PONTIFICIA UNIVERSIDAD CATÓLICA DE CHILE
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TESIS DOCTORAL:

BZIP TRANSCRIPTION FACTORS AND TRANSCRIPTIONAL REGULATORY NETWORKS IN THE NEUROSPORA CIRCADIAN SYSTEM

Por:

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ABBREVIATIONS

ACE	activating clock element
bd	*band*
bHLH	basic helix-loop-helix
bZIP	basic leucine zipper
C-box	clock-box
C-terminus	carboxy-terminus
CAMK-1	calcium/calmodulin dependent protein kinase-1
CCD	charge-coupled device
ccg	clock-controlled gene
cDNA	complementary DNA
ChIP-seq	chromatin immunoprecipitation followed by high-throughput DNA sequencing
CK	casein kinase
DD	constant darkness
DNA	deoxyribonucleic acid
EMSA	electrophoretic mobility shift assay
FFC	FRQ/FRH complex
FLO	FRQ-less oscillator
FRH	FRQ-INTERACTING RNA HELICASE
FRQ	FREQUENCY
FWD-1	F-box WD-40 repeat containing protein-1
FWO	FREQUENCY/WHITE COLLAR COMPLEX oscillator
GO	gene ontology
HOG	high-osmolarity glycerol
IUPAC	International Union of Pure and Applied Chemistry
kb	kilobase
LD	light/dark
LL	constant light
LOV	light oxygen voltage
LUC	luciferase
MAPK	mitogen activated protein kinase
mRNA	messenger ribonucleic acid
N-terminus	amino terminus
ORF	open reading frame
OS	osmotic stress
PAR bZIP	proline and acidic amino acid-rich basic leucine zipper
PBM	protein-binding microarray
PLRE	proximal light regulatory element
PWM	position weight matrix
SDS	sodium dodecyl sulfate
TF	transcription factor
TTFL	transcriptional/translational feedback loop
WC	white collar
WCC	WHITE COLLAR complex
WT	wild type

ABSTRACT

Circadian clocks are endogenous cellular timekeepers that confer daily rhythms to a large number of biological processes. These clocks are present in various organisms across different evolutionary lineages, in which they regulate close to 24-hours rhythms in gene expression, physiology and behavior, enabling individuals to anticipate predictable environmental variations. The ascomycete *Neurospora crassa* has played a key role in the unveiling of the molecular and genetic basis of these time-telling machineries. In Neurospora, as in other eukaryotes, the integration of a series of cellular and molecular processes gives rise to a robust cell-based pacemaker, capable of coordinating rhythmic control of several aspects of their biology. Although a detailed molecular description of the core oscillator or pacemaker is now possible in model eukaryotes, there is limited information on the mechanisms that allow it to regulate rhythmic processes. Such "output pathways", the circuits through which the pacemaker endows different processes with rhythmicity, are the least characterized aspect of circadian systems. In Neurospora, a hierarchical arrangement of transcriptional regulators has been proposed as the main mechanism through which the clock regulates rhythmic gene expression. The different actors involved in such time relay, connecting the oscillator with overt rhythms, are however largely unknown. In addition, despite decades as a research model organism, little is known about transcriptional regulatory networks in this fungus and the vast majority of transcription factors in Neurospora remain uncharacterized. In an effort to improve current knowledge of output pathways, the most neglected aspect of circadian biology, in a clock model system and study transcriptional regulatory networks in a model eukaryote, we set out to characterize the bZIP family of transcriptional regulators in Neurospora, in the

context of its circadian system. We report a complete revision of the list of sequence-specific DNA-binding proteins in this fungus, which resulted in the identification of several novel ones, including many bZIP proteins. As the few transcription factors that have been associated with output pathways in Neurospora have been shown to exhibit clock input, we evaluated whether the expression of bZIP encoding genes in this organism is under control of the circadian clock. By using a luciferase-based, high-throughput screening system, we identified several bZIP encoding genes whose expression is regulated by the circadian pacemaker. A major limitation in the study of transcriptional regulatory networks in Neurospora, such as those underlying clock regulated transcription, stems from the fact that little is known about the sequence preference of its transcription factors. With the goal of identifying and characterizing transcriptional regulatory networks in which the putative Neurospora transcription factors participate, we employed double-stranded DNA microarrays known as protein-binding microarrays, to determine the sequence preference of Neurospora transcription factors. Such an approach allows for rapid, high-throughput and unbiased characterization of the sequence specificity of DNA-binding proteins. This resulted in the determination of the sequence preference of over half of Neurospora predicted transcription factors, information that together with the various molecular tools available in Neurospora, led to the identification of a rhythmically expressed bZIP transcription factor, ADA-1, as a regulator of output pathways in Neurospora, controlling various output genes. In addition, this information allowed for the evaluation of the role of another bZIP transcription factor, ASL-1, in such pathways. Notably, this is the first report aimed at studying DNA-binding specificities on a global scale in the fungal kingdom outside of the yeast clade, representing a powerful resource for the study of transcriptional regulatory networks in filamentous fungi, the largest group

within the fungal kingdom. Indeed, through the use of these data we identified, for the first time, a transcription factor that is required for growth under osmotic stress in Neurospora. Lastly, we report on the identification of a novel process involved in output pathways in Neurospora, namely cell fusion pathways, and we herein show it to be necessary for proper rhythms in a number of genes, including bZIP encoding genes. As a whole, the work reported in this Thesis, represents a major advancement in the study of bZIP proteins and transcriptional regulatory networks in Neurospora.

CHAPTER 1

INTRODUCTION

Rhythms are a part of life and periodicities come in all flavors. Some biological processes take place quite frequently, more than once a day, and are known as ultradian rhythms. The courtship song of a Drosophila male, recurring every minute, and the oscillation of glycolytic intermediates in yeast, with a frequency of several minutes, are examples of such fast cyclical phenomena (Edmunds, 1988). At the other end of the spectrum, we find processes that exhibit very long periods, taking place only once a year. These are known as circannual rhythms. Hibernation cycles, patterns of bird migration and developmental processes such as flowering, exhibit such periodicities (Edmunds, 1988; Gwinner, 1986).

Within this vast range, we observe biological processes that cycle with a period of about a day. These are known as circadian rhythms (derived from the Latin *circa diem*, or "about a day") and underlie a variety of physiological and developmental processes in organisms ranging from cyanobacteria to humans (Bell-Pedersen, et al., 2005; Dunlap, 1999; Dunlap, et al., 2004). These rhythms, an adaptation to the predictable environmental variations brought about by the daily rotation of the Earth, allow organisms to anticipate and respond to such transitions and perform activities at the time of day when they are most advantageous. Indeed, circadian clocks, the molecular timing machineries underlying these oscillations, integrate temporal information and control rhythms in gene expression, biochemical reactions, physiology and behavior in a wide range of organisms and it is generally believed that they confer a selective

advantage. Cumulative evidence obtained from different phyla has supported this notion and highlighted the physiological relevance of circadian clocks (Dodd, et al., 2005; Ouyang, et al., 1998; Paranjpe and Sharma, 2005; Yerushalmi and Green, 2009). Biological clocks are similarly responsible for the correct timing of a variety of physiological processes in humans and misalignment between the endogenous clock and the environment has a variety of clinical consequences (Rey and Reddy, 2013).

These rhythms, a universal feature of living organisms, will be the main focus of the research described in this Thesis.

1.1. Circadian rhythms

Several plant species exhibit daily leaf movements which were thought to be simply a response to the light/dark cycles that accompany the transitions between days and nights. In 1729, French astronomer Jean Jacques d'Ortous de Marian, attempting to test such idea, moved mimosa plants, known to display such leaf movements, to a completely dark basement for several days. Interestingly, he observed that the leaves of these plants continued to display such movements regardless of the absence of external light cues, which led him to conclude that the observed rhythm was not simply a response to the cyclic environment, but an endogenous property of the plant (Sweeney, 1987).

Since this report, credited as the first experiment in chronobiology, other photosynthetic organisms and nearly all life forms have been shown to exhibit endogenous, cell-organized,

daily rhythms in a variety of processes and this field has been the subject of intensive research for the last 60 years (Dunlap, 2008; Mackey, 2007; Pittendrigh, 1993). Circadian clocks coordinate many aspects of biology: asexual spore formation in fungi, eclosion in insects, bioluminescence in dinoflagellates and sleep/wake cycles in humans have all been shown to be under circadian control and research has focused on identifying novel rhythmic processes and characterizing the mechanisms that drive them.

Rhythms must meet three main requirements to be classified as circadian: they must be self-sustained, exhibit entrainment capabilities and also display temperature compensation (Sweeney, 1976), and these traits are explained below. While the circadian clock works in the context of our cyclical world, exposed to constant light/dark and temperature transitions, a rhythm controlled by a truly circadian clock persists in the absence of environmental cues (that is, it is self-sustained), with a period close to 24 h. The period of a particular biological process observed under such constant conditions is known as the free-running period (Figure 1.1). Note that I deliberately mentioned that these rhythms have free running periods *close to*, but not exactly of 24 h. This would not be the case if the rhythm was being driven by particular cue resulting from the 24 hour rotation of the Earth (they would then have a period of 24 h). This, together with the fact that such periods can be altered via gene mutation (Feldman, 1982) and that period length can be inherited (reviewed in (Somers, 1999), clearly established the endogenous and genetically-based nature of these rhythms, although controversy would continue for a while. Indeed, for some time, it was still a subject of discussion whether circadian rhythms were genetically encoded and cell-autonomous or were actually dependant on external factors, such as environmental influences connected somehow

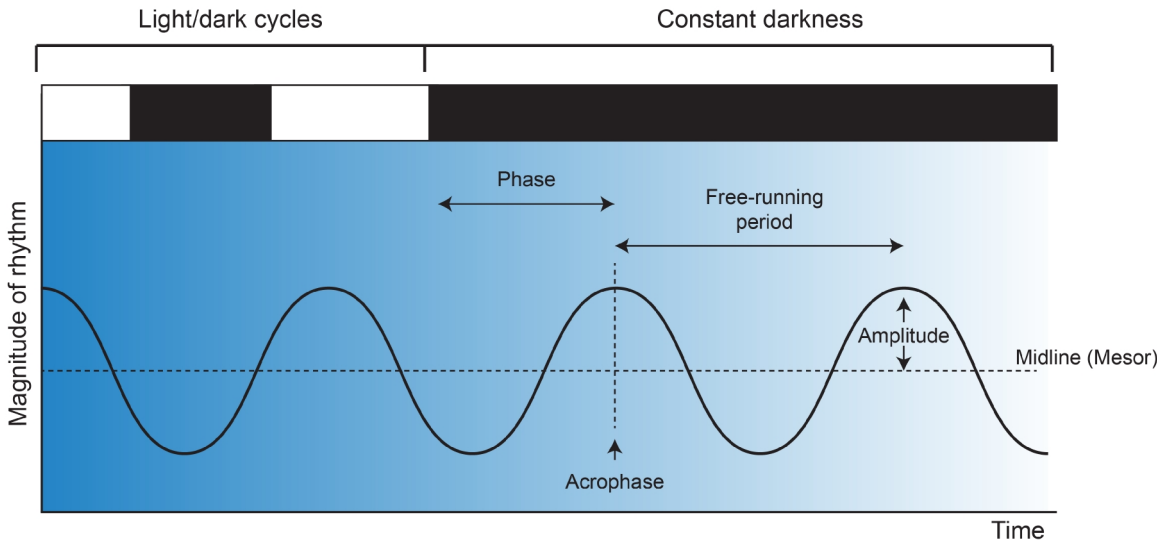

Figure 1.1. Parameters of an oscillatory process. A diagram of a rhythmic process is shown, highlighting typical parameters assessed in circadian biology. In this figure, a rhythm taking place under natural conditions (i.e. light/dark cycles) is then assessed under constant conditions, to evaluate its circadian nature. In this example, such constant conditions refer to constant darkness. Such a regular oscillatory process can be characterized by its midline or mesor (mean level), its amplitude (absolute magnitude between the mesor and the peak or trough of the oscillation), its period (i.e., the duration of a complete cycle), and its phase, which is often reported as the position (in time) of a peak of the wave, called the acrophase, in relation to an external reference point. In this example, the phase of the oscillation is the time at which the first peak occurs, following the start of constant dark conditions.

to the rotation of the Earth or gravitational forces, among others. In an effort to elucidate these issues, the clock-regulated process of spore formation in the filamentous fungus *Neurospora crassa*, one of the most intensively studied clock model systems, was monitored at the South Pole, under constant conditions, on a table with a daily rotation in the direction opposite to that of the Earth's, with no major impact on the rhythm (Hamner, et al., 1962). The idea behind this experiment was to keep tests subjects in a constant position with respect to the stars. Rhythms in other organisms were also tested under these conditions, with similar results. Further, and perhaps more strikingly, this same rhythm in fungal development was monitored in constant darkness during spaceflight. Under these conditions, in the absence of any terrestrial influence, the endogenous nature of circadian rhythms was revealed (Sulzman, et al., 1984).

For the clock to be useful, it should be able to synchronize with environmental cues, so that it can match local time and coordinate processes to take place at the appropriate moments in the context of a day. A clock not only needs to be able to track the amount of time that has passed, but also know exactly what time it is within the day. A system lacking the latter, would only work as an hourglass. Circadian clocks are endogenous, but they truly work as clocks because they can be set, or *entrained*, by external signals, such as temperature and light cues. As circadian clocks do not have exactly 24 h periods, this allows them to work in sync with the 24 h environment, maintaining phase. This allows the clock to know exactly what time of day it is (dawn, midday, dusk, etc.) and rhythmically coordinate different aspects of biology, accordingly.

A reliable clock should operate properly, regardless of environmental temperature. Together with their close-to-one-day cycle length and entrainment abilities, another defining characteristic of rhythms driven by a circadian clock is that they exhibit what is known as "temperature compensation", meaning that the period length is relatively insensitive to ambient temperature changes within a physiological range. If the clock were to run faster at higher temperatures and slower at lower ones (and indeed, the rate of most metabolic processes follow this paradigm), it would just behave as a thermometer, which would not be useful for a clock that must run precisely throughout the day. Indeed, period length shows a Q_{10} (a measure which represents the ratio of the rate of a reaction at temperatures 10 °C apart) of ~ 1, a fascinating, yet still largely mysterious feature of biological clocks. "Nutritional compensation", such that period length is largely unaffected when assayed under different growth media, has also been reported.

Other biological rhythms, including those with long or really short period lengths (compared to one day), those whose period is strongly dependent on temperature and those observed only under cycling conditions, are thus not considered circadian rhythms. This distinction kept research focused on a single mechanism and biological phenomena, allowing the field to advance in the characterization of the nature of these particular rhythms (Dunlap, 2008).

1.2. Circadian clock mechanisms

As mentioned previously, circadian rhythms are widely distributed and they have been studied in a broad range of model organisms. Interestingly, the overall organization and molecular

design underlying the workings of the clock in eukaryotes is conserved (Bell-Pedersen, et al., 2005; Mackey, 2007), even though the genes and proteins involved in the mechanism itself, appear to be unrelated.

Circadian systems are conceptually composed of an oscillator or pacemaker and two signaling pathways: input pathways convey external signals to the oscillator, to allow its synchronization with the environment, and output pathways allow it to temporally regulate diverse cellular processes.

At the core of each circadian system, lays a cell-autonomous and entrainable oscillator composed of "clock genes", those that encode for integral and essential components of the time-keeping mechanism. Such genes were first identified through genetic screens in the 1970s. Mutations in the clock genes *period* (*per*) in the fruit fly *Drosophila melanogaster* (Konopka and Benzer, 1971) and *frequency* (*frq*) in Neurospora (Feldman and Hoyle, 1973), resulted in altered clock properties, including increases or decreases in period length of the rhythms that were assayed at the time in these organisms (eclosion and asexual spore formation, respectively), and even rhythm elimination.

Interestingly, almost 20 years later, these clock genes, or core clock components, were cloned and soon after shown to display daily rhythms in mRNA and protein levels, under both cyclical and constant conditions, with a significant delay between their peaks. In Drosophila, it was shown that missense mutations in the *period* protein could altered *per* mRNA cycling (Hardin, et al., 1990) and that *per* rhythmic mRNA regulation was mainly transcriptional

(Hardin, et al., 1992). This led to the proposal that there was an autoregulatory feedback loop underlying *per* expression and that this was crucial for clock function. Such a feedback loop was soon after explicitly demonstrated in Neurospora, where it was shown that the protein product of the *frq* gene is somehow necessary for *frq* mRNA rhythms and that it negatively regulates the amount of its own transcript, which results in the daily oscillation in *frq* mRNA levels that are necessary for proper clock function in this fungus (Aronson, et al., 1994). This placed *frq* as a key component of the circadian oscillator in Neurospora, just as *per* is for the Drosophila system. Altogether, this evidence suggested that transcriptional/translational feedback loops could indeed be the molecular basis of the observed rhythms.

Theoretical work had suggested that for such a feedback loop to work, positive elements activating the expression of these negatively-acting clock genes, would be required (Goldbeter, 1996). Indeed, transcription factors (TFs) necessary for the activation of such clock genes were later found in Neurospora, mice and Drosophila (Allada, et al., 1998; Crosthwaite, et al., 1997; Darlington, et al., 1998; Gekakis, et al., 1998; Rutila, et al., 1998) and evidence for an inhibitory effect of the clock genes over these positive elements was first reported in the fruit fly (Darlington, et al., 1998). All this evidence helped strengthen the model of autoregulatory negative feedback loops at the core of circadian pacemakers.

These and subsequent work in model systems over the last 20 years, had led to the most generalized model for pacemaker function in eukaryotes, one that is much more complex than the aforementioned early work suggested: the coupled transcriptional/translational feedback

loops (TTFL) model (Figure 1.2) (Brown, et al., 2012; Dunlap, 1999; Mackey, 2007; Zhang and Kay, 2010).

This model posits that an interplay between positive and negative elements form a time-delayed negative feedback loop, which runs at a pace of ~ 24 h per cycle. Positive elements would activate the expression of the negative elements (e.g. *per* and *frq*), which would then build up and after a lag, inhibit the activity of the positive elements, resulting in reduced levels of the negative elements. Ultimately, the negative elements would be degraded, relieving inhibition over the positive elements, allowing the cycle to start anew. This was the originally proposed single transcription–translation oscillator loop (Dunlap, 1999), which would then be expanded to include post-transcriptional and post-translational regulation over core clock components, now known to be crucial for proper clock function (Brown, et al., 2012; Kojima, et al., 2011; Lim and Allada, 2013; Pegoraro and Tauber, 2011). In addition, novel loops were found that are essential for clock robustness (Bell-Pedersen, et al., 2005; Young and Kay, 2001).

These loops, composed of newly identified clock proteins, are intimately intertwined with the ones involving the originally identified core clock components and have led to the current model of "interlocked loops" (Figure 1.2). This remains as the most common and experimentally supported model for the architecture and functioning of eukaryotic circadian pacemakers and is able to account successfully for a large body of experimental evidence, notwithstanding that it has recently been suggested that the actual time-keeping mechanism

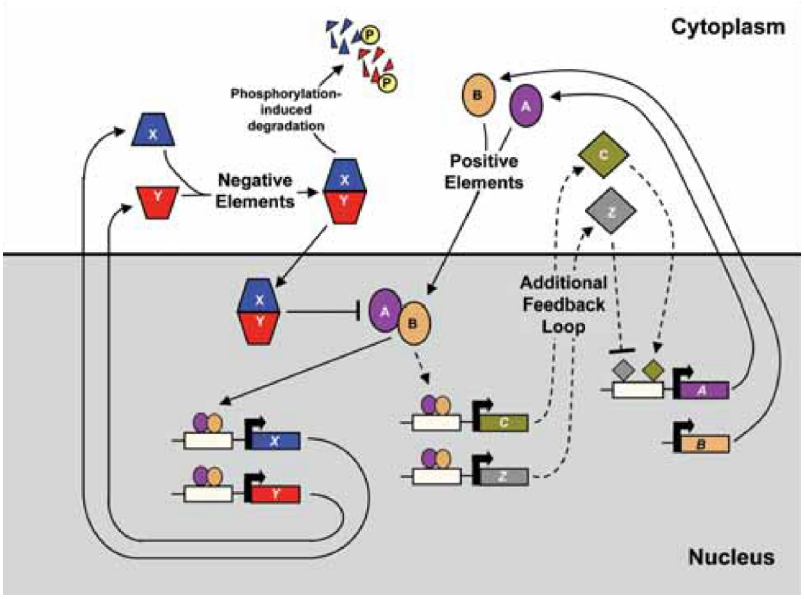

Figure 1.2. The coupled transcriptional/translational feedback loops (TTFL) model. The most common model for the architecture and functioning of eukaryotic circadian pacemakers, involves a molecular feedback loop in which positive elements, working usually as heterodimers (purple and orange ovals), activate the expression of negative elements (red and blue tetrahedrons), which ultimately inhibit the activity of the positive elements, thus negatively regulating their own expression. Progressive phosphorylation of the negative elements throughout the day, eventually results in their targeting by the ubiquitin/proteasome pathway and their degradation, which relieves their inhibitory action over the positive elements, which then allows the cycle to start over. Coupled to this loop, there are additional loops that are important for the robustness of the system, where additional components may be present, that regulate the expression of the positive elements. Figure is from Mackey, 2007.

may be metabolic, rather than transcriptional, in nature (Edgar, et al., 2012; O'Neill and Reddy, 2011; O'Neill, et al., 2011).

Such molecular design underlying pacemaker function has been shown to be conserved among eukaryotes (Bell-Pedersen, et al., 2005), which has made working in model systems a powerful way to dissect the gears and cogs of circadian clocks.

1.3. Neurospora as a research system

The filamentous fungus *Neurospora crassa* (herein referred to as Neurospora) has been studied for decades and is undoubtedly, the best understood filamentous fungus.

