNICATION/SIGNAL TRANSDUCTION MECHANISM__43 CELL TYPE DIFFERENTIATION	YES
NCU05269	heat shock protein hsp88	32 CELL RESCUE, DEFENSE AND VIRULENCE	
NCU03980	probable t-complex-type molecular chaperone, epsilon subunit	32 CELL RESCUE, DEFENSE AND VIRULENCE	
NCU08701	related to verrucotoxin alpha	32 CELL RESCUE, DEFENSE AND VIRULENCE	
NCU05733	acriflavine sensitivity control protein ACR-2	32 CELL RESCUE, DEFENSE AND VIRULENCE	
NCU06660	probable RIC1 protein	32 CELL RESCUE, DEFENSE AND VIRULENCE	YES
NCU07167	related to phenylcoumaran benzylic ether reductase	32 CELL RESCUE, DEFENSE AND VIRULENCE__42 BIOGENESIS OF CELLULAR COMPONENTS	YES
NCU04130	related to lincomycin-condensing protein lmbA	32 CELL RESCUE, DEFENSE AND VIRULENCE	YES

NCU # or Gene I.D.	Known or Predicted Function[1]	FunCat Classification	Predicted ccg[2]
NCU08469	related to transesterase	32 CELL RESCUE, DEFENSE AND VIRULENCE	
NCU01484	rho-type GTPase	32 CELL RESCUE, DEFENSE AND VIRULENCE	
NCU06017	thiosulfate sulfurtransferase	32 CELL RESCUE, DEFENSE AND VIRULENCE	
NCU03370	probable CAP20 - virulence factor	32 CELL RESCUE, DEFENSE AND VIRULENCE	
NCU01857	DEAD/DEAH box RNA helicase	32 CELL RESCUE, DEFENSE AND VIRULENCE	
NCU06682	plasma membrane proteolipid 3	32 CELL RESCUE, DEFENSE AND VIRULENCE	
NCU05706	related to glutathione S-transferase	32 CELL RESCUE, DEFENSE AND VIRULENCE	
NCU01320	related to microsomal glutathione s-transferase 3	32 CELL RESCUE, DEFENSE AND VIRULENCE	
NCU05730	related to pathogenicity cluster PEP2	32 CELL RESCUE, DEFENSE AND VIRULENCE	
NCU03339	probable glutathione reductase (NADPH)	32 CELL RESCUE, DEFENSE AND VIRULENCE	
NCU01735	related to YRO2 protein	32 CELL RESCUE, DEFENSE AND VIRULENCE	
NCU05317	chitinase	32 CELL RESCUE, DEFENSE AND VIRULENCE	
NCU02997	related to nodulation protein nodB	32 CELL RESCUE, DEFENSE AND VIRULENCE	

NCU # or Gene I.D.	Known or Predicted Function[1]	FunCat Classification	Predicted ccg[2]
NCU09579	retinol dehydrogenase 12	34 INTERACTION WITH THE ENVIRONMENT	
NCU00467	probable COP9 signalosome subunit 5 CSN5	34 INTERACTION WITH THE ENVIRONMENT__41 DEVELOPMENT (Systemic)	
NCU09353	related to tol protein	34 INTERACTION WITH THE ENVIRONMENT__43 CELL TYPE DIFFERENTIATION	
NCU05957	Heterokaryon incompatibility	34 INTERACTION WITH THE ENVIRONMENT	
mito_195	predicted ND1 intron protein	38 TRANSPOSABLE ELEMENTS, VIRAL AND PLASMID PROTEINS__70 SUBCELLULAR LOCALIZATION	
NCU05137	non-anchored cell wall protein-1; extracellular serine-rich protein, putative [Metarhizium anisopliae ARSEF 23]	42 BIOGENESIS OF CELLULAR COMPONENTS	YES
NCU04352	probable chitin synthase	42 BIOGENESIS OF CELLULAR COMPONENTS_01 METABOLISM__	
NCU05667	anchored cell wall protein-3; Developmentally Regulated MAPK Interacting Protein	42 BIOGENESIS OF CELLULAR COMPONENTS	
NCU01689	probable suppressor protein of mitochondrial histone mutant	42 BIOGENESIS OF CELLULAR COMPONENTS__70 SUBCELLULAR LOCALIZATION	
NCU07776	anchored cell wall protein-5; Extracellular membrane	42 BIOGENESIS OF CELLULAR COMPONENTS	YES