The first historical report of Neurospora dates back to 1843, when it was reported as a contaminant of French bakeries, producing bright orange asexual spores (or conidia, see below). This fungus, colloquially described as the “bread mold”, was known as *Monilia sitophila*. Several years later, in the mid-1920s, the modern history of this fungus started, with the work of Bernard O. Dodge, who discovered sexual structures in this fungus and placed it on a new genus: Neurospora. Dodge, at the New York Botanical Garden, worked out the basic genetics of the organism and later persuaded T.H. Morgan, the father of Drosophila genetics (then at Columbia University), to take some Neurospora stocks to the new Division of Biology at the California Institute of Technology (Caltech) and test them, arguing that Neurospora was a more favorable organism for genetic studies than was Drosophila. Soon thereafter, Carl C.

Lindgren, then a graduate student, took over the Neurospora work at Caltech (Beadle, 1966; Tatum, 1961).

This initial work (1930-1940s) confirmed Neurospora as an appropriate model organism for genetic studies: it grows mainly as a haploid (which eases the identification of recessive, loss-of-function mutations) and exhibits orthodox genetics, easy culturing, simple growth requirement and susceptibility to mutagenesis. Soon after, the potential of Neurospora for biochemical studies was realized. The subsequent landmark work by George W. Beadle and Edward Tatum, on the isolation of metabolic mutants in Neurospora (Beadle and Tatum, 1941) and following reports, opened the door to the development of the field of biochemical genetics and molecular biology and pioneered the use of microorganisms for such studies (Brambl, 2009; Horowitz, 1991). Altogether, these studies fully established this fungus as a model organism for biochemical and genetics research and is currently a prime research subject for a variety of biological processes (Davis and Perkins, 2002). Neurospora has a variety of molecular tools available and an extensive mutant (Perkins, et al., 2001) and knockout collection (available from the Fungal Genetics Stock Center) (McCluskey, et al., 2010), along with several regulatable promoters and selectable markers. Ultimately, this has positioned Neurospora as a reference organism within the filamentous fungi, although it continues to be a source of new discoveries not only applicable to this group of organisms (Loros and Dunlap, 2006).

1.4. Neurospora biology

Neurospora is a filamentous fungus (Phylum *Ascomycota*) widely distributed in nature (Davis, 2000; Turner, et al., 2001). Interestingly, it is one of the first colonists in areas of burnt-over vegetation, which reflects both the heat requirement that ascospores, the products of sexual reproduction (see below), have for germination (an observation made by Dodge) and the fact that Neurospora can readily use cellulose as a carbon source.

In their natural habit, the products of a sexual cross, the ascospores, are usually activated by the heat from fires. This allows the ascospores to germinate. Neurospora then spends most of its life growing vegetatively as a multinucleate, haploid mycelium composed of filaments (hyphae) with incompletely separated cells, which allows free flow of cytoplasmic components between them (Figure 1.3).

Upon certain environmental cues (e.g. desiccation, carbon source deprivation), a complex developmental program ensues, which leads to the differentiation of the surface mycelia to aerial hyphae, which then, through budding and segmentation, results in the production of asexual spores, or conidia. Conidia are usually multinucleate (usually 2-3 nuclei per conidia) and hydrophobic. As conidia are usually held together by fragile connective threads, they are ideally suited for wind dispersal and are in fact, the main mean of dispersal of Neurospora. Once conidia have found a suitable growth substrate, they germinate and start the vegetative cycle anew. This asexual cycle is known as macroconidiation (Figure 1.3). As mentioned

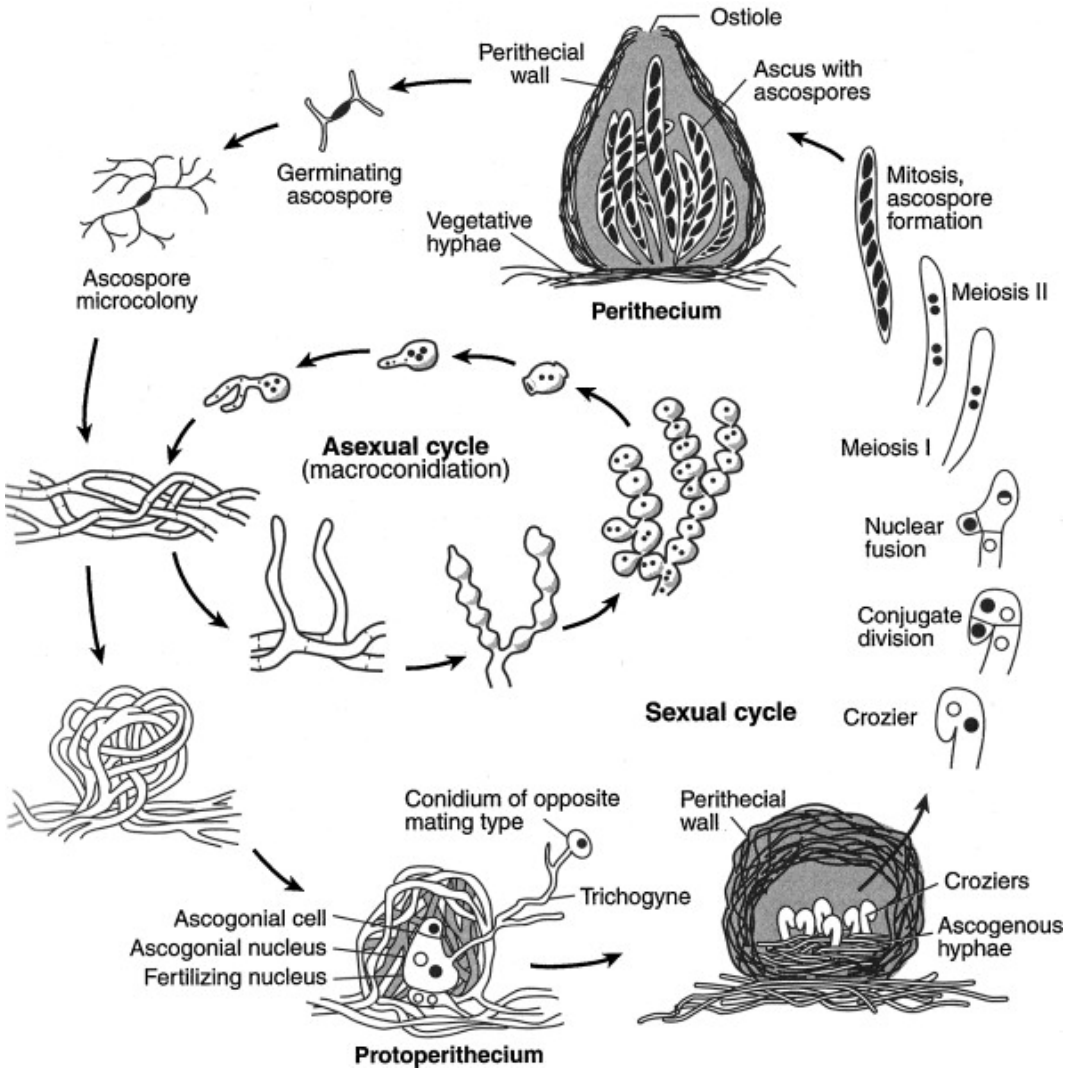

Figure 1.3. The Neurospora life cycle. The asexual cycle (inner circle) starts with the germination of asexual spores (conidia) and their development into a mycelium composed of multinucleate hyphae, with incomplete cell walls. Upon certain environmental cues, aerial hyphae develops from the mycelium and results in the formation of multinucleate conidia through branching, budding and segmentation. The sexual cycle (outer circle) starts with the development of a protoperithecium under particular growth conditions, which contains the ascogonial cell. A trichogyne emerges from the ascogonial cell and contacts a conidia or other fertilizing agent from the opposite mating type. A nucleus from such agent is transported through the trichogyne to the ascogonial cell. Both haploid nuclei undergo several mitotic duplications in a developing mass of ascogenous hyphae, which is accompanied by growth of the perithecial wall. Croziers then develop at hyphal tips and nuclear fusion occurs. Meiosis, followed by mitosis then take place within a developing specialized cell called ascus. The resulting eight nuclei, when enclosed by rigged cell walls, are called ascospores and are ultimately shot through the perithecium ostiole into the environment. Figure is reprinted from Davis (2000). See text for details.

previously, this developmental switch is also under control of the circadian clock in this fungus.

Although Neurospora is typically found growing vegetatively through this asexual and haploid cycle, it also has a short-lived diploid stage, resulting from a sexual cross, which is immediately followed by meiosis and the production of ascospores. Neurospora is a heterothallic species, meaning that it requires the parents (two different individuals) to be of two different mating types. Individuals with the same mating type cannot mate.

When nutrients become scarce, particularly under conditions of reduced nitrogen and carbon, vegetative Neurospora of either mating type, is able to enter the sexual cycle program by forming a multicellular structure known as protoperithecium (composed of interwoven hyphae), which hosts the female gamete, the ascogonial cell (Figure 1.3). A specialized structure known as trichogyne, an extension of the ascogonial cell, emerges through the protoperithecium and responds to pheromone signals emitted by conidia (or even mycelia) from the opposite mating type. The trichogyne grows towards the conidia until cell fusion occurs, which is then followed by transportation of a haploid nucleus from the conidia to the ascogonial cell. The haploid nuclei then undergo several rounds of replication in a developing mass of *ascogenous hyphae*, which exists within an enlarging perithecium. At the tips of these hyphae, a structure known as *crozier* develops, where nuclei of opposite mating types pair up and undergo simultaneous divisions. In a cell enclosing two of these nuclei of opposite mating types (known as *ascus initial*), nuclear fusion takes place, completing the fertilization process. This results in the only (and transient) diploid stage of the Neurospora life cycle. The diploid

nucleus then immediately undergoes meiosis and the resulting 4 nuclei undergo an additional mitotic division, resulting in 8 nuclei. As these meiotic and mitotic divisions take place, the ascus initial becomes the ascus. An individual mature perithecium can host hundreds of asci (plural of ascus), each derived from a single nuclear fusion event. When asci are mature (~ 2 weeks), ascospores are shot into the environment through an opening in the perithecium known as ostiole. Ascospores require a further 5-7 day maturation stage, after which they can be heat-activated for their germination. Each individual ascospore is covered by a hard rigged spore wall with nerve-like ornamentations (striations), which give the genus its name (Neurospora).

1.5. Neurospora as a model system for circadian research

A very robust and visually attractive phenotype observable in Neurospora is the easily assayable daily rhythm in asexual spore production. This physically evident manifestation of the workings of the internal clock was the main reason Neurospora entered the world of circadian research. It was the ease with which circadian conidiation could be visualized and genetically tracked in this fungus, that allowed its positioning at the forefront of circadian studies.

Classical genetic analysis in Neurospora, together with the isolation of mutants with altered circadian properties, helped define the genetic basis of these rhythms (as described earlier), while access to its genome sequence (Borkovich, et al., 2004; Galagan, et al., 2003) and the consolidation of a community-driven Functional Genomics program (Dunlap, et al., 2007),

have contributed to the advancement of the molecular dissection of the Neurospora circadian system (Baker, et al., 2012; Montenegro-Montero and Larrondo, 2013).

As mentioned in the previous section, Neurospora is normally found in its vegetative state, growing as a mycelium composed of haploid multinucleated hyphae. Upon certain cues, a developmental switch is triggered and particular sets of genes change their expression patterns leading to the formation of aerial hyphae, followed by segmentation into asexual spores (conidia) (Springer, 1993). This developmental process has been found to exhibit a daily cycle and to display all the previously discussed characteristics of a truly circadian rhythm in the laboratory, that is, sustained ~24 h period in constant conditions (i.e. it is not just a mere response to an environmental variable, but an endogenously-controlled process), it can be synchronized or entrained by cyclical temperature or light signals and it exhibits temperature and nutritional compensation (Dunlap and Loros, 2005).

Traditionally, this developmental rhythm has been studied in the laboratory, in the context of circadian rhythms, using the "Race Tube" assay, in which a hollow glass tube containing a layer of agar media is inoculated with Neurospora at one end (Figure 1.4A). Initial growth is achieved under constant light, after which the race tubes are transferred to the dark, synchronizing the clock of the growing hyphae to "subjective" dusk, as it reproduces the situation of the sun setting down. Under these conditions of constant darkness (DD) and constant temperature (normally 25 °C), the hyphae will grow ("race") down the tube and at a specific time of day, determined by the circadian clock, the mycelia will differentiate into aerial hyphae and conidia. Indeed, it is at the late subjective night (~12 h after subjective

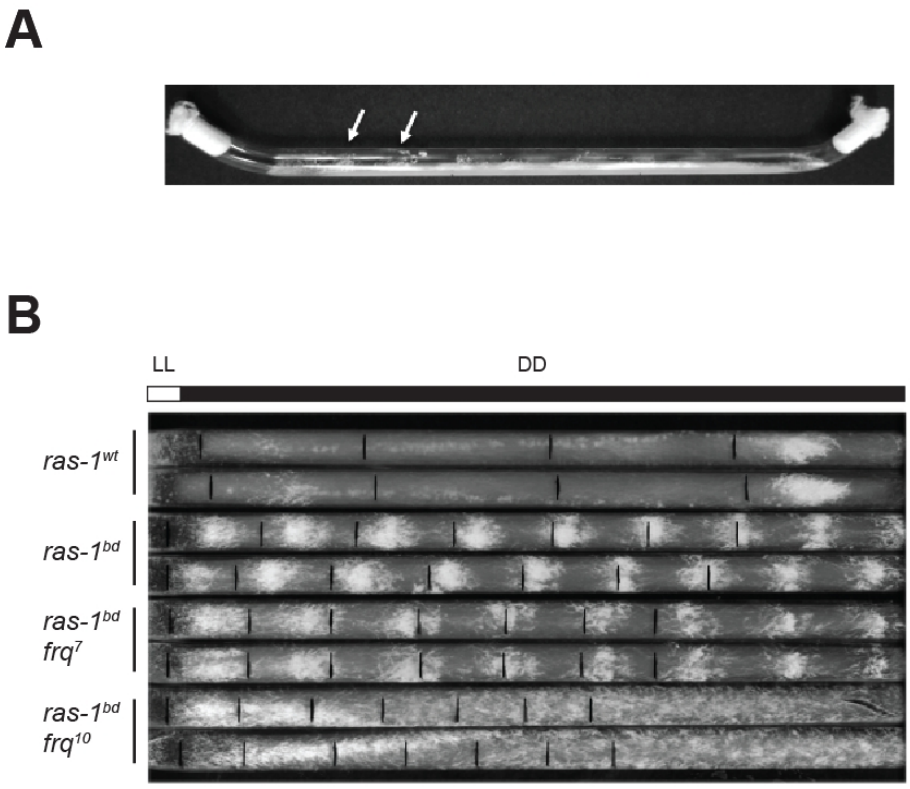

Figure 1.4. The Race Tube assay reveals circadian rhythms in conidiation under laboratory conditions. A) Lateral photograph of a race tube, a hollow glass tube containing solid media in which Neurospora is analyzed as it grows linearly exhibiting periodic conidiation (indicated by the arrows). B) To examine the circadian characteristics of a strain, race tubes are grown at constant temperature (generally 25 °C) first in the presence of light (LL) and then transferred to constant darkness (DD). Under safe red lights (which do not affect the clock), the position of the growth front is marked every 24 hours and the period of the strain is calculated by the position of the conidial bands relative to the marks. In these assays, high levels of CO_2 accumulate in the race tubes, which inhibit conidiation in the WT, so that rhythmic banding cannot be observed under these conditions. Aeration of the culture, thus allows banding to be observed in a WT strain (not shown). A strain harboring the *ras-1*^{bd} mutation however, overcomes this limitation and allows for the clear rhythm in conidiation to be observed without aeration (*bd* refers to the allele of the *ras-1* gene, see text). This *ras-1*^{bd} (or simply *bd*) mutation has since then been adopted as part of the "WT" genotype of strains used in chronobiology studies in Neurospora (see text). Mutations in the core clock component *frequency* can change the period of asexual spore formation, yielding, for example, strains with periods close to 30 h (*frq*^{7}) or totally arrhythmic strains (*frq*^{10}). Figure is from Montenegro-Montero and Larrondo (2013).

dusk) that tissue at the growth front progresses into aerial hyphae and conidia, producing what looks like a puff or "band" of asexual spores in the race tube.

In order to study these rhythms, the position of the growth front is marked every 24 hours under safe-red lights, which do not affect the clock. After 5-7 days, when the cultures have reached the other end of the race tube, the periodicity of conidiation can be determined by evaluating the position of the conidial bands relative to the 24-h reference marks, as shown in Figure 1.4B. Normally, the race tube is scanned and the image subjected to densitometry analysis to retrieve key circadian parameters, such as period and phase (Roenneberg and Taylor, 2000). In Neurospora, the free-running period of conidiation is of ~22 h, with the appearance of the first band (referred to as "phase" of conidiation) generally occurring 11-12 hours after the initial light/dark transition, as previously mentioned (Dunlap and Loros, 2005).

The daily appearance of conidial bands, the first ever evidence of a rhythm in Neurospora, was originally reported in the early 50s (Brandt, 1953) and latter shown to display all circadian properties (Pittendrigh, et al., 1959). During the early 60s, several strains were isolated that helped to better visualize rhythms in this fungus, but it wasn't until the isolation of a particular strain named *band* (*bd*), that Neurospora started to be widely used for circadian studies (Sargent, et al., 1966), since for one part, the conidiation rhythms in this strain were stronger and more pronounced than the ones exhibited by other mutants and additionally, the rhythms in some of the other mutants identified, like *wrist watch* and *clock* (Sussman, et al., 1965) ultimately proved not to be truly circadian. The *bd* mutation has since then been adopted as part of the "WT" genotype of strains used in chronobiology studies in Neurospora. The

mapping and cloning of *bd* revealed a single point mutation in the *ras-1* gene, implicating Ras signaling in circadian output (Belden, et al., 2007). It is worth mentioning that this mutation is not necessary to generate the rhythms, but it enables a clear visualization of the clock-regulated process of spore formation by amplifying a signal from the oscillator to the pathways controlling the conidiation process (Belden, et al., 2007) (Figure 1.4B).

As described in previous sections, important work conducted during the 70s and early 80s, led to the isolation of several *N. crassa* strains displaying altered circadian rhythmicity. Analysis of some of these different mutants with altered clock properties (Feldman, 1982; Gardner and Feldman, 1980; Loros, et al., 1986), particularly those mapping to a particular locus, *frequency*, ultimately led to the heart of the circadian pacemaker and showed that a single gene could control many of the key circadian properties in Neurospora. The cloning of *frq* (McClung, et al., 1989) started off the molecular characterization of the Neurospora circadian machinery, including the study of feedback loops, contributing with findings that later on proved critical for the understanding and further description of the characteristics of mammalian and insect clocks (reviewed in Dunlap, 2008). Moreover, several of the mutants originally isolated during that time, have been essential in forward genetic strategies that have helped understand important aspects of circadian clocks, such as temperature compensation (Mehra, et al., 2009) or the connections between circadian and cell cycles (Pregueiro, et al., 2006).

More recently, the use of molecular reporters such as luciferase (Gooch, et al., 2008; Larrondo, et al., 2012; Morgan, et al., 2003), has helped in moving beyond the phenotypic

assay of rhythmic conidial formation for the study of circadian rhythms in Neurospora, in order to approach this problem in a quantitative and high-throughput manner, as it has been done in other clock model systems (Michael and McClung, 2003; Millar, et al., 1995; Stempfl, et al., 2002; Zhang, et al., 2009)

1.6. The Neurospora FRQ/WCC oscillator, a brief overview

The current molecular description of the best studied Neurospora circadian oscillator, the FRQ/WCC oscillator (FWO) has been put together during the last two decades (Figure 1.5A). Briefly, this oscillator is based on a transcriptional/translational negative feedback loop (TTFL), in which a heterodimeric transcriptional complex, known as the White Collar Complex (the positive element in the loop (Crosthwaite, et al., 1997)), composed of the White Collar 1 (WC-1) and White Collar 2 (WC-2) GATA-type transcription factors, controls the expression of *frq* (a negative element). The resulting protein, FRQ, acts as a kinase-recruiting platform, which leads to the inhibition of its own expression through interaction with WCC and subsequent WCC-inactivating phosphorylation events (see below). Importantly, besides its key role in the Neurospora central oscillator, WC-1 (a flavin containing protein) also acts as a blue light photoreceptor, directly activating the expression of over a hundred genes upon light exposure, including *frq* (Chen, et al., 2009; Froehlich, et al., 2002; He, et al., 2002; Smith, et al., 2010).