NCU # or Gene I.D.	Known or Predicted Function[1]	FunCat Classification	Predicted ccg[2]
	protein, 8-cysteine region, CFEM		
NCU00399	cell wall protein PhiA	42 BIOGENESIS OF CELLULAR COMPONENTS	
NCU05280	DASH complex, subunit Ask1	42 BIOGENESIS OF CELLULAR COMPONENTS	
NCU03875	probable ATPase component of chromatin remodeling complex (ISW1)	42 BIOGENESIS OF CELLULAR COMPONENTS__70 SUBCELLULAR LOCALIZATION	
NCU08193	GPI anchored protein, putative [Metarhizium anisopliae ARSEF 23]	42 BIOGENESIS OF CELLULAR COMPONENTS	
NCU06429	related to alpha-actinin	42 BIOGENESIS OF CELLULAR COMPONENTS__70 SUBCELLULAR LOCALIZATION	
NCU05315	CSE domain	42 BIOGENESIS OF CELLULAR COMPONENTS	YES
NCU07472	related to Ecm39 protein, involved in cell wall biogenesis and architecture	42 BIOGENESIS OF CELLULAR COMPONENTS	
NCU00957	related to extracellular matrix protein precursor	42 BIOGENESIS OF CELLULAR COMPONENTS	
NCU07259	probable GPI-anchor transamidase	42 BIOSYNTHESIS OF CELLULAR COMPONENTS	
NCU00716	non-anchored cell wall protein-5	42 BIOGENESIS OF CELLULAR COMPONENTS	

NCU # or Gene I.D.	Known or Predicted Function[1]	FunCat Classification	Predicted ccg[2]
NCU08541	putative protein	99UNCLASSIFIED PROTEINS	
NCU06911	hypothetical protein	99UNCLASSIFIED PROTEINS	
NCU04493	Ser-Thr-rich glycosyl-phosphatidyl-inositol-anchored membrane family	99UNCLASSIFIED PROTEINS	YES
NCU09693	putative protein	99UNCLASSIFIED PROTEINS	
NCU07311	putative protein	99UNCLASSIFIED PROTEINS	
NCU08949	P-II protein urydylation site	99UNCLASSIFIED PROTEINS	YES
NCU03500	aminotransferase	99UNCLASSIFIED PROTEINS	
NCU01768	conserved hypothetical protein	99UNCLASSIFIED PROTEINS	YES
NCU02573	FAM96B-like protein	99UNCLASSIFIED PROTEINS	
NCU03293	hypothetical protein	99UNCLASSIFIED PROTEINS	YES
NCU04924	cut-1; HAD-superfamily hydrolase	99UNCLASSIFIED PROTEINS	
NCU08351	hypothetical protein	99UNCLASSIFIED PROTEINS	
NCU10360	Galactose oxidase/kelch, beta-propeller	99UNCLASSIFIED PROTEINS	

NCU # or Gene I.D.	Known or Predicted Function[1]	FunCat Classification	Predicted ccg[2]
NCU02602	conserved hypothetical protein	99UNCLASSIFIED PROTEINS	
NCU03753	glucose-repressible gene-1 protein; ccg-1	99UNCLASSIFIED PROTEINS	YES
NCU04276	putative protein	99UNCLASSIFIED PROTEINS	YES
NCU03726	hypothetical protein	99UNCLASSIFIED PROTEINS	
NCU00184	hypothetical protein	99UNCLASSIFIED PROTEINS	YES
NCU04890	hypothetical protein	99UNCLASSIFIED PROTEINS	YES
NCU07588	hypothetical protein	99UNCLASSIFIED PROTEINS	
NCU01566	putative protein	99UNCLASSIFIED PROTEINS	
NCU06611	WD40 repeat	99UNCLASSIFIED PROTEINS	
NCU00042	selenoprotein domain-containing protein	99UNCLASSIFIED PROTEINS	
NCU01144	putative protein	99UNCLASSIFIED PROTEINS	
NCU07154	yippee family protein	99UNCLASSIFIED PROTEINS	
NCU06136	hypothetical protein	99UNCLASSIFIED PROTEINS	YES

NCU # or Gene I.D.	Known or Predicted Function[1]	FunCat Classification	Predicted ccg[2]
NCU06651	AN1-type zinc finger protein; Predicted nucleic acid binding protein containing the AN1-type Zn-finger	99UNCLASSIFIED PROTEINS	YES
NCU03494	partner for incompatibility with het-c	99UNCLASSIFIED PROTEINS	
NCU05055	hypothetical protein	99UNCLASSIFIED PROTEINS	
NCU00864	TIM-barrel enzyme family protein	99UNCLASSIFIED PROTEINS	
NCU00107	mitochondrial inner membrane protease ATP-23	99UNCLASSIFIED PROTEINS	
NCU02019	FAD dependent oxidoreductase	99UNCLASSIFIED PROTEINS	
NCU00180	c6 zinc finger domain containing protein [Grosmannia clavigera kw1407]	99UNCLASSIFIED PROTEINS	
NCU09929	conserved hypothetical protein	99UNCLASSIFIED PROTEINS	YES
NCU06085	LCCL domain-containing protein	99UNCLASSIFIED PROTEINS	YES
NCU04403	ubiquitin domain-containing protein; histone-lysine n-methyltransferase clr4 [Grosmannia clavigera kw1407] 1	99UNCLASSIFIED PROTEINS	
NCU06742	conserved hypothetical protein	99UNCLASSIFIED PROTEINS	YES

NCU # or Gene I.D.	Known or Predicted Function[1]	FunCat Classification	Predicted ccg[2]
NCU08517	putative protein	99UNCLASSIFIED PROTEINS	
NCU03589	hypothetical protein	99UNCLASSIFIED PROTEINS	YES
NCU05547	MFS transporter, putative [Metarhizium acridum CQMa 102]	99UNCLASSIFIED PROTEINS	YES
NCU00015	Peroxiredoxin (PRX)-like 2 family	99UNCLASSIFIED PROTEINS	
NCU07125	cytomegalovirus gH-receptor family protein; Protein of unknown function DUF747, CMV receptor; The protein is involved in the transport of mRNA's from the nucleus to the cytoplasm	99UNCLASSIFIED PROTEINS	
NCU01329	conserved protein hypothetical protein	99UNCLASSIFIED PROTEINS	
NCU06844	putative protein	99UNCLASSIFIED PROTEINS	
NCU06375	metallophosphoesterase domain-containing protein 2	99UNCLASSIFIED PROTEINS	YES
NCU08720	hypothetical protein	99UNCLASSIFIED PROTEINS	
NCU04872	putative protein	99UNCLASSIFIED PROTEINS	
NCU02899	conserved hypothetical protein	99UNCLASSIFIED PROTEINS	