How does this feedback loop work? At the late subjective night, the WCC directly activates *frq* transcription by binding to a specific sequence in the *frq* promoter known as the Clock-box

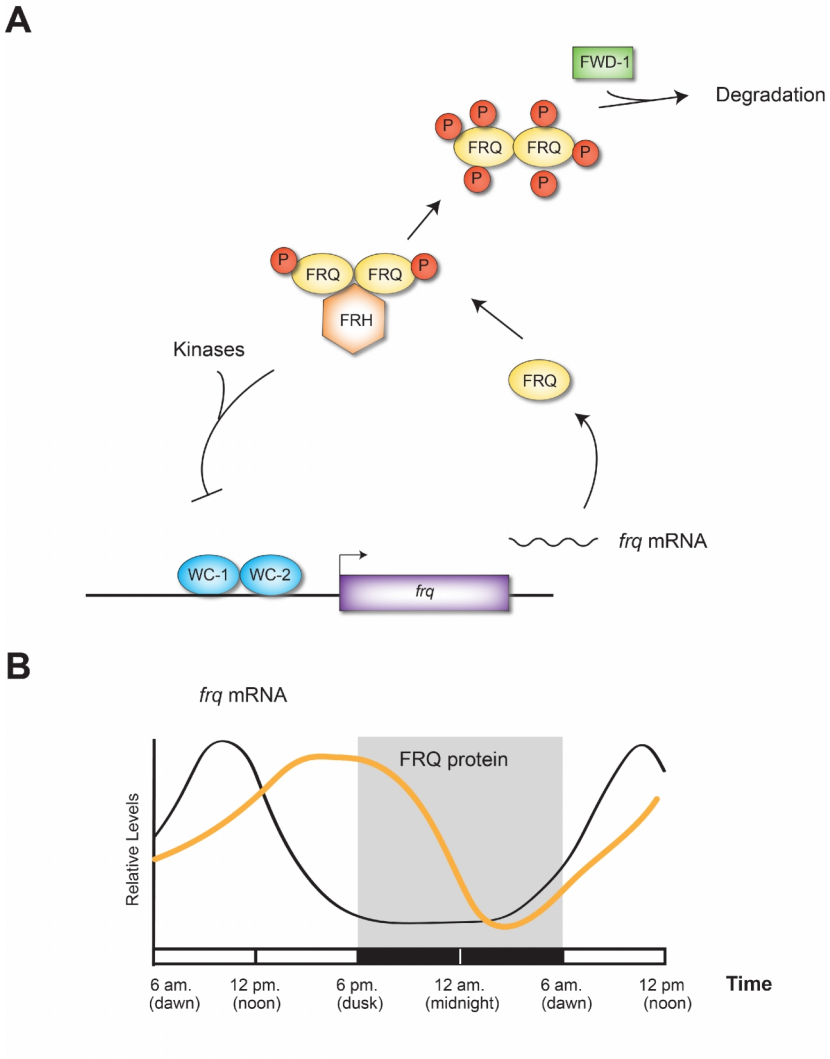
A
FWD-1
Degradation
P
FRQ
FRQ
P
FRH
Kinases
FRQ
frq mRNA
WC-1
WC-2
frq
B
frq mRNA
FRQ protein
Relative Levels
6 am. (dawn)
12 pm. (noon)
6 pm. (dusk)
12 am. (midnight)
6 am. (dawn)
12 pm (noon)
Time

Figure 1.5. Simplified representation of the workings of the FRQ/WCC oscillator (FWO). A) The FWO is composed of positive and negative elements that interact in a transcriptional-translational negative feedback loop (TTFL), generating oscillations in *frq* mRNA and protein levels. At the late subjective night, the White Collar Complex (WCC), composed of the GATA-type transcription factors WC-1 and WC-2, binds the *frq* promoter (at the Clock Box) promoting *frq* transcription. As soon as it is synthesized, FRQ is phosphorylated (P), dimerizes and associates with FRQ-interacting RNA helicase, FRH. This complex, termed FFC (FRQ-FRH complex), interacts with the WCC while serving as a platform for a number of kinases, which then phosphorylate the WCC, decreasing its affinity for DNA binding and consequently shutting down *frq* expression. By the early subjective night, the WCC is inactivated and *frq* transcription is minimal. At the same time, FRQ is becoming highly phosphorylated, which affects its ability to interact with the WCC. Additionally, such high levels of FRQ phosphorylation promote its interaction with the ubiqutin ligase FWD-1, which results in its degradation via the proteasome by the late subjective night. Altogether, this then relieves inhibition over the WCC. The WCC recovers its activity via phosphatases and together with newly synthesized WCC, can once again activate expression from the *frq* locus, in a cycle that under constant conditions occurs every ~ 22 h. Several other proteins, not explicitly shown in the figure, play important roles in this process, such as Casein Kinase I, Casein Kinase II and several phosphatases. Additionally, nuclear and cytoplasmic distribution of WCC and FRQ is important for the proper functioning of the oscillator, which is not shown for clarity (see text). In addition to this loop, additional loops exist, which regulate the levels of the WCC proteins (not shown) and are relevant for clock robustness. B) A diagram depicting the daily oscillations in *frq* mRNA (black line) and protein levels (yellow line). For further details, see Montenegro-Montero and Larrondo (2013).

(*C-box*). This *C-box cis*-element is both necessary and sufficient for *frq* rhythmic expression (Froehlich, et al., 2003). This up-regulation of *frq* expression then leads to the accumulation of *frq* message and FRQ synthesis. As soon as this protein is made, it is phosphorylated and it homodimerizes (Cheng, et al., 2001a) and it physically interacts with FRQ-interacting RNA helicase (FRH), a homolog of the yeast RNA-binding protein Dob1p/Mtr4p (Baker, et al., 2009; Cheng, et al., 2005). Interestingly, down-regulation of FRH completely abolishes circadian rhythmicity (Cheng, et al., 2005). FRH is quite abundant in the cell and not all FRH associates with FRQ, while on the other hand, all FRQ appears to be associated with FRH (Baker, et al., 2009; Cheng, et al., 2005). This protein complex (termed FFC, for *F*RQ-*F*RH *C*omplex, the full negative element of the loop), then enters the nucleus where it can interact with the WCC and promote its phosphorylation by several of its associated kinases, which leads to its subsequent inactivation and clearance from the nucleus (He, et al., 2006; Hong, et al., 2008; Schafmeier, et al., 2008; Schafmeier, et al., 2005). Throughout the day, FRQ is subjected to extensive and ordered phosphorylation events, modifications that are essential to its role in the system (Baker, et al., 2009; Tang, et al., 2009). *In vivo* data, obtained by the use of heavy isotope labeling followed by tandem mass spectrometry, has confirmed phosphorylation events in at least 85 serine/threonine residues, representing ~ 10% of FRQ total residues (Baker et al., 2009). Just before the late subjective night, FRQ reaches its highest level of phosphorylation, which inhibits its ability to interact with the WCC and gets targeted for degradation by the ubiquitin-proteasome pathway (He and Liu, 2005). Reaching such phosphorylation levels as a pre-requisite for the next stage in the cycle (which is, release of FRQ-mediate repression of WCC activity), provides the necessary delay needed for the feedback to last ~ 24 h (Merrow, et al., 1997; Schafmeier, et al., 2006). By the late subjective

night, and in the absence of FRQ, the WCC can once again promote *frq* expression. As a result, *frq* message and protein levels oscillate daily (Figure 1.5B), which can be phenotypically correlated with the rhythmic appearance of asexual spores (Figure 1.4). Several kinases have been implicated in phosphorylating FRQ, including CK-1a, CAMK-1, CK2 and PKA (Baker, et al., 2012; Heintzen and Liu, 2007) and some of these kinases play relevant roles in the circadian systems of other organisms (Gallego and Virshup, 2007; Mizoguchi, et al., 2006).

In addition to the negative feedback loop described above, FRQ can also act in a positive feedback loop, regulating the levels of the WCC proteins, promoting the accumulation of WC-1 through post-translational regulation, and activating *wc-2* expression (Cheng, et al., 2001b; Lee, et al., 2000). This describes an additional loop, interconnected with the one outlined above. In addition to this one, there is another interlocked loop, in which the WC proteins regulate their own expression (Cheng, et al., 2001b; Cheng, et al., 2003). Such interlocked loops are crucial for clock robustness and stability.

It should be noted that the *C-box* is not the only sequence that can be recognized by the WCC in the *frq* promoter. Upon light stimulation, the WCC binds a sequence that is closer to the transcriptional start site called Proximal Light Regulatory Element (PLRE) (Froehlich, et al., 2002). Acute light induction of *frq* expression, which relies on the PLRE (and partially on the *C-box*, which also modestly contributes to this response), allows for entrainment of the clock to environmental time (Froehlich et al., 2002).

In Neurospora, the use of luciferase as a real-time reporter has provided new insights regarding *frq* expression. This tool was adopted in Neurospora rather recently (Morgan et al., 2003) compared to other clock model systems (Brandes, et al., 1996; Geusz, et al., 1997; Kondo, et al., 1993; Millar, et al., 1992), and it was first used to study transcriptional dynamics of a highly expressed output gene. It soon became necessary to conduct a complete codon optimization of the firefly luciferase gene in order to obtain strong signals, such that genes expressed at lower levels (like *frq*), could be efficiently studied (Gooch et al., 2008). By placing this synthetic *luciferase* gene sequence downstream of the *frq* promoter, it is now possible to monitor a core clock component in real time, obtaining high temporal resolution of *frq* dynamics (Cesbron, et al., 2013; Gooch, et al., 2008). This allows for the examination of molecular rhythms under race tube conditions (so that phenotypic and molecular rhythms can be studied separately) (Shi, et al., 2007), or for the evaluation of multiple strains at a time, by adopting a 96-well format (Montenegro-Montero and Larrondo, 2013). Additionally, as it is now trivial to manipulate the Neurospora genome in a locus-specific fashion (Larrondo, et al., 2009), translational fusions to luciferase can be generated for any protein at its endogenous locus, to monitor protein daily dynamics. This was recently done for the *frq* locus and the resulting strain, expressing FRQ-LUC as the only source of FRQ in the cell, not only displays robust conidiation rhythms (suggesting that the fusion resulted in a functional FRQ protein), but also exhibits strong oscillations in bioluminescence, recapitulating the normal ups and downs in FRQ levels (Figure 1.5B) (Larrondo, et al., 2012). This same tagging strategy can be used to study output pathway components, such as transcription factors or rhythmic proteins of unknown function.

For a more comprehensive explanation of the workings of the central oscillator, the reader is referred to detailed recent reviews (Baker, et al., 2012; Diernfellner and Schafmeier, 2011; Guo and Liu, 2010; Heintzen and Liu, 2007; Lakin-Thomas, et al., 2011; Montenegro-Montero and Larrondo, 2013).

As mentioned previously, the circadian system is conceptually composed of an oscillator or pacemaker and both input and output pathways. A detailed description of input pathways, those that convey external signals to the oscillator to allow its synchronization with the environment, is beyond the scope of this Thesis and the reader is referred to comprehensive reviews (Chen, et al., 2010; Heintzen and Liu, 2007). Output pathways, on the other hand, are the main focus of Chapter 2 and will be discussed next.

1.7. Circadian output pathways in Neurospora

In the previous section, we discussed the molecular gears and cogs that constitute the Neurospora central clock, highlighting the striking overall similarity at the organizational level between the central oscillators in different clock model organisms and the underlying transcriptional/translational feedback loops. Despite the undeniable importance of understanding the molecular basis of the circadian machinery (and in fact, this is what most research efforts have historically focused on), it is the wide array of processes that are under clock control (and their regulation) that ultimately impact the biology of organisms. The proper spatial and temporal regulation of gene expression has important implications for a variety of processes, including cell growth, differentiation and responses to environmental

cues. Understanding how the time-of-day information is passed on from the central clock to regulate daily processes, and identifying the different activities regulated by the clock, would then provide valuable information regarding the adaptive value of circadian rhythms (Yerushalmi and Green, 2009) and has, in fact, become the subject of intense research in the last few years.

1.7.1. Searching for clock-controlled genes

The first systematic screen for clock-regulated genes in any clock model organism was performed in Neurospora (Loros, et al., 1989), which led to the identification of two genes which peaked in the early subjective morning. These "clock-controlled genes" (or *ccgs*, a term coined in Neurospora and now widely used in the circadian literature), were soon after shown to be regulated by the clock, at least partly, at the level of transcription (Loros and Dunlap, 1991). These genes, *ccg-1* and *ccg-2*, have been the most studied clock-regulated genes in Neurospora (reviewed in Montenegro-Montero and Larrondo, 2013). A distinction between "clock genes" and clock-controlled genes is warranted. As mentioned above, "clock genes" refers to genes whose product is essential for the clock mechanism (e.g. *frq*, the *wc* genes). Clock-controlled genes, on the other hand, are rhythmically expressed genes which depend on the oscillator for rhythmicity, but whose ablation does not impact the workings of the clock. Some clock-controlled genes have been found to feed back onto the oscillator or input pathways and when inactivated, can alter some properties of the clock, although it can continue working. These are referred to as "clock modulators", which somehow complicates the nomenclature of clock-associated genes.

The *ccg-1* gene encodes for a protein of unknown function, but conserved among filamentous fungi, while *ccg-2* (also known as *eas*) encodes for a hydrophobin that coats the mature conidia and provides the hydrophobicity necessary for their air dispersal. Its deletion results in an "*eas*ily wettable" phenotype, in which asexual conidia adopt a dark color and mix readily with water (Bell-Pedersen, et al., 1992). Since then, screens for *ccgs* have been performed in Neurospora using a variety of experimental approaches, including differential hybridization of time-specific libraries (Bell-Pedersen, et al., 1996), exhaustive cDNA sequencing (Zhu, et al., 2001) and microarrays (Correa, et al., 2003; Dong, et al., 2008; Nowrousian, et al., 2003). As a whole, these studies have suggested that ~20% of the Neurospora transcriptome may be under circadian control (usually assessed using liquid cultures, see below), with transcripts levels peaking at all phases of the day, but with most of them peaking at the late subjective night to early morning (Correa, et al., 2003).

A few things should be noted about the extent of circadian control derived from these studies though, which could modify this current estimate, either by reducing it or most likely, by increasing it. Regarding the first possibility, while microarray studies have successfully identified genes which had been previously classified as clock-controlled through classical biochemical approaches (i.e. *ccg-1*, *ccg-2*, etc.), and thus partly validating the approach, most of these newly identified genes have not been validated through independent methodologies/studies and a fraction could easily reflect false positives.

Further, most circadian studies in Neurospora have been done in the *ras-1*^{bd} background, a strain that while eases the visualization of the clock-controlled process of conidiation and has

been crucial for the advancement of Neurospora as a clock model, is a mutant in which endogenous signals appear to be amplified to unnatural levels (for instance, it displays enhanced light- and clock-regulated gene expression, see Belden, et al., 2007). New molecular circadian studies in the *ras-1* WT background will help get a clear view of the extent of circadian gene expression, especially now that banding-independent phenotypes, such as luciferase expression, can be analyzed in Neurospora (Cesbron, et al., 2013; Gooch, et al., 2008).

Regarding the second possibility, that is, that the extent of clock control is underestimated, it has become evident not only in Neurospora but in other organisms as well, that the catalogue of *ccgs* reported in each study is heavily dependent on growth conditions (for instance, low vs. high glucose, Correa, et al., 2003 and Nowrousian, et al., 2003), tissues assayed, entrainment protocols, sampling frequency and algorithms used to identify rhythmic genes, among other factors (Doherty and Kay, 2010). This then, may lead to underestimations regarding the number of genes under clock control, particularly from noisy microarray data sets. This issue is particularly relevant in Neurospora, since microarray data has provided most of the information regarding the extent of clock control of gene expression in this organism (no circadian RNA-seq or proteomics studies have yet been published). Meta-analyzes integrating data from different tissues and conditions have shown that it is not simply a matter of determining whether a particular gene is rhythmic or not, but that certain genes may only be detected as rhythmic under certain circumstances and in particular tissues (Covington, et al., 2008; Keegan, et al., 2007; Miller, et al., 2007; Ptitsyn, et al., 2006). Notably, this has been shown to be true even for core clock components (Hardin, 1994). This may reflect the

different inputs that each gene has, under different conditions, which could override (or mask) their control by the clock. When integrating data then, from different studies using various experimental settings, the number of cycling transcripts usually increases. In plants for instance, a detailed study under a variety of thermocycles, photocycles, and free-running conditions, has suggested that as much as 90% of the Arabidopsis transcriptome cycles in at least one condition (Michael, et al., 2008). Similar conclusions were obtained through meta-analysis of circadian expression from different murine tissues, in combination with the use of different algorithms (Ptitsyn and Gimble, 2011). This may be an important factor in Neurospora, where clear developmental transitions occur in solid media, but are not evident in submerged cultures, which is the most common growth condition used for the study of circadian gene expression. Thus, it is possible that the repertoire of Neurospora *ccgs* known to date represents just a fraction of the ones occurring in nature when this saprophyte is growing on the bark of dead trees. Finally, limitations of microarray technology can make the detection of low-amplitude rhythms in mRNA levels, difficult.

Studies recently performed in mammalian systems have shown that only a small fraction of the genes that exhibit rhythms at the mRNA level, have corresponding transcriptional rhythms, highlighting a role for post-transcriptional mechanisms on controlling rhythmic mRNA accumulation (Koike, et al., 2012; Le Martelot, et al., 2012; Menet, et al., 2012). A recent study in flies also supports an important role for post-transcriptional regulation in rhythmic gene expression (Rodriguez, et al., 2013). Additionally, these studies report that there is a group of genes that display transcriptional rhythms, but do not appear to show oscillations at the mRNA level. The use of techniques that just explore steady-state levels of mRNAs to

assess the extent of circadian control in an organism, may then underestimate just how many genes have clock input at some level. Studies performed in Arabidopsis, using enhancer trapping, have shown that clock regulation may be more widespread that expected based on steady-state mRNA abundance studies (Michael and McClung, 2003). Further, it has been shown that only about half of the proteins that exhibit rhythms in accumulation in mouse liver, have corresponding cyclically expressed mRNAs (Reddy, et al., 2006). These studies suggest that looking solely at a single step of gene expression, may greatly limit our perception of the extent of clock control. The recent introduction of luciferase translational fusions to the Neurospora field (Larrondo, et al., 2012), has allowed for the study of rhythmic protein expression profiles of several *ccgs* in Neurospora under a variety of genetic backgrounds and at a global scale (Montenegro-Montero and Larrondo, 2013). This approach may help identify proteins that may exhibit circadian rhythms despite constant mRNA levels. Similarly, transcriptional fusions to luciferase may help identify genes that are rhythmically transcribed, despite seemingly flat mRNA levels.

Although RNA-based approaches may miss the full extent of clock control, they have been very informative about clock regulation in all clock model systems, including Neurospora. The ease with which these global experiments can be performed has propelled its use in the circadian field. The number of circadian microarray studies conducted in Neurospora however, heavily lags behind what has been done in plants, insects and mammals and so a lot remains to be uncovered about the Neurospora circadian system. These studies have nevertheless revealed that the extent of processes under circadian control is wide and includes DNA damage repair, sexual and asexual development, intermediary metabolism, cell cycle and

transport, just to name a few (Dunlap and Loros, 2004; Lakin-Thomas, et al., 2011; Vitalini, et al., 2006), similar to what has been described in other organisms (Ceriani, et al., 2002; McClung, 2011; Miller, et al., 2007). Notably, the glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH), used as a reference gene in quantitative PCR assays in different organisms, has been reported to be under circadian control, first in Neurospora (Bell-Pedersen, et al., 1996) and later in several other organisms (Fagan, et al., 1999; Kamphuis, et al., 2005; Temme, et al., 2000). It is important to highlight that this was the first example of circadian regulation of a gene with a wide phylogenetic distribution.

Of particular interest is the circadian control of stress responses, an area that appears to have a clock input in all circadian model organisms (Ceriani, et al., 2002; Covington, et al., 2008; Hardeland, et al., 2003; Kreps, et al., 2002). This make sense in light of the proposed adaptive value of circadian clocks, as it would allow organisms to anticipate and prepare for stressful conditions, such as oxidative and osmotic stress, brought about by sunrise. Particularly, the expression of genes encoding antioxidant enzymes appears to be under clock control in Neurospora (Correa, et al., 2003; Yoshida, et al., 2011). Rhythms in the expression of genes with antioxidant roles have also been reported in plants and mammals (Covington, et al., 2008; Hardeland, et al., 2003). Further, osmotic stress responses have also been characterized for its clock input in Neurospora (Lamb, et al., 2011; Vitalini, et al., 2007).

In one of the first studies using microarrays to evaluate rhythmic gene expression in Neurospora, Correa et al. (2003) not only reported several novel circadian regulated genes, but also described the expression of three genes that appeared to oscillate in a FRQ-independent

manner. Research was only thereafter pursued for one of them, *ccg-16*, which peaks in the evening. The *ccg-16* mRNA appears to oscillate with a temperature compensated period (albeit within a smaller range) via a WCC-requiring *frequency*-less oscillator (FLO) (de Paula, et al., 2006), but no defined regulators or components have yet been reported for this oscillator.

One area that could benefit from large-scale expression studies is the identification of *cis*-regulatory elements, which could later be integrated into circadian transcriptional networks for system biology approaches. As mentioned previously, the number of microarray experiments aimed at studying circadian rhythms in Neurospora is small and these reports have only surveyed a small number of growth conditions, so their contribution to this area is limited. This, together with the fact that the sequence preference of TFs is largely unknown in Neurospora, poses a great limitation to our ability to characterize these regulatory circuits. The use of global approaches will help in this regard, allowing the characterization of transcriptional networks in the Neurospora circadian system (see Chapter 2 and Montenegro-Montero and Larrondo, 2013).

1.7.2. Transcriptional regulation and output pathways

The idea that clock-regulated transcription would be a major way for the clock to control output pathways was first proposed in the Neurospora system (Loros, et al., 1989) and has since then, been shown to be true not only in Neurospora, but in other organisms as well. The most obvious candidates for mediating such rhythmic transcriptional regulation are the TFs

involved in the workings of the central oscillator, that is, the positive elements: the WCC in Neurospora and its analogs, CLK/CYC in Drosophila and CLOCK/BMAL in mammals. They can confer rhythmicity to the negative elements of the respective clocks and could, in principle, also bestow this trait onto other genes. The genome-wide mapping of direct targets of these transcriptional regulators then, can be used to identify novel clock-controlled genes. Indeed, this has been done in insects (Abruzzi, et al., 2011), mammals (Hatanaka, et al., 2010; Koike, et al., 2012; Rey, et al., 2011), plants (Huang, et al., 2012) and also Neurospora (Smith, et al., 2010), which has shed some light on the circadian transcriptional regulatory landscape. Most rhythmic genes however, lack binding sites for these TFs, suggesting that other regulators are involved in their rhythmicity. The TFs that participate in such relay of time-of-day information to regulate the expression of rhythmically expressed genes however, are largely unknown and their identification and characterization has proceeded slowly. Indeed, in Neurospora as in other clock model organisms, output pathways have been the least characterized aspect of their circadian systems and little is known about the different regulatory factors involved in connecting the oscillator with overt rhythms (Doherty and Kay, 2010; Montenegro-Montero and Larrondo, 2013). Similarly, the extent of clock control at the transcriptional level, which would be the result of the activity of such TFs, has not been studied in Neurospora.