NCU # or Gene I.D.	Known or Predicted Function[1]	FunCat Classification	Predicted ccg[2]
NCU03712	MoxR-like ATPases	99UNCLASSIFIED PROTEINS	
NCU07149	conserved hypothetical protein	99UNCLASSIFIED PROTEINS	
NCU02577	hypothetical protein	99UNCLASSIFIED PROTEINS	YES
NCU08390	Acetyltransferases, including N-acetylases of ribosomal proteins [Translation, ribosomal structure and biogenesis]	99UNCLASSIFIED PROTEINS	
NCU02061	present in yeast cell wall integrity and stress response component proteins; domain present in WSC proteins, polycystin and fungal exoglucanase; carbohydrate-binding WSC	99UNCLASSIFIED PROTEINS	YES
NCU06653	hypothetical protein	99UNCLASSIFIED PROTEINS	
NCU04324	conserved hypothetical protein	99UNCLASSIFIED PROTEINS	
NCU03543	autophagy protein DOMAIN	99UNCLASSIFIED PROTEINS	
NCU09681	related to hydroxyproline-rich glycoprotein precursor	99UNCLASSIFIED PROTEINS	
NCU08757	Alcohol dehydrogenase, zinc-binding	99UNCLASSIFIED PROTEINS	
NCU07557	hypothetical protein	99UNCLASSIFIED PROTEINS	

NCU # or Gene I.D.	Known or Predicted Function[1]	FunCat Classification	Predicted ccg[2]
NCU06644	Ran Binding Protein 1	99UNCLASSIFIED PROTEINS	
NCU02652	Ligand-binding SRPBCC domain of an uncharacterized subfamily of proteins	99UNCLASSIFIED PROTEINS	YES
NCU04852	hypothetical protein	99UNCLASSIFIED PROTEINS	
NCU06286	hypothetical protein	99UNCLASSIFIED PROTEINS	
NCU06133	hypothetical protein	99UNCLASSIFIED PROTEINS	
NCU08476	conserved hypothetical protein	99UNCLASSIFIED PROTEINS	
NCU09702	endo-beta-1,6-galactanase	99UNCLASSIFIED PROTEINS	
NCU05864	Endoplasmic reticulum targeting sequence	99UNCLASSIFIED PROTEINS	YES
NCU04580	vacuolar protein sorting protein Vps66; acyltransferase [Glomerella graminicola M1.001]	99UNCLASSIFIED PROTEINS	
NCU04811	hypothetical protein	99UNCLASSIFIED PROTEINS	YES
NCU05005	HHE domain-containing protein	99UNCLASSIFIED PROTEINS	YES
NCU04931	conserved hypothetical protein	99UNCLASSIFIED PROTEINS	YES

NCU # or Gene I.D.	Known or Predicted Function[1]	FunCat Classification	Predicted ccg[2]
NCU05647	hypothetical protein	99UNCLASSIFIED PROTEINS	
NCU00162	lectin family integral membrane protein [Aspergillus niger CBS 513.88]	99UNCLASSIFIED PROTEINS	
NCU08241	hypothetical protein	99UNCLASSIFIED PROTEINS	
NCU03192	hypothetical protein	99UNCLASSIFIED PROTEINS	YES
NCU04905	S-adenosylmethionine-dependent methyltransferases (SAM or AdoMet-MTase)	99UNCLASSIFIED PROTEINS	
NCU07963	conserved hypothetical protein	99UNCLASSIFIED PROTEINS	YES
NCU01098	ankyrin repeat protein	99UNCLASSIFIED PROTEINS	
NCU00226	MFS transporter	99UNCLASSIFIED PROTEINS	
NCU04744	dihydrodipicolinate synthetase	99UNCLASSIFIED PROTEINS	YES
NCU05634	endosomal peripheral membrane protein [Glomerella graminicola M1.001]	99UNCLASSIFIED PROTEINS	
NCU01148	methyltransferase	99UNCLASSIFIED PROTEINS	
NCU06478	Methyltransferase type 12; S-adenosylmethionine-dependent methyltransferases (SAM or AdoMet-MTase)	99UNCLASSIFIED PROTEINS	