The basic region/leucine zipper (bZIP) transcription factors constitute one of the largest families of TFs in eukaryotes (Weirauch and Hughes, 2011). Members of this family contain a basic α-helical DNA-contacting region, linked to a dimerization interface consisting of a leucine zipper motif (Pabo and Sauer, 1992; Vinson, et al., 2002). The leucine zipper motif

was first discovered as a conserved sequence pattern in several eukaryotic transcription factors (Landschulz, et al., 1988), and since then, bZIP transcription factors have been found in a wide variety of organisms, playing relevant roles (Gachon, 2007; Hurst, 1994; Jakoby, et al., 2002; Toone, et al., 2001; Vinson, et al., 2006).

The bZIP domain consists of 60-80 amino acid residues and as mentioned, is subdivided into a basic, arginine- and lysine-rich motif, involved in sequence-specific interactions with DNA through the major groove, and a leucine zipper motif, an amphipatic α-helical structure characterized by a heptad repeat of leucines over a region of 30-40 residues. It usually also contains a conserved repeat of other hydrophobic residues (often valine or isoleucine) occurring three residues to the N-terminal side of each of the leucines in the heptads (Kerppola and Curran, 1991; Vinson, et al., 2006).

The bZIP monomer consists of a long α helix, in which the basic region is located on the N-terminus, followed by the leucine zipper motif (Figure 1.6). These TFs require either homo- or heterodimerization for DNA binding. Dimerization results from the interaction between the leucine zipper motifs of two monomers, which interact in a parallel orientation forming a left-handed coiled-coil. This arrangement of intertwined α-helices produces a repeating structure in each helix, which creates a hydrophobic surface on one side of each α-helix, with a leucine every second turn (Figure 1.6). This constitutes the dimerization interface, in which the hydrophobic sides of the amphipatic α-helices of the two monomers face each other and interact, stabilizing the parallel coiled-coil arrangement mentioned above. Additionally,

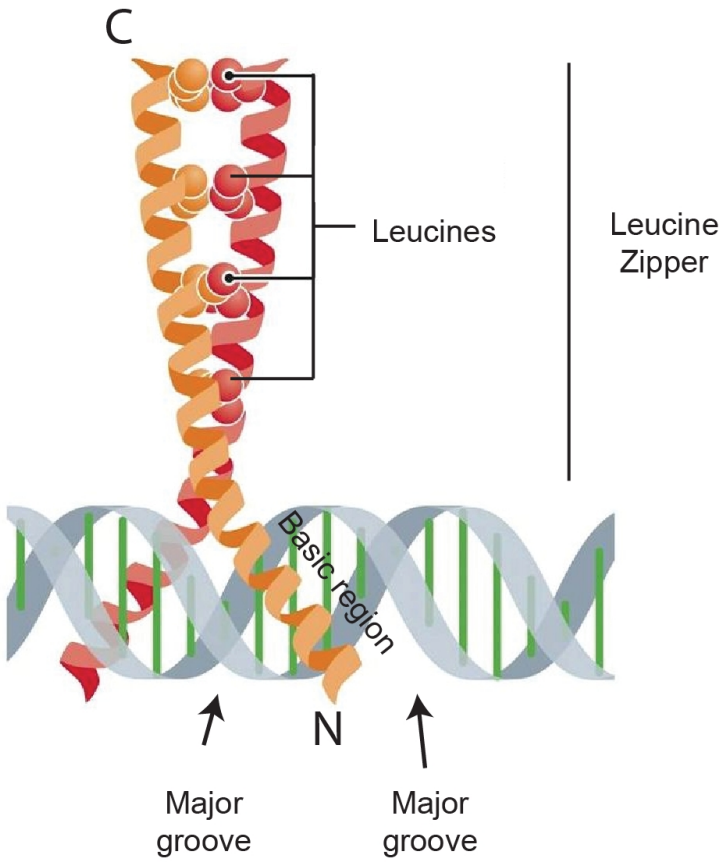

Figure 1.6. The bZIP transcription factors. Schematic representation of a bZIP protein dimer bound to DNA. The bZIP α-helices are shown in red and orange, reach representing one of the interacting monomers. The hydrophobic interface where the leucines lie between the interacting α-helices, is depicted with colored spheres. Leucine zippers are characterized by a heptad repeat of leucines and as α-helices in a coiled-coil structure have a periodicity of 3.5 residues per helical turn, each leucine is present in the hydrophobic surface every second turn. In the turns in between, other hydrophobic residues are present facing the other helix (not shown). The leucine zipper and the basic region, the latter involved in sequence-specific DNA contacts through the major groove, are labeled. The N and C represent the N- and C-terminus of the protein, respectively. Figure is a modified version of the one in (Brown, 2011).

oppositely charged residues interact inter-helically, contributing to structure stabilization and helping to regulate the specificity of bZIP protein dimerization. Altogether, this creates a Y-shaped structure in which the basic regions reach into the major groove of the DNA double helix (Figure 1.6). The bZIP transcription factors, through this dimer structure, bind short palindromic or pseudo-palindromic sequence motifs (Fujii, et al., 2000; Pabo and Sauer, 1992; Vinson, et al., 2006).

The bZIP transcription factors were originally divided into different classes, depending on their overall sequence identity, dimerization properties and their DNA sequence preferences (Fujii, et al., 2000; Kerppola and Curran, 1991; Vinson, et al., 2002). For instance, the TFs in the ATF/CREB family of bZIP proteins, usually prefer adjacent half-sites (i.e. TGACGTCA), while members of the AP-1 family, prefer overlapping and asymmetric half-sites (TGAC/GTCA). With the identification and characterization of several new members of bZIP TFs from numerous species however, the class boundaries have been blurred.

A detailed analysis of the Neurospora genome sequence (Borkovich, et al., 2004), suggested the presence of 17 bZIP encoding genes and only few of these have been studied. Among the ones that have been more extensively characterized, we find CPC-1, a homolog of the yeast GCN4 protein, which is involved in the global transcriptional response to amino acid starvation (as its yeast counterpart) (Paluh and Yanofsky, 1991; Tian, et al., 2007) and CYS-3, involved in the regulation of sulfur-related genes (Fu, et al., 1989; Li and Marzluf, 1996). At the beginning of this Thesis project, information regarding other bZIP transcription factors in Neurospora was nonexistent. At present, gene expression data is available from a few bZIP TF

mutant strains (Lamb, et al., 2012; Tian, et al., 2010) and growth phenotypes have been found for a subset of those (Colot, et al., 2006), but details regarding their regulation and DNA-binding specificities, are mostly unknown. Some of these 17 TFs are only "predicted bZIP TFs" and no information is available on their sequence preference or even whether they can actually bind DNA in a sequence-specific manner and regulate gene expression. The vast majority of bZIP proteins in Neurospora then, remain uncharacterized, both regarding to their putative targets and how they are integrated within the cell's regulatory circuits. Additionally, some bZIP TFs that are present and have been characterized in several fungi, have not been reported in Neurospora, questioning the completeness of the bZIP list in this fungus. It should be mentioned that this not only applies to the bZIP family of transcriptional regulators: despite several decades as a research model organism, our knowledge of transcriptional regulatory networks in Neurospora is limited.

In Chapter 2 of this Thesis, we set out to evaluate the role of bZIP TFs in the Neurospora circadian system. We first revisited the list of transcriptional regulators in this fungus, which led to the identification of several novel putative bZIP proteins and then, through the use of a luciferase-based high-throughput screening system, we identified several bZIP encoding genes whose expression is regulated by the circadian clock. This system, coupled to genetic approaches, also allowed us to delve into how such control is mediated. In addition to the reporting of several bZIP encoding genes with rhythmic input, our strategy led to the identification of a bZIP protein involved in regulating circadian output pathways in Neurospora and allowed us to evaluate the role of another one which has been suggested to participate in such pathways. To identify and characterize regulatory networks in which these

and all putative Neurospora transcription factors participate, we used double-stranded DNA microarrays, known as protein-binding microarrays (PBMs), containing all possible 10-base sequences, to examine their binding specificities and in that way, predict possible targets on a genome-wide level. Notably, this is the first study aimed at studying DNA-binding specificities on a global scale in the fungal kingdom, outside of the yeast clade. Interestingly, in addition to its use in studying circadian control of gene expression, this knowledge resulted in the identification of the first TF shown to be required for growth under osmotic stress in Neurospora, highlighting the relevance of this dataset which we anticipate will be a powerful resource for mapping transcriptional networks in filamentous fungi.

As a whole, the work reported on this Thesis, represents a major advancement in the study of bZIP proteins and transcriptional regulatory networks in Neurospora.

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CHAPTER 2

BZIP TRANSCRIPTION FACTORS AND CIRCADIAN REGULATORY NETWORKS IN NEUROSPORA

As some of the topics that will be discussed in the introductory section of this Chapter have already been covered in Chapter 1, they will only be briefly described and are herein included solely for context.

2.1. Introduction

Circadian clocks control the time-of-day-specific expression of hundreds of genes in a wide variety of organisms (Bell-Pedersen, et al., 2005; Dunlap, 1999; Dunlap, et al., 2004). The resulting temporal coordination of physiology and behavior allows them to anticipate and prepare for the predictable environmental variations that are brought about by the daily transitions between days and nights and to schedule diverse cellular processes to optimal times throughout the day.

Circadian systems are conceptually composed of an endogenous and cell-based oscillator or pacemaker and two signaling pathways: input pathways convey external signals to the oscillator, to allow its synchronization with the environment, and output pathways, which allow it to temporally regulate diverse cellular processes. A wealth of knowledge has

accumulated over the last two decades on the molecular details underlying the workings of the oscillator in some model eukaryotic species, including *Neurospora crassa* (Lakin-Thomas, et al., 2011), *Drosophila melanogaster* (Hardin, 2011), *Arabidopsis thaliana* (McClung, 2011) and mammalian model systems (Lowrey and Takahashi, 2011). Even though it is undeniably important to understand the molecular basis of the core circadian machinery and input pathways, it is the wide array of processes that are under clock control and their regulation that ultimately impact the biology of organisms and comparatively, the "output pathways", which mediate the rhythmicity of clock-controlled genes, have been far less characterized. Indeed, even though the question of which genes appear to be under clock control has been extensively (albeit not comprehensively) addressed in these circadian model systems (Adams and Carre, 2011; Ceriani, et al., 2002; Correa, et al., 2003; Dong, et al., 2008; Gachon, et al., 2004), few details are known of the regulatory circuits that relay the time information from the pacemaker to regulate their rhythmic expression.

The ascomycete *Neurospora crassa* has one of the best-understood circadian systems, in which a molecular negative feedback loop involving FRQ and the WCC form the basis of its most characterized oscillator (Heintzen and Liu, 2007; Lakin-Thomas, et al., 2011; Montenegro-Montero and Larrondo, 2013). In Neurospora, it has been estimated that 20% of the steady-state transcriptome is regulated by the circadian clock, with peak phases occurring throughout the day (Correa, et al., 2003; Dong, et al., 2008). The corresponding genes include those associated with rhythms in sexual and asexual development, metabolism and stress responses, among others (Vitalini, et al., 2006). As in other clock model organisms, few of these genes have been studied in detail and the pathways emerging from the oscillator

responsible for the rhythms in these genes, are largely unknown. Simply stated, we have a general idea of which genes are rhythmically expressed, but we do not know how the clock is endowing these genes with rhythmicity.

The various genetic and molecular tools available for research in Neurospora, have nonetheless set the stage for the initial study of these pathways and continue to allow dissection of these regulatory circuits.

In the late 1980s, the first ever systematic screen for clock-controlled genes was performed, using Neurospora as a model (Loros, et al., 1989). This screen identified two genes peaking in the early subjective morning, *ccg-1* and *ccg-2*. In an effort to characterize the output pathways controlling these genes, Vitalini et al. (2004) conducted a genetic screen, in which they identified a number of mutant strains exhibiting low and arrhythmic expression of *ccg-1* (Vitalini, et al., 2004). The subsequent mapping of one of these mutations led to the identification of *rrg-1*, a gene encoding a response regulator, as a mediator involved in the time-of-day information relay pathway from the central oscillator to the rhythmic expression of *ccg-1* (Vitalini, et al., 2007). RRG-1 is an upstream regulator of the osmotic stress (OS) MAPK pathway in Neurospora, a conserved signaling pathway involved in orchestrating global responses to hyperosmolarity (Jones, et al., 2007; Kamei, et al., 2013). This pathway is homologous to one of the most intensively studied MAPK pathways in fungi, the yeast high-osmolarity glycerol (HOG) pathway, for which many upstream and downstream regulators are known (Saito and Posas, 2012).

Vitalini et al. (2007) showed that the OS pathway is an output of the clock that controls the rhythmic expression of two *ccgs* (*ccg-1* and *ccg-9*). The Neurospora circadian clock appears to regulate the daily activation of the OS pathway by controlling the rhythmic phosphorylation of a key element of the pathway, the OS-2 MAPK, which would in turn regulate, via phosphorylation, downstream effectors, including transcription factors and other modulators that could then control the rhythmic expression of target genes. In Neurospora, such rhythmic activation of the pathway is dependent on a functional RRG-1 (Vitalini, et al., 2007).

Lamb et al. (2011) later showed that several components of the OS pathway are rhythmically expressed, which revealed a direct connection between this pathway and the clock. Notably, the authors also reported a time-of-day difference in glycerol accumulation in response to salt stress, supporting the idea of a pathway-priming role for the clock (Lamb, et al., 2011). Note however, that a functional clock is not required for survival under salt stress, suggesting that the clock appears to have co-opted this conserved pathway (Vitalini, et al., 2007). This link between the circadian clock and MAPK pathways has also been described in other organisms (de Paula, et al., 2008).

The downstream regulators of the OS pathway that control the rhythms in expression of the pathway's target genes are currently under study. ASL-1 (ascospore lethal-1), also referred to as ATF-1 (Yamashita et al., 2008), a bZIP transcription factor known to be required for the OS-2-dependent expression of *ccg-1* in response to osmotic stress and the antifungal fludioxonil, has been shown to bind the *ccg-1* promoter *in vitro* (Yamashita, et al., 2008), which makes it an attractive candidate for modulating *ccg-1* rhythms. A number of other

transcription factors, some of which are predicted to function in the OS pathway based on what is known in yeast, have also been suggested to mediate rhythmicity of the *ccgs* that are under control of the OS pathway. This however, remains to be evaluated.

Interestingly, there is evidence that the OS-pathway is not the only MAPK pathway with a circadian input in Neurospora. Recently, it was reported that two other MAPKs, MAK-1 and MAK-2, are also rhythmically phosphorylated in Neurospora (Bennett, et al., 2013). In the absence of MAK-1, which is the homolog of the yeast Slt2 MAPK (involved in cell wall integrity) (Chen and Thorner, 2007; Park, et al., 2008), the steady-state levels of about 500 genes are affected. Notably, more than 25% of these genes have previously been identified as *ccgs*, suggesting that this pathway plays a role in the output circuitry. Indeed, detailed analysis of two morning-specific *ccgs*, including *ccg-1*, revealed that their robust rhythmicity relies on this pathway (Bennett, et al., 2013). The downstream TFs mediating such rhythms are however, unknown, similar to the situation with the OS pathway.

Together with *ccg-1*, *ccg-2* has also been one of the most studied *ccgs* in Neurospora. The rhythmic expression profile of *ccg-2* appears to be altered in the *rrg-1* mutant, although not in all clones, suggesting a complex control over its expression (Vitalini, et al., 2007). A *cis*-regulatory element present in its promoter, termed activating clock element (ACE), has been shown to be both necessary and sufficient for its rhythmicity (Bell-Pedersen, et al., 1996). Interestingly, protein factors present in nuclear extracts, distinct from the WCC, have been shown to bind specifically to this sequence in a time-of-day-specific manner (Bell-Pedersen, et al., 2001), although the identity of these proteins is still unknown. Interestingly, an 8

nucleotide element, very similar to the core of the ACE, was identified in the promoter of several rhythmic genes (Correa, et al., 2003). As very little information regarding the DNA-binding preference of Neurospora TFs is available, this motif has not been able to be matched to a TF and thus the regulators operating via these elements, remain unknown.

Together with the ACE, the only other *cis*-regulatory element shown to be involved in transcriptional clock control in Neurospora is the *C-box*, which is rhythmically bound by the WCC in the *frq* (Belden, et al., 2007a; Froehlich, et al., 2003) and *os-4* (Lamb, et al., 2011) promoters and is sufficient for clock-regulated expression (Froehlich, et al., 2003; Gooch, et al., 2008). This sequence is also present in the promoter region of some (but not all) clock-regulated genes (Correa, et al., 2003; Dong, et al., 2008) and has been experimentally shown to be a binding site for the WCC for a few of them, although just in the context of light-induced gene expression (He and Liu, 2005; Olmedo, et al., 2009). As a whole, little is known about the identity of *cis*-elements regulating rhythmic transcription and the TFs that operate via them, not only in Neurospora, but in all clock model organisms.

The idea that transcriptional regulation would be a major way for the circadian clock to rhythmically control output pathways was first proposed in the Neurospora system (Loros, et al., 1989). Since then, it has been assumed, mostly based on what was known about the regulation of core clock components (see Chapter 1), that transcription would drive rhythms of most output cycling mRNAs, not only in Neurospora, but in other organisms as well. Transcriptional regulation has indeed been shown to play a role in controlling the expression of output genes (Lamb, et al., 2011; Loros and Dunlap, 1991; Smith, et al., 2010), but the

extent to which transcriptional regulation accounts for global rhythmic mRNA levels in Neurospora is not known and neither is the set of genes that are rhythmically transcribed (which does not necessarily include all genes exhibiting rhythms at the mRNA level, as discussed in Chapter 1).

The most natural candidates for mediating such rhythmic transcriptional regulation are the transcription factors involved in activating the negative elements in the central oscillator. In Neurospora, this job belongs to the White Collar Complex, composed of the GATA-type transcription factors WC-1 and WC-2 (Crosthwaite, et al., 1997). Their rhythmic binding confers rhythmicity to *frq* (Belden, et al., 2007b), the negative element of the system, and *os-4* (Lamb, et al., 2011) and could in principle, also bestow this trait onto other genes. The genome-wide mapping of direct targets of these transcriptional regulators then, could be used to identify novel clock-controlled genes and indeed, this has been done in Neurospora (Smith, et al., 2010). As has been mentioned before, besides its role as a positive element in the Neurospora circadian system, WC-1 is also the major blue light receptor in this fungus (Ballario, et al., 1996; Chen, et al., 2009).

In order to identify genes that are directly regulated by the WCC in Neurospora, which could give important insights into light- and clock-regulated transcription, chromatin immunoprecipitation coupled to high-throughout sequencing assays (ChIP-seq) with an anti-WC2 antibody, were performed after a brief light pulse (Smith, et al., 2010). Over 400 enriched genomic regions were identified, with about half of them in known or predicted promoters. Even though most of these regions were associated with genes encoding for

unclassified proteins, enrichment for genes linked to processes such as cell cycle, transcription, and responses to the environment was found. As a way to characterize the transcriptional networks associated with light and clock regulation of gene expression (because WC-1, through the WCC, acts on both processes), Smith et al. (2010) focused on the WCC binding sites near predicted transcription factor genes. Notably, they identified several known or putative transcription factor genes with significant WCC binding in their promoters or nearby regions. Most of these putative TFs however, have not been characterized and it is unknown whether they play any role in the Neurospora circadian system or if they can even bind DNA and regulate gene expression (that is, if they are indeed bona fide TFs).

In any case, considering that rhythmic genes peaking at all phases of the day have been found in Neurospora and the fact that there are genes that respond early to a light pulse and others, late (Chen, et al., 2009), it is unlikely that the WCC is directly and solely responsible for their expression profiles in response to clock and light signals, respectively. The identification of these second-tier transcription factors suggests a flat hierarchical regulatory network in which the WCC regulates the expression of a second set of transcriptional regulators which could, in turn, mediate rhythmic expression of some of their target genes (Smith, et al., 2010). The fact that only a few of the identified *ccgs* have an obvious binding site for the WCC and that *ccgs* have been found to peak at different phases of the day, supports this notion. Indeed, for light responses, such a hierarchical arrangement has been described in Neurospora (Chen, et al., 2009) and it also appears to underlie circadian regulatory networks (Vitalini, et al., 2006) (see below).

In principle, one could envision a simple transcriptionally-based system that would allow different *ccgs* to peak at different times of the day. Morning activation or night-time repression could lead to peaks during the day, while daytime activation or morning repression could lead to peaks during the night. Using a synthetic biology approach to study the design principles underlying the mammalian clock, Ueda and colleagues have shown that such a design could explain the aforementioned phases (Ukai-Tadenuma, et al., 2008). Further, they observed that simple combinations of transcriptional activators and repressors could drive rhythmic expression at other phases. In Neurospora, direct targets of the WCC would fit the "morning activation" scheme and would thus peak during the day (e.g. *frq*). The network controlling *ccg* expression in Neurospora however, is complex, and as mentioned, not all morning genes are directly regulated by the WCC (e.g. *ccg-2*), suggesting that other factors are involved in their rhythmic expression. Second-tier transcriptional activators (Smith, et al., 2010), could be responsible for the rhythmic expression of these other morning genes. Smith et al. (2010) characterized one of the identified TFs that could play a role in morning-specific gene expression, ADV-1, and reported it to be a rhythmically expressed target of the WCC and to be required for circadian rhythms in asexual development. Its target genes and sequence preference however, are unknown. Interestingly, they showed that in the absence of ADV-1, the clock functions normally, suggesting that it operates in output pathways.