NCU # or Gene I.D.	Known or Predicted Function[1]	FunCat Classification	Predicted ccg[2]
NCU01483	hypothetical protein	99UNCLASSIFIED PROTEINS	
NCU07784	SAM binding domain-containing protein (methyltransferase domain)	99UNCLASSIFIED PROTEINS	
NCU06855	The GYF domain is named because of the presence of Gly-Tyr-Phe residues. The GYF domain is a proline-binding domain in CD2-binding protein.	99UNCLASSIFIED PROTEINS	
NCU06915	hypothetical protein	99UNCLASSIFIED PROTEINS	
NCU05493	WD40 repeat-like	99UNCLASSIFIED PROTEINS	YES
NCU09314	hypothetical protein	99UNCLASSIFIED PROTEINS	YES
NCU08155	putative protein	99UNCLASSIFIED PROTEINS	
NCU08797	hypothetical protein	99UNCLASSIFIED PROTEINS	
NCU00729	developmentally Regulated MAPK Interacting protein [Glomerella graminicola M1.001]	99UNCLASSIFIED PROTEINS	YES
NCU01801	putative protein	99UNCLASSIFIED PROTEINS	
NCU02261	hypothetical protein	99UNCLASSIFIED PROTEINS	

NCU # or Gene I.D.	Known or Predicted Function[1]	FunCat Classification	Predicted ccg[2]
NCU01565	hypothetical protein	99UNCLASSIFIED PROTEINS	
NCU00995	putative protein	99UNCLASSIFIED PROTEINS	
NCU09874	Oligopeptide transporter OPT superfamily	99UNCLASSIFIED PROTEINS	
NCU05692	OHCU decarboxylase domain	99UNCLASSIFIED PROTEINS	
NCU08077	hypothetical protein	99UNCLASSIFIED PROTEINS	
NCU02694	Regulator of chromosome condensation, RCC1	99UNCLASSIFIED PROTEINS	
NCU05825	EGF-like region	99UNCLASSIFIED PROTEINS	
NCU03506	hypothetical protein	99UNCLASSIFIED PROTEINS	YES
NCU02121	transcriptional regulator; PHD-finger domain-containing protein	99UNCLASSIFIED PROTEINS	YES
NCU05954	putative translation initiation factor IF-2; Validated	99UNCLASSIFIED PROTEINS	
NCU01113	protein kinase	99UNCLASSIFIED PROTEINS	
NCU01039	hypothetical protein	99UNCLASSIFIED PROTEINS	
NCU03652	SNF2 family helicase/ATPase	99UNCLASSIFIED PROTEINS	

NCU # or Gene I.D.	Known or Predicted Function[1]	FunCat Classification	Predicted ccg[2]
NCU00411	hypothetical protein	99UNCLASSIFIED PROTEINS	
NCU09072	putative protein	99UNCLASSIFIED PROTEINS	
NCU07751	hypothetical protein	99UNCLASSIFIED PROTEINS	YES
NCU03208	hypothetical protein	99UNCLASSIFIED PROTEINS	YES
NCU04894	hypothetical protein	99UNCLASSIFIED PROTEINS	
NCU06258	hypothetical protein	99UNCLASSIFIED PROTEINS	
NCU07449	putative protein	99UNCLASSIFIED PROTEINS	YES
NCU05498	putative protein	99UNCLASSIFIED PROTEINS	YES
NCU00870	Tetratricopeptide	99UNCLASSIFIED PROTEINS	YES
NCU06718	zinc knuckle domain-containing protein; retinoblastoma-binding protein [Metarhizium acridum CQMa 102]	99UNCLASSIFIED PROTEINS	
NCU10045	pectinesterase	99UNCLASSIFIED PROTEINS	
NCU07962	Extracellular membrane protein, 8-cysteine region, CFEM	99UNCLASSIFIED PROTEINS	