Evening-specific expression, as mentioned, could instead result from daytime activation, morning repression or a complex combination of activators and repressors. Recently, the first characterization of rhythmic control pathways leading to the expression of evening genes in Neurospora was reported. Brunner and colleagues reported that *conidial separation 1* (*csp-1*),

a gene encoding for a protein with similarities to the yeast transcriptional repressors NRG1 and NRG2 (Vyas, et al., 2005) and known to be a rhythmically expressed target of the WCC (Lambreghts, et al., 2009; Smith, et al., 2010), acts as a morning repressor, leading to evening-specific expression of a subset of its targets (Sancar, et al., 2011) (reviewed in Montenegro-Montero and Larrondo, 2013). This is the first report of a molecular mechanism that results in evening expression of *ccgs* in Neurospora and further supports the hierarchical model outlined before, where second-tier activators and repressors could accounts for expression of *ccgs* in a variety of phases. The identification then, of TFs within the Neurospora circadian system, can help understand how the clock signal is transmitted to allow genes to be expressed at different phases of the day.

Even though most studies aimed at understanding the mechanisms underlying the rhythmic control of these genes have historically focused on their transcriptional regulation (and we have done so in Chapter 1 and will also do in this Chapter), clock control could in principle, take place at any other stage of gene expression, namely, mRNA processing, export, stability and subcellular localization, as well as at the translational and post-translational levels. Regulation at these levels also contributes to the rhythmicity and phase observed for clock-controlled genes. Indeed, evidence for such regulation has been described in clock model systems, including Neurospora (Garbarino-Pico and Green, 2007; Kojima, et al., 2011; Mehra, et al., 2009; Partch, et al., 2013; Staiger and Koster, 2011; Wang, et al., 2013; Zhang, et al., 2011), impacting not only the circadian output transcriptome and proteome, but also the oscillator itself. Thus, together with transcriptional regulation, a complex view emerges where a combination of regulatory mechanisms at different levels of gene expression could lead to

the observed phases and amplitudes (Du, et al., 2014; Partch, et al., 2013). As the focus of the work reported in this Thesis (as in most studies in the circadian field) is clock transcriptional regulation, this will not be discussed further and the interested reader is referred to the cited reviews.

ADV-1 and CSP-1 are only two the many TFs that could work downstream of the WCC, participating in the relay of time information from the central oscillator. Obvious additional candidates are the other TFs that are direct targets of the WCC. These reported direct targets however, were identified in light pulse experiments and the targets of the WCC under circadian regimes (and in different growth conditions) may be different (Montenegro-Montero and Larrondo, 2013). This opens the door to the interesting quest for transcriptional regulators operating in output pathways, an elusive aspect of molecular chronobiology. In addition, other TFs that may not be direct targets of the WCC, could also be rhythmically expressed at any level, as part of complex circadian regulatory networks, and modulate output pathways.

The basic region/leucine zipper (bZIP) transcription factors constitute one of the largest families of TFs in eukaryotes (Weirauch and Hughes, 2011). Members of this family contain a basic α-helical DNA-contacting region, linked to a dimerization interface consisting of a leucine zipper motif (Pabo and Sauer, 1992; Vinson, et al., 2002). Since the discovery of the motif in several eukaryotic TFs (Landschulz, et al., 1988), bZIP transcription factors have been found in a wide variety of organisms, where they play important regulatory roles (Gachon, 2007; Hurst, 1994; Jakoby, et al., 2002; Toone, et al., 2001; Vinson, et al., 2006). Certain members of the bZIP family of TFs can homo- and heterodimerize, greatly expanding

their repertoire of specific DNA-binding sites and their potential regulatory functions (Miller, 2009).

Members of this family of TFs have been associated with circadian systems in different organisms. In mammals, members of a bZIP family subclass called PAR bZIP (for *p*roline and acidic amino *a*cid-*r*ich *b*asic leucine *zip*per), are rhythmically expressed and play a role in output pathways, regulating genes involved in glucose and lipid metabolism and participating in the circadian control of xenobiotic detoxification in the liver and other peripheral organs (Gachon, 2007; Gachon, et al., 2011). In addition, transcriptional repressors known as PAR-related bZIPs, also play a role in this circadian system (Mitsui, et al., 2001). In Drosophila, proteins related to both the PAR bZIP and PAR-related bZIP proteins, are also rhythmically expressed and are regulatory elements in the fly's circadian system, participating in the central oscillator and output pathways (reviewed in Hardin, 2011). Rhythmic members of this subclass have also been identified in zebrafish (Ben-Moshe, et al., 2010), suggesting that a role for this subclass of bZIP TFs in circadian systems, is conserved among Metazoans (Blau and Young, 1999). The expression of other bZIP TFs, that do not belong to the PAR bZIP subclass, has also been shown to be controlled by the circadian clock in animals (Hattar, et al., 2002; Ma, et al., 2011; Rudic, et al., 2005).

Although it has been reported that core clock components directly regulate the expression of some of the PAR bZIP genes (reviewed in Gachon, 2007), and target genes have been identified for some of these bZIP proteins, the mechanisms controlling the rhythmic expression of the majority of the identified oscillatory bZIP TFs in animals and the identity of

their targets, are largely unknown, consistent with the previously mentioned general lack of knowledge regarding output pathways in clock model systems.

Rhythmicity in the expression of bZIP TFs is not restricted to animals. In Arabidopsis, several bZIP TFs have been shown to exhibit rhythms at the mRNA level (Hanano, et al., 2008), and they could participate in any aspect of the Arabidopsis circadian system. In addition, it has been shown that bZIP TF LONG HYPOCOTYL 5 (HY5) operates in output pathways in this organism, regulating the proper rhythmic expression of the *Lhcb1*1* gene (Andronis, et al., 2008). A survey of clock-controlled genes in soybean seeds identified two bZIP TFs with rhythmic mRNA levels and this study also found typical bZIP-binding motifs over-represented in the promoter regions of the identified rhythmic genes (Hudson, 2010), suggesting that bZIP TFs could mediate circadian gene expression in this system. As in animals, the pathways and regulatory circuits involved in controlling the rhythmicity of the bZIP TFs in plants remain largely uncharacterized and so are their targets.

In Neurospora, 17 bZIP TFs have been predicted based on its genome sequence (Borkovich, et al., 2004) and many of them remain uncharacterized, both in regards to their regulation, function and to their DNA sequence preference. This list of 17 TFs is most likely incomplete, as it lacks, for example, the homolog of the yeast Hac1 protein, a highly conserved bZIP TF involved in the unfolded protein response (Gardner, et al., 2013; Montenegro-Montero, et al., manuscript in preparation).

No detailed study has been performed to address whether any of the Neurospora bZIP TFs is rhythmically expressed or if they play any role in the Neurospora circadian system. Unpublished microarray data (Deborah Bell-Pedersen, personal communication) suggests that some members are rhythmic at the mRNA level, but this has not been independently validated and it is unknown whether this would result from rhythmic transcription, post-transcriptional regulation or a combination of both. Additionally, no regulators have been identified.

Despite several decades as a research model organism and the variety of molecular tools available, our knowledge of transcriptional regulatory networks in Neurospora is very limited. Knowledge of the sequence preference of the ~180 predicted TFs in Neurospora (Borkovich, et al., 2004) is scarce and this represents a fundamental limitation in our ability to analyze and interpret transcriptional regulatory networks in this fungus which serves as a model system for a variety of biological processes (Davis and Perkins, 2002).

With the general goal of characterizing output pathways in a model clock system and studying transcriptional regulatory networks in a model eukaryote, we set out to characterize the bZIP TF family in Neurospora, in the context of the circadian system. We first revisited the list of transcriptional regulators in this fungus, which led to the identification of several novel TFs, including many putative bZIP proteins. By using a luciferase-based high-throughput screening system, we identified several bZIP encoding genes whose expression is regulated by the circadian clock. To identify and characterize regulatory networks in which these and all putative Neurospora transcription factors participate, we used double-stranded DNA microarrays, known as protein-binding microarrays (PBMs) (Berger, et al., 2006; Weirauch, et

al., 2013), which contain all possible 10-base sequences, to examine their binding specificities and in that way, predict possible targets on a genome-wide level. This resulted in the determination of the sequence preference of over half of Neurospora predicted TFs. Such information, together with the various molecular tools available in Neurospora, led us to the identification of a rhythmically expressed bZIP TF, ADA-1, as a regulator of output pathways in Neurospora, controlling various output genes. In addition, this information allowed for the evaluation of the role of another bZIP TF, ASL-1, in such pathways.

This is the first study aimed at studying DNA-binding specificities on a global scale in the fungal kingdom outside of the yeast clade and represents a powerful resource for the study of transcriptional regulatory networks in Neurospora and related fungi. We further highlight the usefulness of this dataset, by reporting the first TF required for growth under osmotic stress in Neurospora.

Lastly, the ease with which genetic studies can be undertaken in Neurospora led us to identify that genes required for cell fusion, an important element for the establishment and development of fungal colonies (Fleissner, et al., 2008; Glass and Fleissner, 2006), are part of output pathways that are necessary for the proper rhythmic expression of some bZIP encoding genes. This approach also shed light on the possible molecular basis of a common mutant phenotype present in various strains of the Neurospora knockout collection, which in unrelated to the specific gene deleted.

As a whole, the work reported on this Chapter, represents a major advancement in the study of bZIP proteins and transcriptional regulatory networks in Neurospora.

2.2. Materials and Methods

2.2.1. Identification of putative sequence-specific DNA-binding proteins in Neurospora crassa

We obtained all protein sequences from the Neurospora Genome Database Release 5 (http://www.broadinstitute.org/annotation/genome/neurospora/MultiHome.html) and we scanned them for putative DNA-binding domains (DBDs) using the 81 Pfam (Finn, et al., 2010) models listed in (Weirauch and Hughes, 2011) and the HMMER tool v. 3.0 (Eddy, 2009), with the default detection thresholds. Each protein was classified into a family based on its identified DBD. Gene nomenclature throughout this Thesis follows (Perkins, 1999).

2.2.2. Phylogenetic analysis of the bZIP DNA-binding protein family in ascomycete fungi

The full protein sequence of the predicted bZIP proteins from seven different ascomycete fungal species where retrieved from CisBP (http://cisbp.ccbr.utoronto.ca/) and aligned using MAFFT (Katoh, et al., 2002) with default settings. The ProtTest 2.4 Server was used to determine the best model of protein evolution for phylogenetic reconstruction (Abascal, et al., 2005). The phylogeny was built in Mega 4.0 (Tamura, et al., 2007) using Maximum Likelihood and the reliability of the tree branching was assessed using 100 bootstrap replicates. Tree was built and edited with the iTOL web tool (Letunic and Bork, 2007) (http://itol.embl.de/). Phylogenetic analyses were performed in collaboration with Dr. Alex Slater (PUC-Chile).

2.2.3. Identification of recent duplications among bZIP encoding genes in ascomycete fungi

Genes were classified as paralogs if the predicted proteins were the best bi-directional hits (excluding self-hits) in an all-versus-all BlastP search using all the predicted bZIP proteins from seven fungal species (See Section 2.3.2), with an E-value cutoff of 1×10^{-9}, and if the alignments spanned a minimum of 60% of each protein, which is the same intuitive approach used in (Woods, et al., 2013).

2.2.4. Protein binding microarrays (PBMs) and data processing

For the characterization of the *in vitro* DNA-binding preference of Neurospora proteins, we designed primers to clone the region encompassing all the predicted DBDs, plus the 50 flanking endogenous amino acids on either side (or until the termini of the protein). Due to its length, the primer list is not included and is available upon request. The sequences were amplified from a cDNA pool by PCR and then independently cloned into a T7-GST vector for expression in *E. coli*. Information on the plasmid backbones is provided at the following website, as a web supplement to our recently accepted manuscript (http://hugheslab.ccbr.utoronto.ca/supplementary-data/CisBP/). The PBM experiments were performed in the Hughes Lab at The University of Toronto. PBM laboratory methods were identical to those described in (Lam, et al., 2011; Weirauch, et al., 2013). Each construct was analyzed in duplicate on two different arrays with differing probe sequences (termed ME and HK). Microarray data was processed as in (Weirauch, et al., 2013). Detailed methods, including criteria for deeming an experiment successful and motif derivation for each

Neurospora protein, can be found in our recently accepted manuscript, available here (Weirauch, et al., accepted in *Cell*, http://hugheslab.ccbr.utoronto.ca/supplementary-data/CisBP/).

2.2.5. Phylogenetic analysis of DNA-binding proteins in Neurospora

The sequence of the DNA-binding domain (as determined by HMMER, see above) of all proteins for which a DNA-binding motif was determined in this study using PBMs, was retrieved from CisBP (http://cisbp.ccbr.utoronto.ca/) and used for the analysis. Phylogenetic analysis was done as for the bZIP proteins, detailed above, and the derived motifs were placed at the end of the branch for each DNA-binding protein. Tree was built and edited with the iTOL web tool (Letunic and Bork, 2007) (http://itol.embl.de/).

2.2.6. Cloning of promoter regions for reporter studies at the his-3 locus and Neurospora transformation

For the study of bZIP promoters, the sequence 3 kb upstream of the predicted start codon of each bZIP encoding gene was taken as promoter region for the generation of the reporter constructs. When there was another predicted ORF within that region, the inter-ORF sequence was used instead. For a few cases, a deviation from this protocol was used, as detailed below: for three reporter constructs (for the *NCU06399*, *NCU00329* and *NCU00499* genes), we selected 3 kb of upstream sequence as the promoter region, even though the inter-ORF region was less than 3 kb. For the reporter construct of *NCU05637*, we selected a ~ 1.5 Kb fragment

as the promoter region, based on the transcription start site of the upstream gene, rather than its translation start site. The difference is only of about 300 nt. For the reporter construct of *NCU16494*, only 2 kb of upstream sequence was taken as promoter region. This was an educated guess, based on our extensive analyses of reporter constructs in Neurospora, in which we had not observed major differences between using 2 or 3 kb (unpublished observations). This construct was generated by the end of this study, hence the educated guess. For *NCU01345*, a ~1 kb fragment was selected as promoter. Detailed mapping can be derived from the primer sequences used for cloning (Table 1).

Sequences were cloned upstream of a codon-optimized luciferase gene sequence (Gooch, et al., 2008) via yeast recombinational cloning (Oldenburg, et al., 1997), using a pRS426-derived plasmid (pLL07, Figure 2.1), which allows integration of the constructs into the *his-3* locus in *Neurospora crassa* by homologous recombination. The backbone vector pLL07 was digested with *Eco*RI and *Xba*I and mixed with a PCR fragment harboring the promoter region of interest, described above. These were co-transformed into yeast BY4741 for recombinational cloning (for yeast transformation methods, see below).

Strains FGSC #9720 (frq^{wt}; $ras1^{wt}$; *his-3*) and 94-1 (frq^{9}; $ras1^{bd}$; *his-3*), were transformed by electroporation with linear plasmids harboring the constructs of interest, as previously described (Margolin, et al., 1997). Transformants were picked and cultured on 1X Vogel's salts (Vogel, 1956), 2% sucrose, 1.5% agar, supplemented with luciferin (15 μM), the latter to identify luciferase^{+} transformants using a Turner D-20e luminometer.

Table 1. Primers used for transcriptional fusion constructs. The primers listed here were used to generate transcriptional fusions between the promoter region of the genes mentioned and the luciferase coding region, at the *his-3* locus. Lowercase denotes promoter-specific sequences, while uppercase denotes homologous recombination regions between promoter fragments and the vector.

Gene	Primer Name	Orientation	Sequence (5' to 3')
NCU07379	oL66	FW	CGGAATTATACGATTTAGGTGACTGCAGGtgatgggcttgatggggaag
	oL67	RC	TTGATGTTCTTGGCGTCCTCCATATTCTAGggtggcagggtggagatacg
NCU01856	oL74	FW	CGGAATTATACGATTTAGGTGACTGCAGGcaagcagttagagcaacgtgc
	oL75	RC	TTGATGTTCTTGGCGTCCTCCATATTCTAGgatgggtatcggcgtccacg
NCU04211	oL68	FW	CGGAATTATACGATTTAGGTGACTGCAGGgttgtacgtcaagggtccttc
	oL1464	RC	TTGATGTTCTTGGCGTCCTCCATATTCTAGttatgtaacgatggaaaagg
NCU08891	oL62	FW	CGGAATTATACGATTTAGGTGACTGCAGGttttgtggtggttgttgttg
	oL63	RC	TTGATGTTCTTGGCGTCCTCCATATTCTAGggcacacagtgttgtctgtcg
NCU00223	oL60	FW	CGGAATTATACGATTTAGGTGACTGCAGGggttttcggcctagtgtcgtc
	oL61	RC	TTGATGTTCTTGGCGTCCTCCATATTCTAGcttgtctgccatgtccttcttc
NCU01994	oL70	FW	CGGAATTATACGATTTAGGTGACTGCAGGggccgtgtagtgaatagatc
	oL71	RC	TTGATGTTCTTGGCGTCCTCCATATTCTAGggtcttgtaagctagttgatg
NCU03536	oL72	FW	CGGAATTATACGATTTAGGTGACTGCAGGgtccgctttcatcgccgttc
	oL73	RC	TTGATGTTCTTGGCGTCCTCCATATTCTAGtttgacgatggtttgtgtgg
NCU06399	oL64	FW	CGGAATTATACGATTTAGGTGACTGCAGGctatgacgccgacttgatcgc
	oL65	RC	TTGATGTTCTTGGCGTCCTCCATATTCTAGcattccgggcactggttgatatc
NCU00329	oL1183	FW	CGGAATTATACGATTTAGGTGACTGCAGGggatgtggaggtacaatttc
	oL1184	RC	TTGATGTTCTTGGCGTCCTCCATATTCTAGgtcccggagcctgtaattg
NCU05637	oL56	FW	CGGAATTATACGATTTAGGTGACTGCAGGaatgcaaagtgcaagtggatgc
	oL57	RC	TTGATGTTCTTGGCGTCCTCCATATTCTAGagtcgatgattgttgggtttaag
NCU00499	oL39	FW	CGGAATTATACGATTTAGGTGACTGCAGGcaagcactgccgtgatactgc
	oL40	RC	TTGATGTTCTTGGCGTCCTCCATATTCTAGggtgggctatttgaatgtgc
NCU04050	oL35	FW	CGGAATTATACGATTTAGGTGACTGCAGGattttgacatcgtgatggttgc
	oL36	RC	TTGATGTTCTTGGCGTCCTCCATATTCTAGcttgttgccctgctttccgtg
NCU08744	oL1462	FW	CGGAATTATACGATTTAGGTGACTGCAGGGggtcgtccaattggttgttac
	oL1463	RC	TTGATGTTCTTGGCGTCCTCCATATTCTAGgtcggagtggcccgtgatgg
NCU01459	oL37	FW	CGGAATTATACGATTTAGGTGACTGCAGGggggtcggagatcggatgaagc
	oL38	RC	TTGATGTTCTTGGCGTCCTCCATATTCTAGggcgtcctggtgattctggtg
NCU03905	oL18	FW	CGGAATTATACGATTTAGGTGACTGCAGGgctactgtagggatttggtc
	oL19	RC	TTGATGTTCTTGGCGTCCTCCATATTCTAGtttcggttgtcttgtattgg
NCU08055	LC-281	FW	CGGAATTATACGATTTAGGTGACTGCAGGaaggaggactgactgcggat
	LC-282	RC	TTGATGTTCTTGGCGTCCTCCATATTCTAGgggcggttgtggtggtgccg
NCU01345	LC-279	FW	CGGAATTATACGATTTAGGTGACTGCAGGactgtgcttgctggtactgc
	LC-280	RC	TTGATGTTCTTGGCGTCCTCCATATTCTAGgatgtcaatccaaaacttgc
NCU06965	oL602	FW	CGGAATTATACGATTTAGGTGACTGCAGGggtacatgtagtgctaccc
	oL603	RC	TTGATGTTCTTGGCGTCCTCCATATTCTAGcttgtgctttttgctgagaag
NCU04058	oL598	FW	CGGAATTATACGATTTAGGTGACTGCAGGtcgcccatggtcttacgagg
	oL599	RC	TTGATGTTCTTGGCGTCCTCCATATTCTAGgtgcgtgtgtgttgcagatg
NCU01074	oL1509	FW	CGGAATTATACGATTTAGGTGACTGCAGGtgcaacacaacgtgtcgaggtc
	oL595	RC	TTGATGTTCTTGGCGTCCTCCATATTCTAGgattcgatatgaccttctgtc
NCU01204	oL1502	FW	CGGAATTATACGATTTAGGTGACTGCAGGcttgctgtgttgctatggaat
	oL597	RC	TTGATGTTCTTGGCGTCCTCCATATTCTAGtgtgtcggtattaggtgtttg
NCU07146	oL1503	FW	CGGAATTATACGATTTAGGTGACTGCAGGtcccatggtcttgaggatacg
	oL605	RC	TTGATGTTCTTGGCGTCCTCCATATTCTAGtgtcgatagtcgtcgatgcag
NCU07312	oL606	FW	CGGAATTATACGATTTAGGTGACTGCAGGcgccccgttggtgtcatgtg
	oL607	RC	TTGATGTTCTTGGCGTCCTCCATATTCTAGccaccacctactggtcgctac
NCU16494	oL1507	FW	CGGAATTATACGATTTAGGTGACTGCAGGgaggggggttgcacaccacgag
	oL1508	RC	TTGATGTTCTTGGCGTCCTCCATATTCTAGctggctgcaacgatttggtc
NCU03905	oL1465	FW	CGGAATTATACGATTTAGGTGACTGCAGGtgcccctggctgttgtttgc
(p1.8)	oL19	RC	TTGATGTTCTTGGCGTCCTCCATATTCTAGtttcggttgtcttgtattgg
NCU03905	oL1466	FW	CGGAATTATACGATTTAGGTGACTGCAGGggtcaaacaaaaagtggctt
(p1.5)	oL19	RC	TTGATGTTCTTGGCGTCCTCCATATTCTAGtttcggttgtcttgtattgg
NCU03753	oL219	FW	CGGAATTATACGATTTAGGTGACTGCAGGctgctattcagatttcatct
(p631)	oL131	RC	TTGATGTTCTTGGCGTCCTCCATATTCTAGtttggttgatgtgaggggttg
NCU03753	oL220	FW	CGGAATTATACGATTTAGGTGACTGCAGGcagacaacgggtgaaggacg
(p575)	oL131	RC	TTGATGTTCTTGGCGTCCTCCATATTCTAGtttggttgatgtgaggggttg
NCU03753	oL220	FW	CGGAATTATACGATTTAGGTGACTGCAGGcagacaacgggtgaaggacg
(p575mut)	oL1312	RC	AAGGAGATACcaCATAATCCCAACGCGGATggtatgttccggccatctcg
NCU03753	oL1311	FW	ATCCGCGTTGGGATTATGtgGTATCTCCTTcttcttcacatgattccatc
(p575mut)	oL131	RC	TTGATGTTCTTGGCGTCCTCCATATTCTAGtttggttgatgtgaggggttg