NCU # or Gene I.D.	Known or Predicted Function[1]	FunCat Classification	Predicted ccg[2]
NCU08235	K+-dependent Na+/Ca+ exchanger DOMAIN	99UNCLASSIFIED PROTEINS	
NCU07718	GCN5-related N-acetyltransferase	99UNCLASSIFIED PROTEINS	
NCU01783	phosphatidylinositol N-acetylglucosaminyltransferase	99UNCLASSIFIED PROTEINS	YES
NCU08996	hypothetical protein	99UNCLASSIFIED PROTEINS	
NCU09853	Thioesterase superfamily	99UNCLASSIFIED PROTEINS	
NCU03121	AAA+ ATPase, core	99UNCLASSIFIED PROTEINS	
NCU09348	kelch domain-containing protein	99UNCLASSIFIED PROTEINS	
NCU03020	IdgA domain-containing protein; Carbohydrate/purine kinase	99UNCLASSIFIED PROTEINS	
NCU00268	hypothetical protein	99UNCLASSIFIED PROTEINS	
NCU05226	ABC transporter	99UNCLASSIFIED PROTEINS	
NCU06650	secretory phospholipase A2	99UNCLASSIFIED PROTEINS	
NCU10039	set domain; secretory pathway	99UNCLASSIFIED PROTEINS	
NCU02869	hypothetical protein	99UNCLASSIFIED PROTEINS	YES

NCU # or Gene I.D.	Known or Predicted Function[1]	FunCat Classification	Predicted ccg[2]
NCU06589	hypothetical protein	99UNCLASSIFIED PROTEINS	
NCU06265	conserved hypothetical protein	99UNCLASSIFIED PROTEINS	YES
NCU00397	Lipolytic enzyme, G-D-S-L	99UNCLASSIFIED PROTEINS	YES
NCU02528	G-patch domain; domain is found in RNA binding proteins, and is also found in proteins that contain RNA binding domains, suggesting this domain may have an RNA binding function. This domain has seven highly conserved glycines.	99UNCLASSIFIED PROTEINS	
NCU09714	hypothetical protein	99UNCLASSIFIED PROTEINS	YES
NCU03003	conserved hypothetical protein	99UNCLASSIFIED PROTEINS	
NCU07643	Carbamoyl-phosphate synthase L chain, ATP-binding	99UNCLASSIFIED PROTEINS	YES
NCU02625	hypothetical protein	99UNCLASSIFIED PROTEINS	
NCU00881	putative protein	99UNCLASSIFIED PROTEINS	
NCU08826	hypothetical protein	99UNCLASSIFIED PROTEINS	
NCU00633	Glycoside hydrolase, family 1	99UNCLASSIFIED PROTEINS	

NCU # or Gene I.D.	Known or Predicted Function[1]	FunCat Classification	Predicted ccg[2]
NCU05934	putative protein	99UNCLASSIFIED PROTEINS	YES
NCU04490	hypothetical protein	99UNCLASSIFIED PROTEINS	
NCU06127	putative protein	99UNCLASSIFIED PROTEINS	
NCU03825	phosphoprotein phosphatase [Metarhizium anisopliae ARSEF 23] targeted to mitochondria	99UNCLASSIFIED PROTEINS	
NCU02617	kelch repeat-containing protein	99UNCLASSIFIED PROTEINS	
NCU09535	FAD dependent oxidoreductase	99UNCLASSIFIED PROTEINS	
NCU01038	similar to beta-lactamase family protein	99UNCLASSIFIED PROTEINS	
NCU08405	IBR finger domain protein	99UNCLASSIFIED PROTEINS	
NCU06050	hypothetical protein	99UNCLASSIFIED PROTEINS	
NCU08028	conserved hypothetical protein	99UNCLASSIFIED PROTEINS	
NCU01844	conserved hypothetical protein	99UNCLASSIFIED PROTEINS	
NCU01091	F-box and ankyrin domain protein	99UNCLASSIFIED PROTEINS	