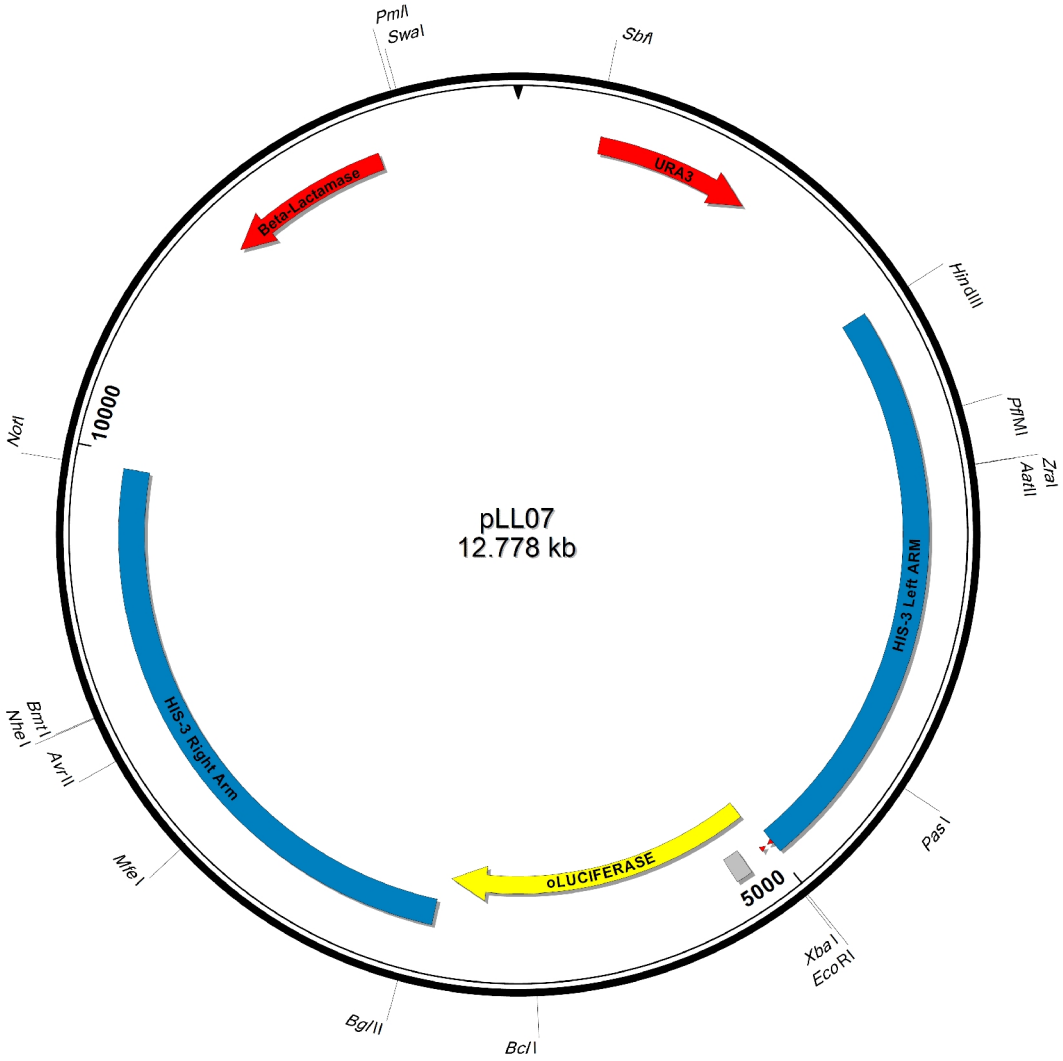


Figure 2.1. Map of pLL07. This pRS426-derived shuttle plasmid is used for cloning DNA segments upstream of a codon-optimized luciferase gene (*oLUCIFERASE*), between the *Eco*RI and *Xba*I sites (shown as red arrowheads). The construct can then be used for integrative transformation in Neurospora, where it will recombine at the *his-3* locus, restoring histidine prototrophy. Figure was generated using DNASTAR Lasergene 7.0.0.

For *NCU03905* and *NCU03753*/*ccg-1* promoter resection studies, the DNA segments mentioned in the corresponding Sections (Sections 2.3.6 and 2.3.9, respectively), were cloned as described above. Detailed mapping can be derived from the primer sequences used for cloning (Table 1). All constructs were sequence-confirmed via Sanger sequencing and transformed into FGSC #9720, as detailed above. The resulting plasmids were p2600, p1800 and p1500 for *NCU03905* and p631 and p575 for *NCU03753*/*ccg-1*.

For the generation of plasmid p575mut, a plasmid bearing a mutated version of the *NCU03753*/*ccg-1* promoter in p575, the backbone vector pLL07 was digested with *Eco*RI and *Xba*I and mixed with two overlapping PCR fragments, which together encompass the same *NCU03753*/*ccg-1* promoter length as the one in the p575 plasmid. The first fragment contains the -575, -290 region of the *NCU03753*/*ccg-1* promoter in p575, but with an AC→ TG mutation in a predicted CRE site (-305, -296). The second, contains the -320, -1 region of the *NCU03753*/*ccg-1* promoter in the p575 plasmid (note the 30 nt overlap between the two fragments, which allows for recombination), and also includes the mutation. These were co-transformed into yeast BY4741 for recombinational cloning, as detailed above. This plasmid was sequence-confirmed via Sanger sequencing and transformed into FGSC #9720, as described above.

Three additional constructs were generated in pLL07 which served as controls. The first contains a highly resected *frequency* promoter region upstream of the luciferase gene, in which the clock box (*C-box*), a *cis* element that is both necessary and sufficient for *frequency* rhythmic expression (Froehlich, et al., 2003), is included (Larrondo, et al., unpublished data).

This is essentially the same region as the one in the AF45 construct in (Froehlich, et al., 2002). This construct is referred to as the *C-box* construct throughout this chapter. The second control construct, bears a minimal promoter region upstream of the luciferase gene. A highly resected *frequency* promoter region, bearing the 196 nt upstream the start codon and with the region endowing light-responsiveness mutated (Froehlich, et al., 2002), was used (Larrondo, et al., unpublished data). The third one contains the ~1.7 kb region upstream of the start codon of *NCU08457* (Bell-Pedersen, et al., 1992) in front of the luciferase gene. Under our conditions, this construct is non-rhythmically transcribed according to our criteria (see below).

2.2.7. Constructs designed for integrative transformation at loci other than his-3 and Neurospora transformation

For integrative transformations modifying genes of interest at their endogenous loci, the knock-in sequence (e.g. luciferase gene for translational fusion, sequence for endogenous promoter replacement, etc.), a selective marker and 1 kb of both upstream and downstream sequence from the desired recombination spot, were fused together via yeast recombinational cloning into the *Eco*RI and *Xho*I sites of pRS426 (Figure 2.2). All constructs were sequence-confirmed via Sanger sequencing. Neurospora transformation was performed as in (Colot, et al., 2006). Primary transformants were homokaryonized via sexual crossing. For these integrative transformations, the following strains were used: I) for luciferase translational fusions, XG1-1 (*Δmus52::hph, a*) and II) for promoter replacement, FGSC #9719, (*Δmus52::bar, a*). In these strains, the gene encoding *mus-52* has been replaced by a selectable marker. These *mus-52*-deficient strains exhibit high levels of homologous recombination and

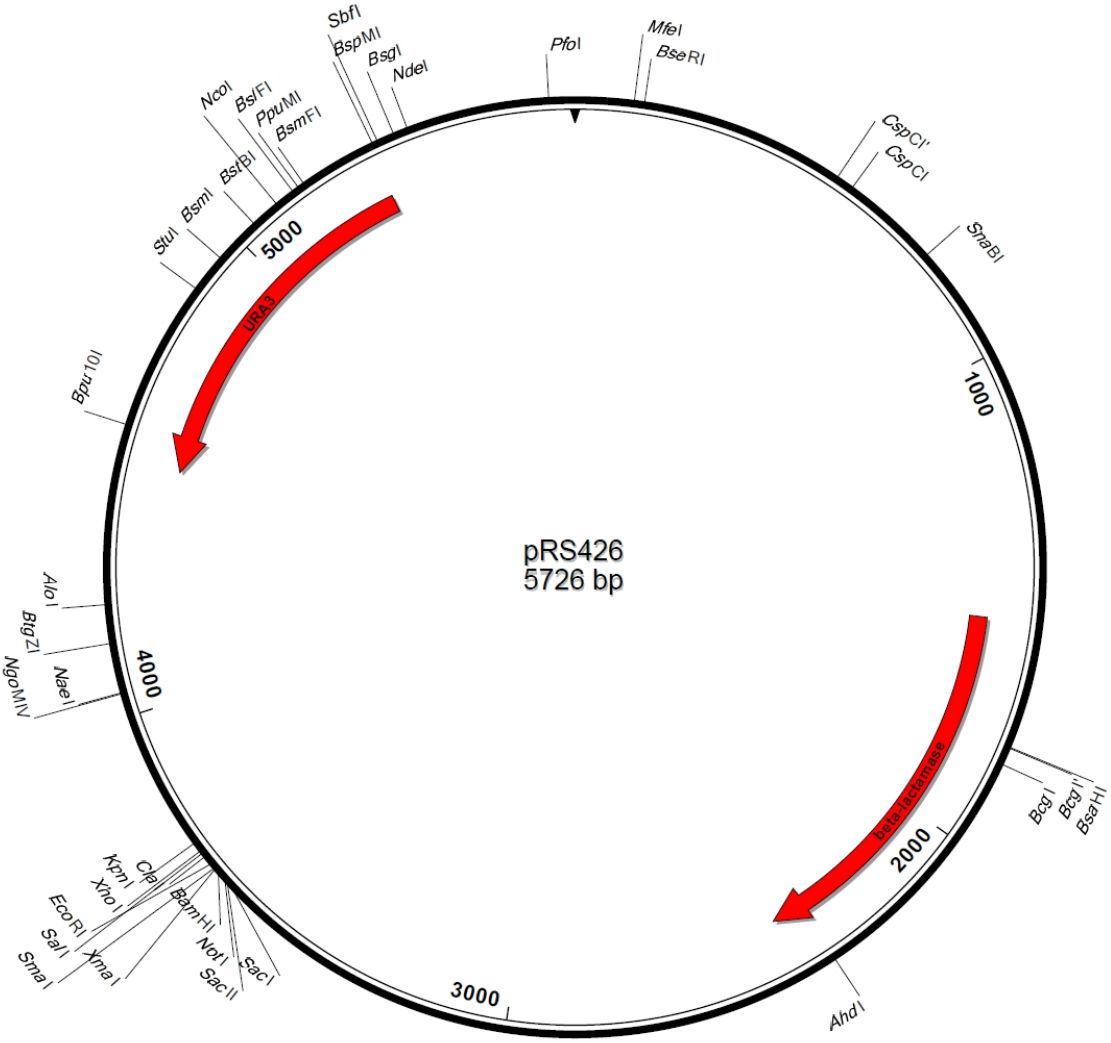


Figure 2.2. Map of pRS426. Shuttle vector used in this study for generating constructs for translational fusions and promoter replacements via yeast recombinational cloning. Figure was generated using DNASTAR Lasergene 7.0.0.

virtually no ectopic insertions are observed when used for integrative transformation (Ninomiya, et al., 2004). The bacterial *hph* gene, encoding hygromycin phosphotransferase, confers dominant resistance to hygromicin. On the other hand, the bacterial bialaphos-resistance (*bar*) gene, encoding phosphinothricin acetyltransferase, confers dominant resistance to the herbicide Ignite (also known as Bialaphos or BASTA) (Pall, 1993). To identify hygromycin resistant transformants, colonies were picked and cultured on Vogel's Media (1X Vogel's salts, 2% sucrose and 1.5% agar), supplemented with 200 µg/mL hygromycin. For Ignite resistant transformants, colonies were picked and cultured on 1X Vogel's salts (lacking NH_4NO_3), 2% sucrose and 1.5% agar, supplemented with 400 µg/mL Ignite.

For luciferase translational fusions (designed to tag the C-terminal region of the protein of interest), the backbone vector pRS426 was digested with *Eco*RI and *Xho*I and mixed with four overlapping PCR fragments, each one detailed below, following the strategy described in (Larrondo, et al., 2012).

For *NCU01345*: a 1 kb 5' ORF fragment excluding the stop codon (Forward 5'-GCGGATAACAATTTCACACAGGAAACAGCcatcagcatcaacaacaatc-3'; Reverse 5'-GGGCCCTTCTTGATGTTCTTGGCGTCCTCtgagaatcggcgaggagcaac-3'), the luciferase ORF sequence (designed to recombine in-frame, Forward 5'- gaggacgccaagaacatcaa-3'; Reverse 5'-tcagagcttggacttgccgc-3'), the *bar* gene selection marker (Forward 5'-GCCAAGAAGGGCGGCAAGTCCAAGCTCTGAaggtcatcaacctttacta-3'; Reverse 5'-tctagactcgacagaagatg-3') and 1 kb 3' flank downstream of the stop codon (Forward 5'-

CCTTCAATATCATCTTCTGTCGAGTCTAGAcgatggggtgcggaggttg-3'; Reverse 5'-GTAACGCCAGGGTTTTCCCAGTCACGACGcccagggattgattattgta-3').

These fragments were co-transformed into yeast BY4741 for recombinational cloning, as detailed above. Uppercase denotes homologous recombination regions between fragments or between fragments and vector.

For *NCU01345* promoter replacement constructs, the backbone vector pRS426 was digested with *Eco*RI and *Xho*I and mixed with four overlapping PCR fragments: 1 kb fragment upstream (and not including) the *NCU01345* start codon (Forward 5'-GCGGATAACAATTTCACACAGGAAACAGCatgtatgggaaataacaccg-3'; Reverse 5'-GCTCCTTCAATATCATCTTCTGTCTCCGACgatgtcaatccaaaacttgc-3'), the *hph* cassette (Forward 5'-gtcggagacagaagatgata-3'; Reverse 5'-gttggagatttcagtaacgt-3') (Colot, et al., 2006), a 2 kb *sod-1* promoter region (Forward 5'-ATCCACTTAACGTTACTGAAATCTCCAACgagtccttaccgtgaatagg-3'; Reverse 5'-tttgacggatttgaaagtgc-3') (Chen, et al., 2010) and a 1 kb fragment encompassing the first 1000 nt of the *NCU01345* coding region (Forward 5'-TTCTACCCAAGCACTTTCAAATCCGTCAAAatggggtcgtctactggagc-3'; Reverse 5'-GTAACGCCAGGGTTTTCCCAGTCACGACGatgcgggtcaaagggtccagtc-3').

These fragments were co-transformed into yeast BY4741 for recombinational cloning, as detailed above. Uppercase denotes homologous recombination regions between fragments or between fragments and vector.

2.2.8. Yeast transformation and plasmid retrieval

Yeast was transformed essentially as described in (Gietz and Woods, 2002). Yeast plasmid extraction was performed using the Zymoprep™ Yeast Plasmid Miniprep I kit (Zymo Research) and plasmids were then propagated in *E. coli* using the Subcloning Efficiency™ DH5α™ Competent Cells (Invitrogen). Bacterial minipreps were done using the Zyppy™ Plasmid Miniprep Kit (Zymo Research). All constructs were sequence-confirmed via Sanger sequencing.

2.2.9. In vivo bioluminescence measurements

Strains expressing luciferase were grown on maltose media (1X Vogel's salts, 0.5% maltose, 2% agar) (Schneider, et al., 2009) supplemented with luciferin (25 μM), in a 96-well plate format in a Percival Scientific incubator I-36LL for 24 h in constant light (LL) for culture synchronization. After this time, cultures were placed in a Percival Scientific incubator I-36NL equipped with a CCD (Charge-coupled Device) camera (PIXIS1024B, Princeton Instruments) in constant darkness (DD) for at least 4 days. Bioluminescence data recording was done using WinView v. 2.5.23.0 (Princeton Instruments) in a Dell Studio Hybrid 140G computer. Growth temperature was 25°C under both light regimes. Bioluminescence was measured in 20 minutes intervals, with a camera exposure time of 5 minutes.

2.2.10. Analysis of bioluminescence data

Data was analyzed in MATLAB v. R2010a (Mathworks) using Spectrum Resampling (Costa, et al., 2013), a period fitting algorithm with a graphical MATLAB interface, which imports periodic time series data from Microsoft Excel files. Bioluminescence traces were classified as circadian, if they satisfied the following criteria: a) their period was within 20-30 hr in constant conditions and displayed a relative amplitude error (RAE) lower than 0.2. RAE is a measure of the strength of the rhythm, where RAE = 0 defines a perfect sine wave and RAE = 1 defines a rhythm that is not statistically significant (Plautz, et al., 1997); b) rhythm was dependent on FRQ and c) mesor value exceeded that found when using a minimal promoter. The first 12 h were excluded, to allow for the inactivation of luciferase synthesized during entrainment. At least three biological replicates were tested per reporter strain, each with at least three technical replicates.

2.2.11. Strains and growth conditions

The general conditions for Neurospora growth and maintenance, as well as routine manipulative procedures, have previously been described (Davis and de Serres, 1970) and so the interested reader is encourage to review that reference for specific details. Crosses were performed on Westergaard and Mitchell's synthetic crossing media (Westergaard and Mitchell, 1947) and resulting ascospores where analyzed on media containing 1X Vogel's salts, 2% w/v yeast extract, 1X FGS (0.05% fructose, 0.05% glucose, 2% sorbose), and 1% agar, supplemented with hygromycin (200 μg/mL) when necessary. When Ignite was used as a

selective agent in a sexual cross, ascospores were analyzed on Vogel's salts lacking NH_4NO_3, 1X FGS (0.05% fructose, 0.05% glucose, 2% sorbose), 1% agar and 400 µg/mL Ignite. Ascospores were picked under a NIKON SMZ-645 dissecting microscope and cultured on Vogel's Media supplemented with the appropriate selective agent.

Knockout strains (KO) were obtained from the Fungal Genetics Stock Center (Kansas City, Missouri USA, http://www.fgsc.net/), which were generated through the Neurospora Functional Genomics project (grant P01GM68087). The method used to generate the KO strains was a PCR-based gene deletion strategy leading to a start- to stop- codon deletion of each of the ORFs in the Neurospora genome (Colot, et al., 2006). As part of the deletion process, each ORF was replaced with the *hph* gene, conferring resistance to hygromycin. A complete list of the KO strains available can be obtained here: http://www.fgsc.net/ncrassa.html. The only exception is the *NCU01856* KO strain, which we generated for this study using a *bar*-resistance cassette (Montenegro-Montero, et al., in preparation). Strains used for particular experiments, are detailed in their corresponding Methods section.

2.2.12. Phenotypic assays

For all phenotypic assays, 10^5 conidia from 7 day-old slants cultures were inoculated on the center of Petri dish plates containing the media described in the corresponding Figure legends. Unless otherwise stated, all phenotypic assays were performed in DD at 34 °C for 3 days (as in Vitalini, et al., 2007), after which plates were placed in LL for 24 h at 25 °C before imaging.

For phenotypic assays in slants (See Section 2.3.10), strains were grown on slants in Vogel's Media for 6 days in constant light at 25 °C.

Fludioxonil (SCHOLAR® 230 SC, Syngenta) was a kind gift by Bernardo Latorre (School of Agronomical Sciences, P. Universidad Católica de Chile).

The Δ*asl-1* strain was a kind gift by Deborah Bell-Pedersen (Department of Biology, Texas A&M University). As this strain harbors the *ras-1*^{bd} allele, the WT strain used as a comparison in all assays including this strain, was 328-4 (*ras-1*^{bd}, A).

The following strains were used in phenotypic assays: WT (FGSC #2489), Δ*os-2* (FGSC #17933), Δ*nuc-1* (FGSC #11448), Δ*cut-1* (FGSC #20351). Detailed information about these strains can be found at http://www.fgsc.net/.

2.2.13. Culture conditions for gene expression analysis upon osmotic stress.

The strain used for osmotic stress assays was FGSC #9720, transformed with the constructs described in Section 2.3.9. Conidia (10^5 conidia/mL) were inoculated in flasks containing 80 mL of liquid Vogel's media (1X Vogel's salts, 2% (w/v) sucrose, 0.5% (w/v) arginine and 50 ng/mL biotin). Flasks were placed on orbital shakers (125 rpm) in incubators (Percival Scientific) in LL and 25 °C for 24 h. After 24 h, flasks were moved to DD in incubators (Percival Scientific) for another 24 h, after which 5 M NaCl was added directly to the culture medium to a final concentration of 1 M for 1 h. Mycelia were then harvested, dried by vacuum

filtration and wrapped in aluminium foil. Mycelia was then rapidly frozen in liquid nitrogen and stored at -80°C.