NCU # or Gene I.D.	Known or Predicted Function[1]	FunCat Classification	Predicted ccg[2]
NCU00005	conserved hypothetical protein	99UNCLASSIFIED PROTEINS	YES
NCU05319	LysM domain-containing protein	99UNCLASSIFIED PROTEINS	YES
NCU02547	putative protein	99UNCLASSIFIED PROTEINS	
NCU08509	hypothetical protein	99UNCLASSIFIED PROTEINS	YES
NCU05030	putative protein	99UNCLASSIFIED PROTEINS	
NCU05083	hypothetical protein	99UNCLASSIFIED PROTEINS	
NCU09725	hypothetical protein	99UNCLASSIFIED PROTEINS	
NCU08654	hypothetical protein	99UNCLASSIFIED PROTEINS	
NCU04409	ankyrin repeat protein	99UNCLASSIFIED PROTEINS	
NCU09190	hypothetical protein	99UNCLASSIFIED PROTEINS	YES
NCU08680	hypothetical protein	99UNCLASSIFIED PROTEINS	YES
NCU02656	conserved hypothetical protein	99UNCLASSIFIED PROTEINS	YES
NCU08641	AAA+ ATPase, core	99UNCLASSIFIED PROTEINS	

NCU # or Gene I.D.	Known or Predicted Function[1]	FunCat Classification	Predicted ccg[2]
NCU04862	hypothetical protein	99UNCLASSIFIED PROTEINS	
NCU02378	integral membrane protein	99UNCLASSIFIED PROTEINS	
NCU08866	hypothetical protein	99UNCLASSIFIED PROTEINS	
NCU01649	hypothetical protein	99UNCLASSIFIED PROTEINS	YES
NCU01011	hypothetical protein	99UNCLASSIFIED PROTEINS	
NCU04477	hypothetical protein	99UNCLASSIFIED PROTEINS	
NCU01536	predicted G-protein coupled receptor (Metarhizium acridum)	99UNCLASSIFIED PROTEINS	
NCU07254.2	Cytochrome b561 / ferric reductase transmembrane like	99UNCLASSIFIED PROTEINS	YES
NCU07912.2	hypothetical protein	99UNCLASSIFIED PROTEINS	
NCU08168	CyanoVirin-N Homology domains are found in the sugar-binding antiviral protein cyanovirin-N (CVN) as well as filamentous ascomycetes and in the fern Ceratopteris richardii.	99UNCLASSIFIED PROTEINS	YES
NCU06143	conserved hypothetical protein	99UNCLASSIFIED PROTEINS	

NCU # or Gene I.D.	Known or Predicted Function[1]	FunCat Classification	Predicted ccg[2]
NCU00878	hypothetical protein	99UNCLASSIFIED PROTEINS	
NCU01424	Glutathione-dependent formaldehyde-activating, GFA	99UNCLASSIFIED PROTEINS	
NCU00960	hypothetical protein	99UNCLASSIFIED PROTEINS	
NCU03960	hypothetical protein	99UNCLASSIFIED PROTEINS	
NCU10572	short chain oxidoreductase	99UNCLASSIFIED PROTEINS	
NCU09639	hypothetical protein	99UNCLASSIFIED PROTEINS	
NCU09162	conserved hypothetical protein	99UNCLASSIFIED PROTEINS	
NCU07976	hypothetical protein	99UNCLASSIFIED PROTEINS	YES
NCU09401	hypothetical protein	99UNCLASSIFIED PROTEINS	YES
NCU01672	DUF726 domain-containing protein	99UNCLASSIFIED PROTEINS	
NCU05826	putative protein	99UNCLASSIFIED PROTEINS	
NCU00859	putative protein	99UNCLASSIFIED PROTEINS	YES
NCU06147	hypothetical protein	99UNCLASSIFIED PROTEINS	

[1] Known or predicted function/domains identified by MIPS FunCat or NCBI Blast.
[2] Putative ccgs identified in previous microarray studies. See text for details.