2.2.14. RNA extraction and reverse transcription

Total RNA was prepared essentially as described by Kramer (Kramer, 2007), with minor modifications. We added a second chloroform extraction and a second ethanol wash, which in our hands, resulted in RNA with a higher 260/230 ratio. The concentration of each RNA sample was measured using the Nanodrop 2000 Spectrophotometer (Thermo Scientific). All of the RNA samples had a 260/280 ratio > 1.9 and a 260/230 ratio of > 1.9. RNA integrity was verified on 1% agarose gel with ethidium bromide staining. Prior to cDNA synthesis, RNA samples were treated with RQ1 RNase-free DNase (Promega) according to manufacturer's instructions. The absence of contaminating genomic DNA in the RNA samples was checked with real-time PCR (qPCR) using RNA as template, using the StepOnePlus™ system (Applied Biosystems) in a 96-well plate format (Applied Biosystems, cat. number 4346907). These -RT reactions contained SensiMix SYBR Hi-ROX mix (Bioline Inc. USA) (6.25 μL), 0.25 μL of a mix of specific forward and reverse primers for *NCU04173*/*actin* (10 μM each, see Table 2), 1 μL nuclease-free water and 5 μL of RNA (2.5 ng/μL). The cycling conditions were as follows: 10 min at 95 °C and 40 cycles of 15 s at 95 °C, 15 s at 66 °C and 15 s at 72 °C, followed by melt curve analysis (ran from 60 °C to 95 °C with 0.3 °C increments). For most of the -RT samples, no Cq was recorded. In the few samples where one was recorded, it was at least 15 cycles higher than that obtained from the corresponding cDNA sample, at the same

Table 2. Primers for qPCR analyses.

Gene	Primer Name	Orientation	Sequence (5' to 3')
NCU04173	oL101	FW	TTACCGAGGCTCCCATCAAC
	oL102	RC	GGCCTGGATGGAGACGTAGA
oLUC	oL315	FW	CGTCACCTCCCACCTCCC
	oL316	RC	CCGGAGGAGTTCATGATGAGG

concentration. We thus considered these RNA samples free from gDNA that could make a significant contribution to the cDNA quantification.

Reverse transcription was performed on 0.5 μg of purified RNA using SuperScript III (Invitrogen) and anchored oligo-dT, according to manufacturer's instructions. The reaction was subsequently diluted 10 times with nuclease-free water (Invitrogen), to 2.5 ng/μL cDNA (total RNA equivalents) and stored at – 20 °C.

2.2.15. Real-time PCR.

Every primer pair was subjected to optimization in order to obtain reaction efficiencies of 90-100% and $r^2 > 0.99$. Different annealing temperatures and primer and $MgCl_2$ concentrations were tested for each primer pair until the aforementioned criteria was met. For gene expression studies, PCR reactions contained the SensiMix SYBR Hi-ROX mix (Bioline Inc. USA) (6.25 μL), 0.25 μL of a mix of specific forward and reverse primers (10 μM each), 1 μL nuclease-free water and 5 μL of cDNA (0.08 ng/μL). Cycling conditions were as described above, although 56 °C and 66 °C were used as the annealing temperatures for the *luciferase* and *NCU04173* primer pairs respectively, which were determined to be optimal under our conditions (not shown). Assay efficiency was assessed using serial dilutions of synthetic 60 bp single-stranded unmodified oligonucleotides composed of the first and last 30 bases of the "true" amplicon. Ten-fold serial dilutions of these oligonucleotides were prepared in nuclease-free water with 10 ng/μL yeast tRNA (Life Technologies) as carrier. Extensive comparative analyses between double-stranded full length amplicons and short synthetic single-stranded

oligonucleotides (as described above) for efficiency determination, have yielded no significant differences (Hellemans, et al., 2013). The formula $E = (10^{-1/slope}-1)$ was used to calculate the amplification efficiency. Specificity was evaluated by both melt curve and agarose gel analyses. Primers were designed using either Primer3 (Untergasser, et al., 2012) or OligoArchitect (Sigma). Every qPCR run in this study included corresponding non-template controls to evaluate reagent contamination. Data was analyzed using the REST (Relative Expression Software Tool) suite (v. 2009), derived from (Pfaffl, et al., 2002) and plotted with GraphPad Prism v. 6.01 (GraphPad Software, Inc.). All qPCR reactions were run in the StepOnePlus™ system (Applied Biosystems), as described above.

2.2.16. Genomic DNA extraction.

For genomic DNA extraction, conidia were inoculated on 4 mL of liquid Vogel's Media for 24 h at 30 °C, on a shaker (200 rpm). Mycelia were harvested, dried and snap frozen with liquid nitrogen. Mycelia were ground and processed as in (Larrondo, et al., 2001).

To test for the presence of the *ada-3* locus in the WT, FGSC #11070 and xc1008-52 and xc1008-71 strains (referred to as Clones 52 and 71, respectively), we used the following primers: Forward 5'- GCCTCGGAGGGGAGTCCAT-3'; Reverse 5'- GTCGCACCGCCTCAAGTTC-3'. A PCR targeting the promoter region of *NCU03753*/*ccg-1* was used as a control, to test for the integrity of the genomic DNA analyzed (using primers oL219-oL131, see Table 1).

2.2.17. Genome scanning with Position Weight Matrices (PWMs)

We extracted the 3 kb upstream region of each annotated gene in Neurospora (Neurospora genome release 5) and used a custom perl script (courtesy of Matt Weirauch, Cincinnati Children's Hospital Medical Center) to scan them using our PBM-derived PWMs (hosted at CisBP v. 0.6). The script scores sequences with a given PWM using the standard log-odds scoring system (Stormo, 2000). For each scan, we classified as "putative targets" those with scores equal or better than 85% of the highest possible score for that PWM. The script is available upon request. For the identification of putative TF target genes associated with particular Gene Ontology (GO) categories, we first obtained the GO list from the *Neurospora crassa* Database. The genes in each GO category were overlapped with the gene list of putative targets for each TF using the hypergeometric distribution (analysis done in collaboration with Matt Weirauch). The cutoff used was $p > 0.01$. The Neurospora GO categories associated with osmotic stress used were GO:0042538, GO:0009651, GO:0006970, GO:0033554 and GO:0006972.

2.3. Results

In the first part of this Chapter, we describe the identification of bZIP proteins in Neurospora and the determination of those under circadian control. In the following section, we report the determination of the sequence specificity of Neurospora transcription factors. In addition, we described how we used that data for the characterization of transcriptional networks controlling rhythmic expression of bZIP proteins. Finally, in the last part of this chapter, we report on the use of the data generated in previous sections to identify novel transcription factors involved in diverse processes in Neurospora.

2.3.1. Identification of bZIP TF proteins in Neurospora

In order to study the bZIP family of transcriptional regulators in Neurospora, we first had to identify all the genes encoding members of this family. Although two previous TF lists have been compiled (Borkovich, et al., 2004; Tian, et al., 2010), we decided to re-analyze the data, as we were confident that these lists were not exhaustive. For instance, neither of them included the putative ortholog of the highly conserved yeast *HAC1* gene, which encodes for a bZIP transcription factor (TF) involved in the unfolded protein response (Gardner, et al., 2013; Mori, et al., 1996; Walter and Ron, 2011). Indeed, orthologs of this TF have been characterized in several species, including many fungi (Joubert, et al., 2011; Mulder, et al., 2004; Richie, et al., 2009; Saloheimo, et al., 2003). We have identified and characterized a putative ortholog of this gene in Neurospora (*NCU01856*) and shown it to be a bona fide sequence-specific DNA-binding protein involved in the unfolded protein response and to be

required for growth in complex carbon sources (Montenegro-Montero, et al., manuscript in preparation).

We decided to scan the whole genome for all types of putative DNA-binding proteins and not just restrict the search to bZIP TFs, in order to revise early lists of putative transcription factors in Neurospora (Borkovich, et al., 2004) and to have a clearer view of sequence-specific transcriptional regulators in this model organism.

To identify TFs, we scanned the amino acid sequences of known or predicted Neurospora proteins for each of the 81 different types of DNA-binding domains (DBDs) for which there is an available Pfam model (Weirauch and Hughes, 2011) (see Materials and Methods), identifying a total of 273 putative TFs (Table 3). This number is close to the median among fungi (203 TF per genome) (Charoensawan, et al., 2010) and represents ~3% of the predicted protein coding genes in Neurospora. Our findings represent an important improvement on the information provided by Tian et al. (2010) and Borkovich et al. (2004) regarding TF annotation, which reported a total of 178 and 174 TFs, respectively. It should be noted that while these two numbers look alike, the overlap between those lists is only of 128 TFs. The overlap between the three lists is not that different from that number, amounting to 116 TFs (Table 3).

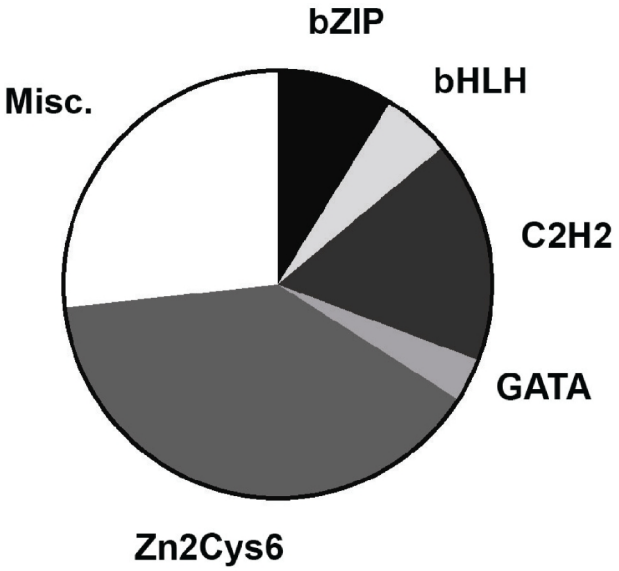
We followed the classification criteria in (Borkovich, et al., 2004) and organized the TFs in 6 families: Zn2Cys6 fungal-specific binuclear cluster, C2H2 zinc finger, Basic Leucine Zipper (bZIP), Basic helix-loop-helix (bHLH), GATA and Miscellaneous factors, the latter including

Table 3. Predicted transcription factor coding genes in the Neurospora genome

Borkovich et al. 2004		Tian et al. 2010		This study			
NCU00017	NCU04851	NCU00017	NCU04673	NCU00017	NCU03266	NCU06707	NCU11359
NCU00019	NCU04866	NCU00019	NCU04731	NCU00019	NCU03273	NCU06744	NCU11569
NCU00038	NCU05051	NCU00038	NCU04827	NCU00024	NCU03352	NCU06799	NCU11571
NCU00054	NCU05064	NCU00090	NCU04851	NCU00038	NCU03416	NCU06874	NCU11723
NCU00090	NCU05210	NCU00144	NCU04866	NCU00090	NCU03417	NCU06907	NCU11965
NCU00097	NCU05242	NCU00155	NCU05022	NCU00100	NCU03481	NCU06919	NCU12126
NCU00144	NCU05250	NCU00285	NCU05024	NCU00144	NCU03489	NCU06920	
NCU00217	NCU05257	NCU00289	NCU05051	NCU00155	NCU03536	NCU06965	
NCU00233	NCU05285	NCU00340	NCU05064	NCU00223	NCU03552	NCU06971	
NCU00285	NCU05294	NCU00344	NCU05207	NCU00282	NCU03593	NCU06975	
NCU00289	NCU05383	NCU00445	NCU05242	NCU00285	NCU03643	NCU06990	
NCU00329	NCU05411	NCU00499	NCU05257	NCU00289	NCU03649	NCU06994	
NCU00340	NCU05536	NCU00694	NCU05285	NCU00329	NCU03686	NCU07039	
NCU00385	NCU05637	NCU00749	NCU05294	NCU00340	NCU03699	NCU07139	
NCU00499	NCU05767	NCU00808	NCU05308	NCU00344	NCU03725	NCU07146	
NCU00631	NCU05909	NCU00902	NCU05383	NCU00392	NCU03728	NCU07246	
NCU00694	NCU05970	NCU01097	NCU05411	NCU00445	NCU03892	NCU07312	
NCU00749	NCU05993	NCU01122	NCU05685	NCU00499	NCU03905	NCU07374	
NCU00808	NCU05994	NCU01154	NCU05733	NCU00694	NCU03931	NCU07379	
NCU00902	NCU06028	NCU01209	NCU05767	NCU00749	NCU03938	NCU07392	
NCU00945	NCU06068	NCU01238	NCU05891	NCU00833	NCU03960	NCU07411	
NCU01097	NCU06173	NCU01312	NCU05909	NCU00902	NCU03975	NCU07430	
NCU01122	NCU06186	NCU01345	NCU05993	NCU00945	NCU04001	NCU07543	
NCU01154	NCU06205	NCU01386	NCU06028	NCU01074	NCU04022	NCU07568	
NCU01345	NCU06213	NCU01459	NCU06145	NCU01097	NCU04050	NCU07575	
NCU01386	NCU06399	NCU01478	NCU06173	NCU01122	NCU04058	NCU07587	
NCU01459	NCU06407	NCU01629	NCU06186	NCU01145	NCU04079	NCU07669	
NCU01478	NCU06411	NCU01640	NCU06205	NCU01154	NCU04179	NCU07675	
NCU01629	NCU06487	NCU01706	NCU06407	NCU01204	NCU04211	NCU07705	
NCU01871	NCU06503	NCU01871	NCU06487	NCU01209	NCU04353	NCU07728	
NCU01954	NCU06656	NCU01994	NCU06503	NCU01238	NCU04359	NCU07788	
NCU01994	NCU06744	NCU02142	NCU06656	NCU01243	NCU04390	NCU07834	
NCU02017	NCU06799	NCU02173	NCU06744	NCU01312	NCU04459	NCU07855	
NCU02094	NCU06907	NCU02182	NCU06799	NCU01345	NCU04561	NCU07863	
NCU02142	NCU06919	NCU02203	NCU06907	NCU01386	NCU04619	NCU07893	
NCU02173	NCU06971	NCU02214	NCU06919	NCU01414	NCU04628	NCU07945	
NCU02214	NCU06990	NCU02307	NCU06920	NCU01459	NCU04663	NCU07952	
NCU02307	NCU07007	NCU02315	NCU06944	NCU01461	NCU04671	NCU08000	
NCU02356	NCU07039	NCU02323	NCU06971	NCU01478	NCU04729	NCU08003	
NCU02576	NCU07139	NCU02356	NCU06994	NCU01629	NCU04731	NCU08042	
NCU02666	NCU07374	NCU02432	NCU07007	NCU01640	NCU04770	NCU08049	
NCU02671	NCU07379	NCU02487	NCU07039	NCU01706	NCU04773	NCU08055	
NCU02699	NCU07392	NCU02576	NCU07139	NCU01734	NCU04827	NCU08063	
NCU02713	NCU07535	NCU02621	NCU07180	NCU01856	NCU04830	NCU08159	
NCU02724	NCU07561	NCU02666	NCU07374	NCU01871	NCU04848	NCU08184	
NCU02752	NCU07669	NCU02671	NCU07392	NCU01924	NCU04851	NCU08289	
NCU02768	NCU07705	NCU02699	NCU07543	NCU01958	NCU04866	NCU08294	
NCU02853	NCU07728	NCU02713	NCU07586	NCU01960	NCU04960	NCU08307	
NCU02896	NCU07788	NCU02724	NCU07705	NCU01994	NCU05022	NCU08480	
NCU02934	NCU07900	NCU02752	NCU07728	NCU02012	NCU05024	NCU08512	
NCU02957	NCU07945	NCU02787	NCU07788	NCU02094	NCU05051	NCU08527	
NCU02994	NCU07952	NCU02853	NCU07863	NCU02142	NCU05061	NCU08594	
NCU03033	NCU08000	NCU02896	NCU07952	NCU02173	NCU05064	NCU08634	
NCU03043	NCU08042	NCU02934	NCU08000	NCU02182	NCU05145	NCU08651	
NCU03070	NCU08049	NCU02957	NCU08003	NCU02214	NCU05208	NCU08652	
NCU03073	NCU08055	NCU02994	NCU08042	NCU02307	NCU05242	NCU08658	
NCU03077	NCU08063	NCU03043	NCU08055	NCU02315	NCU05257	NCU08726	
NCU03110	NCU08289	NCU03070	NCU08063	NCU02326	NCU05285	NCU08744	
NCU03120	NCU08294	NCU03110	NCU08294	NCU02356	NCU05294	NCU08807	
NCU03184	NCU08443	NCU03120	NCU08307	NCU02386	NCU05308	NCU08848	
NCU03206	NCU08634	NCU03184	NCU08443	NCU02413	NCU05383	NCU08891	
NCU03244	NCU08651	NCU03206	NCU08594	NCU02487	NCU05411	NCU08932	
NCU03266	NCU08652	NCU03244	NCU08634	NCU02525	NCU05414	NCU08999	
NCU03320	NCU08658	NCU03266	NCU08651	NCU02558	NCU05536	NCU09033	
NCU03356	NCU08726	NCU03320	NCU08652	NCU02576	NCU05635	NCU09068	
NCU03417	NCU08744	NCU03417	NCU08726	NCU02666	NCU05637	NCU09120	
NCU03421	NCU08807	NCU03421	NCU08807	NCU02671	NCU05685	NCU09197	
NCU03489	NCU08848	NCU03536	NCU08848	NCU02695	NCU05733	NCU09205	
NCU03536	NCU08891	NCU03552	NCU08854	NCU02699	NCU05767	NCU09214	
NCU03552	NCU08899	NCU03593	NCU08901	NCU02713	NCU05891	NCU09315	
NCU03593	NCU08901	NCU03643	NCU08999	NCU02719	NCU05909	NCU09329	
NCU03643	NCU09033	NCU03686	NCU09068	NCU02724	NCU05970	NCU09333	
NCU03686	NCU09068	NCU03699	NCU09197	NCU02752	NCU05996	NCU09387	
NCU03699	NCU09205	NCU03725	NCU09205	NCU02787	NCU06028	NCU09496	
NCU03905	NCU09248	NCU03892	NCU09252	NCU02819	NCU06095	NCU09529	
NCU03931	NCU09252	NCU03905	NCU09315	NCU02853	NCU06145	NCU09556	
NCU03962	NCU09315	NCU03931	NCU09329	NCU02896	NCU06173	NCU09576	
NCU03975	NCU09333	NCU04001	NCU09333	NCU02934	NCU06186	NCU09615	
NCU04001	NCU09529	NCU04022	NCU09496	NCU02957	NCU06324	NCU09739	
NCU04050	NCU09549	NCU04050	NCU09529	NCU03033	NCU06399	NCU09915	
NCU04179	NCU09576	NCU04179	NCU09576	NCU03043	NCU06407	NCU09973	
NCU04211	NCU09739	NCU04211	NCU09739	NCU03070	NCU06487	NCU09995	
NCU04359	NCU09804	NCU04353	NCU09804	NCU03077	NCU06503	NCU10006	
NCU04390	NCU09829	NCU04359	NCU10006	NCU03110	NCU06521	NCU10080	
NCU04561	NCU10006	NCU04390	NCU10080	NCU03120	NCU06551	NCU10346	
NCU04619		NCU04459	NCU10284	NCU03126	NCU06560	NCU10570	
NCU04628		NCU04561	NCU10346	NCU03184	NCU06614	NCU10597	
NCU04731		NCU04619	NCU10459	NCU03206	NCU06656	NCU11222	
NCU04827		NCU04628	NCU10679	NCU03248	NCU06701	NCU11295	

all proteins belonging to families that do not fit the other major categories (Figure 2.3).

A total of 23 predicted bZIP proteins were identified in the Neurospora genome using our selected criteria. This number includes *hac-1* (*NCU01856*), which escaped identification in previous analyses, as well as several new ones. We also added *NCU00329* to this list, bringing the total to 24. This gene was previously suggested as a bZIP TF encoding gene that when disrupted, leads to growth phenotypes in Neurospora (Colot, et al., 2006). Our search criteria indeed found this gene, but it did not meet our cutoff. Considering the phenotypic evidence, we decided to include it in our list. The latest published tally included only 9 bZIP TFs (Tian, et al., 2010), all of which are included in our list and were also present in early studies of the Neurospora genome sequence (Borkovich, et al., 2004). Our list resembles more the one published by Borkovich et al. (2004), which listed 17 bZIP proteins, including *NCU00329*. The ones in our list that are missing from (Borkovich, et al., 2004) are the following: *NCU01856*, *NCU06965*, *NCU04058*, *NCU01074*, *NCU01204*, *NCU07146*, *NCU07312* and *NCU11571*. Interestingly, for three of these novel ones, we have evidence for sequence-specific DNA-binding activity, suggesting they are bona fide TFs (See Section 2.3.4). As expected (considering our search method), alignment of the bZIP domain of the 24 identified bZIP proteins, reveals a clear basic region, essential for DNA binding of bZIP TFs, and a leucine zipper motif, involved in homo- and heterodimerization (Figure 2.4) (Hurst, 1994; Landschulz, et al., 1988).



TF Family	Number of predicted members
bHLH	14
bZIP	24
C2H2	46
GATA	9
Zn2Cys6	107
Misc.	73

Figure 2.3. Identification of putative DNA-binding proteins in *Neurospora crassa*. The sequences of all predicted proteins in the *Neurospora crassa* genome were scanned for putative DNA-binding domains using the 81 Pfam models listed in (Weirauch and Hughes, 2011). Each protein was classified into a family based on its identified DBD.

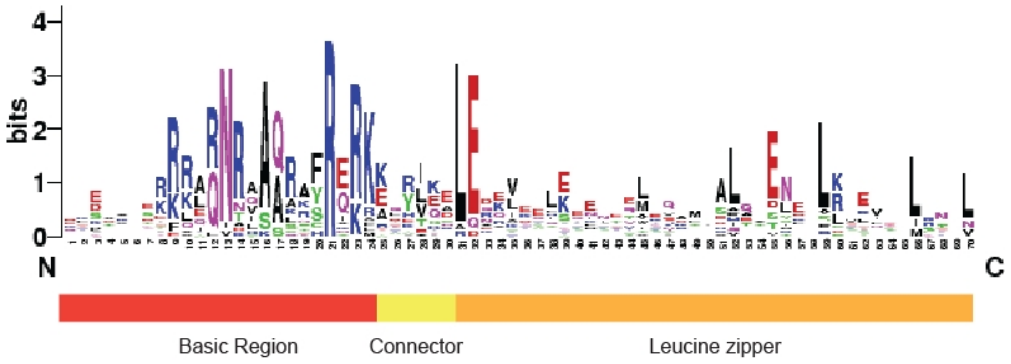


Figure 2.4. Alignment of the bZIP region of the predicted members of the Basic Leucine Zipper family in Neurospora. The identified bZIP domain of the proteins predicted to be members of this family of transcriptional regulators in Neurospora, was aligned and a graphical representation of the pattern present within the multiple sequence alignment is shown. The Figure was generated using Weblogo (http://weblogo.berkeley.edu/logo.cgi). The basic region, the connector (also known as fork, joint or linker) and the leucine zipper region are highlighted.

2.3.2. Phylogenetic analysis of Neurospora bZIP family members

In order to position the identified Neurospora bZIP TFs among its putative orthologs and identify possible relationships among the fungal bZIP proteins, we performed phylogenetic analysis using the full protein sequence of predicted bZIP TFs from several fungal species, including the hemiascomycete *S. cerevisiae* (18 bZIP TFs) and *Candida albicans* (12 bZIP TFs), a species at the base of the hemiascomycete clade, *Yarrowia lipolytica* (11 bZIP TFs), the archiascomycete species *Schizosaccharomyces pombe* (8 bZIP TFs) and the euascomycete species *Aspergillus nidulans* (29 bZIP TFs), *Magnaporthe oryzae* (30 bZIP TFs) and *Neurospora crassa* (24 bZIP TFs) (See Materials and Methods). For a detailed analysis of the phylogenetic relationship between these species see (Fitzpatrick, et al., 2006).

The resulting phylogenetic tree exhibits two separate clades. One of them is small, in the sense that it only includes a few bZIP TFs (Clade I), and the other one is further divided into two large clades (Clades II and III) (Figure 2.5). Clade I includes known or predicted *HAC1* and *GCN4* orthologs. This group lacks *S. pombe* members, consistent with the fact that this fungi lacks both a *HAC1* (Kimmig, et al., 2012) and a *GCN4* (Udagawa, et al., 2008) ortholog. Clade II includes all known members of the well characterized yeast YAP subclass (Fernandes, et al., 1997), involved in stress responses (Rodrigues-Pousada, et al., 2010). The Neurospora AP-1 protein (NcAp-1, encoded by *NCU03905*), involved in oxidative stress responses and a functional ortholog of the yeast *YAP1* gene (Tian, et al., 2010), is present in this clade. Clade III on the other hand, includes all members of the yeast ATF/CREB subclass (Fujii, et al., 2000). The Neurospora bZIP proteins are distributed evenly throughout the tree,

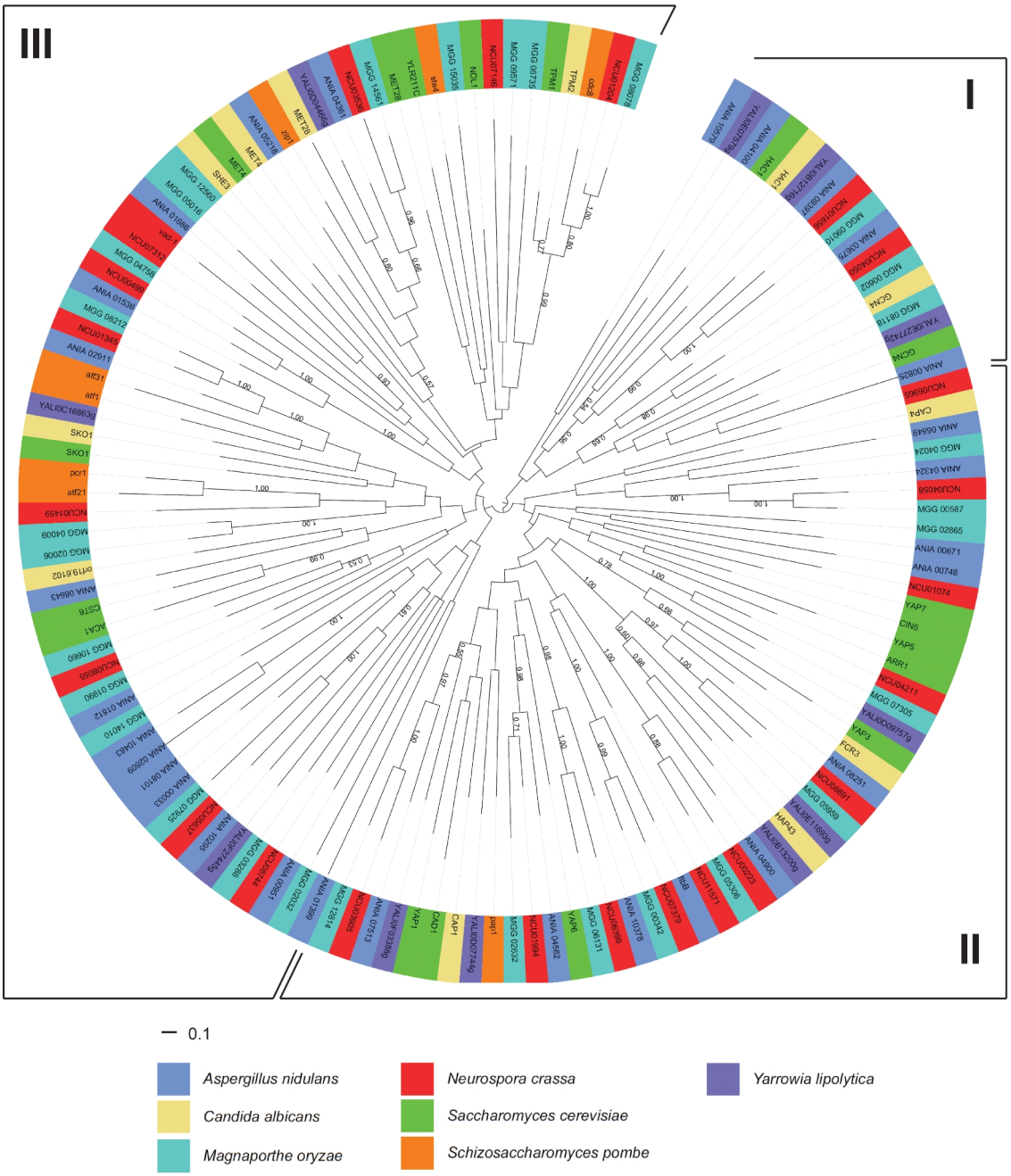


Figure 2.5. Phylogenetic analysis of the bZIP TF gene family in ascomycete species. The complete sequence of all predicted bZIP proteins from 7 ascomycete fungi were retrieved from CisBP and subjected to phylogenetic analysis using Maximum Likelihood (see Materials and Methods). Each TF is color-coded according to the species it belongs to. The scale bar indicates a distance equivalent to 0.1 amino acid substitution per site. 100 bootstrap replicates were performed. The roman numbers indicate different groups, which are described in the main text.

generally clustering, as expected, more closely with proteins from other filamentous fungi, rather than with the yeast proteins.

Among the euascomycete species, no recent duplication events concerning bZIP proteins could be inferred in Neurospora (See Materials and Methods), which is expected as this species has several mechanisms to protect its genome against sequence duplication (Galagan and Selker, 2004), and overall, only two duplications could be detected using our criteria within the analyzed euascomycete fungi. In *M. oryzae* we detected a putative recent gene duplication (*MG02006* and *MG04009*), which clusters together with the product of the Neurospora gene *NCU04059*, a putative ortholog of the *S. pombe* PCR1 TF (Watanabe and Yamamoto, 1996). The same event was identified by (Tian, et al., 2007), supporting our overall sequence analysis, although it should be mentioned that these authors provided no information regarding their analysis for paralog detection. The second putative paralog pair identified was in *Aspergillus nidulans* (ANIA10483 and ANIA02809). Although several proteins cluster together in the same clade, none has been characterized molecularly. This general paucity in duplications among the euascomycete, contrasts with what has been observed for bZIPs in yeasts, in which it is clear that ACA1/CST6 and the YAP family in *S. cerevisiae*, together with some members in the ATF/PCR1 family in *S. pombe*, have expanded via duplication (Figure 2.5). As a whole, our analysis suggests, in agreement with what was reported by Tian et al. (2010), that in fungi, the major expansion of the bZIP family occurred before the divergence of the euascomycete from the aforementioned yeast species.

No studies have been published on the identified genes involved in the aforementioned putative duplications, so their functional relationship, that is, if non-functionalization (i.e. one of the copies has acquired an inactivating mutation), neo-functionalization (i.e. one of the copies has acquired a new function), sub-functionalization (i.e. original function has been subdivided among the duplicated genes) or simple gene conservation (i.e. both copies have retained the original function) has occurred (Innan and Kondrashov, 2010), awaits experimental evidence.

2.3.3. Identification of Neurospora bZIP encoding genes with circadian expression profiles

To identify bZIP encoding genes whose corresponding proteins products could play a role in the Neurospora circadian system, we set out to identify those whose expression was under circadian control. Indeed, the few TFs (only two, see Introduction) that have been identified to participate in output pathways, all have clock input and exhibit rhythmic expression patterns (Sancar, et al., 2011; Smith, et al., 2010). We note however, that it is also possible for TFs without rhythmic profiles, to still impact circadian machinery (for example WC-2; (Cheng, et al., 2001)).

To identify rhythmic bZIP TF, we used transcriptional reporter constructs based on a codon-optimized luciferase gene (Gooch, et al., 2008) (See Materials and Methods). The use of luciferase reporters have been implemented in all clock model systems as it provides a relatively simple way to study temporally regulated transcription (Corellou, et al., 2009; Gooch, et al., 2008; Kondo, et al., 1993; Michael and McClung, 2003; Millar, et al., 1995;

Stempfl, et al., 2002; Welsh, et al., 2005). This tool has greatly simplified experiments in chronobiology and allows high-resolution studies over long time periods (several days), enabling researchers to quantitatively approach this phenomenon and precisely monitor the rhythms in gene expression that underlie circadian clock function.

We generated reporter constructs by fusing this codon-optimized luciferase gene to the promoter region of all 24 identified bZIP TF encoding genes and then evaluated bioluminescence under constant conditions for several days.

Through this approach, we identified 17 promoter regions from bZIP encoding genes whose activity results in circadian rhythms in bioluminescence (for criteria used to determine circadian genes, see Materials and Methods) (Figure 2.6). These rhythms were lost in strains harboring a defective clock, suggesting that these rhythms were indeed driven by a functional FWO. The promoters of all of the identified rhythmic bZIP encoding genes lead to (subjective) daytime increases in bioluminescence, similar to the situation when using the *frq* promoter construct (Figure 2.7B and Gooch, et al., 2008. See also Figure 1.5B). The rest of the bZIP encoding genes (7) had background levels of expression in our setting, so we could not define whether their transcription was controlled by the clock or not. This could be due to their expression being growth condition-dependent or simply because the promoter region chosen could not support above-background expression of the reporter under our conditions.

Considering that microarray approaches have suggested that only ~20% of the transcriptome is under circadian control in Neurospora (Correa, et al., 2003; Dong, et al., 2008), one could

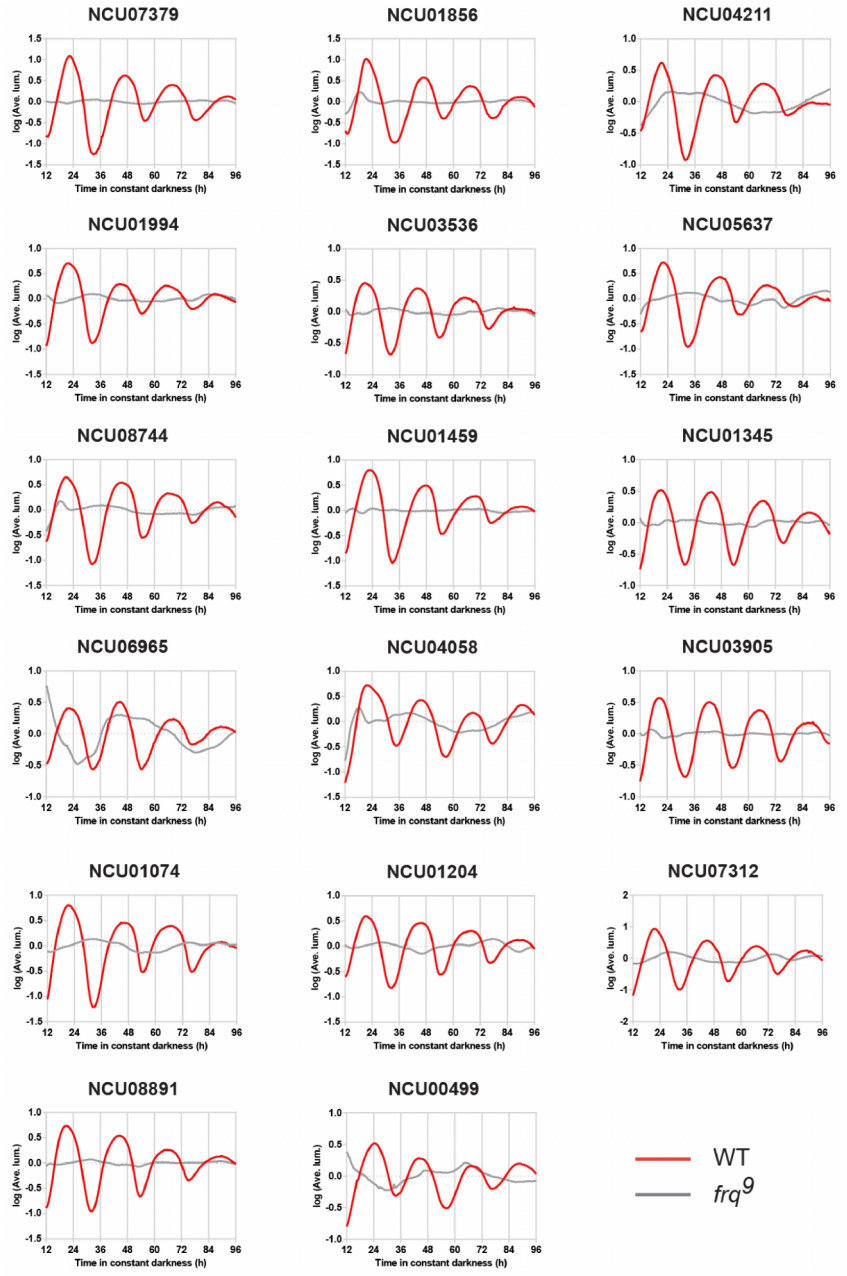


Figure 2.6. Identification of rhythmically expressed bZIP encoding genes in Neurospora. The promoter region of all predicted bZIP encoding genes in Neurospora was fused to luciferase and bioluminescence traces were tracked under constant conditions to identify promoters under circadian control. Luciferase activity was evaluated in clock-functional (WT, red traces) and clock-defective (*frq*^{9}, gray traces) strains to identify true circadian regulation by the FWO. In *frq9* strains, a premature stop codon mutation in the *frq* gene results in a non-functional FRQ protein and consequently, overall arhythmicity in FWO-dependent processes. Data was analyzed using Spectrum Resampling (see Materials and Methods). Traces are representative from at least three biological replicates, each with three technical replicates per run.

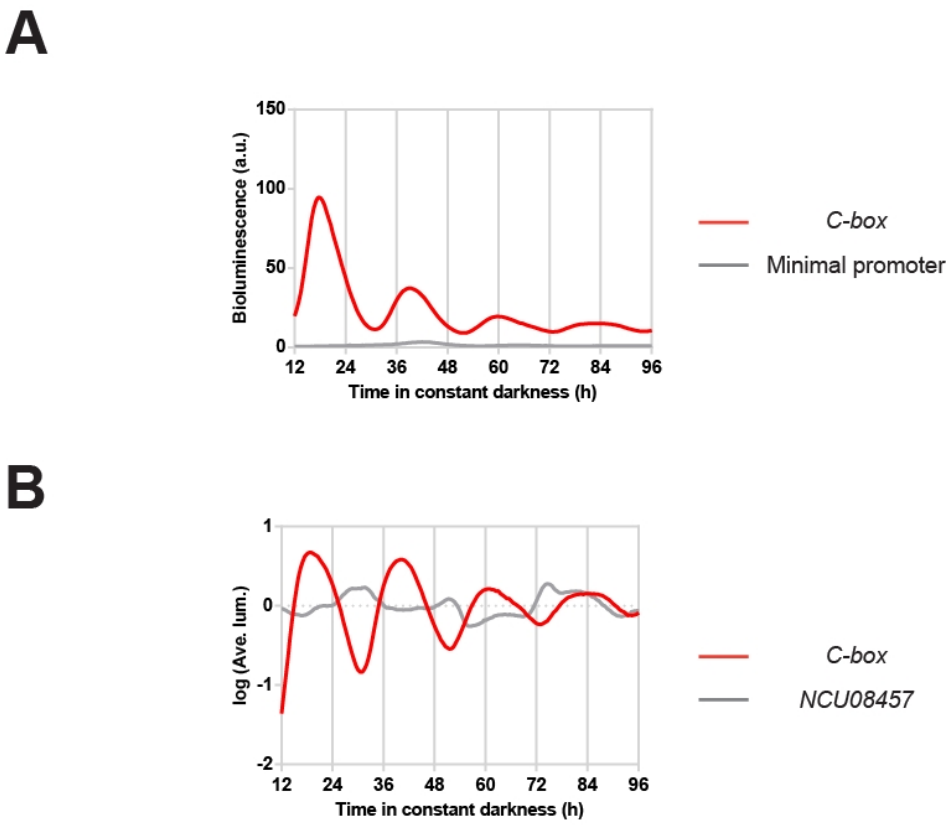


Figure 2.7. Control reporter constructs. A) Analysis of bioluminescence generated by a positive control construct (*C-box*, red trace) and a construct bearing a minimal promoter (gray trace). The *C-box* construct contains *cis* regulatory elements that are sufficient for rhythmic transcription (see Materials and Methods). The promoter region used in the minimal promoter construct is derived from the same gene as the one in the *C-box* construct (*frq*), but lacks the elements required for rhythmicity. Raw bioluminescence data is shown, to highlight the differences in bioluminescence levels generated from each construct. B) The detrended bioluminescence data generated from a construct containing the promoter region of *NCU08457* (grey trace) was compared to the one derived from the *C-box* construct (red trace). Data was analyzed using Spectrum Resampling (see Materials and Methods). The data resulting from the *NCU08457* construct is arrhythmic according to our criteria (see text for details). All constructs were integrated at the *his-3* locus. A.U. arbitrary units.

argue that the large fraction of bZIP encoding genes tested that displayed rhythmic transcription profiles, may simply be an artifact, derived for instance, from the particular locus chosen for the genome integration of the constructs (the *his-3* locus). In other words, it could be argued that the *his-3* locus may have rhythmic input and consequently, any reporter integrated at this locus, would be identified as rhythmic. Several lines of evidence however, suggest that this is not the case. First, the profiles generated by each of the constructs are different. The phase and waveforms do not match between the constructs, suggesting specific circadian parameters associated with each sequence cloned upstream of the reporter (compare for instance, the phase of *NCU00499* and *NCU01204* in Figure 2.6, or of any of them to the one exhibited by *frq*, as shown in Figure 2.7B). Second, genetic perturbation impacts each reporter differently. Particular mutations affect rhythmicity of certain reporters at the *his-3* locus, but not of others (see Sections 2.3.6 and 2.3.10). Third, integration of a construct bearing a minimal promoter at the *his-3* locus, results in low levels of bioluminescence, which can be observed by plotting the raw data (Figure 2.7A) and fourth, and possibly more relevant, not all constructs targeted at the *his-3* locus oscillate (Figure 2.7B).

Altogether, this suggests that indeed a large fraction of the bZIP TF encoding genes exhibit rhythmic transcription profiles in Neurospora. It should be mentioned that it was early noticed, in other systems, that rhythms in transcription do not necessarily translate into rhythms of mRNA or protein levels (Millar and Kay, 1991; Wuarin, et al., 1992; Wuarin and Schibler, 1990). In fact, more recent evidence has shown that over 50% of the genes that exhibit cycling transcription, lack a rhythm for the corresponding mRNA (Koike, et al., 2012; Le Martelot, et al., 2012; Menet, et al., 2012). Further, recent global studies have shown that only a small

fraction of the genes that do exhibit rhythms at the mRNA level, have corresponding transcriptional rhythms, highlighting a role for post-transcriptional mechanisms on controlling rhythmic mRNA accumulation (Koike, et al., 2012; Le Martelot, et al., 2012; Menet, et al., 2012). Similarly, only about half of the proteins that exhibit rhythms in accumulation in mouse liver, have corresponding cyclically expressed mRNAs (Reddy, et al., 2006). Due to these issues, it is difficult to asses, using a single approach, whether all the steps of gene expression of a particular gene that has been shown to have clock input are rhythmic, or just a few. Considering our experimental setting (i.e. reporter constructs), which is amenable to high throughput functional genomics (Millar, et al., 1995; Montenegro-Montero and Larrondo, 2013; Zhang, et al., 2009), we decided to focus on a particular level of gene expression, namely transcription, and use that as the starting point of our global analysis to characterize the position the bZIP proteins within the circadian transcriptional chart in Neurospora.

Our rationale was the following: our study has revealed that several bZIPs encoding genes are being rhythmically transcribed. That is a fact, independent of whether the mRNA is rhythmic or if even the protein or its activity, oscillate daily. This rhythm is the result of a functional oscillator and ultimately, of its associated transcriptional networks, as the readout of our experiments is transcriptional regulation. In light of our results, the question we thus decided to focus on was how the rhythmic signal was being channeled through to the rhythmic bZIP encoding genes, or in other words, what are the output pathways that allow these genes to be transcribed with a circadian rhythm? This is a question we could address with our reporter system in a global manner. Again, this is regardless of whether the mRNA or the final protein levels of the bZIP gene in question oscillate or not and we do not focus on those aspects. As

discussed above, that is a different problem and different actors and different stages of gene expression are involved, and the approaches to study it, need to be different (Koike, et al., 2012; Kojima, et al., 2012; Le Martelot, et al., 2012; Menet, et al., 2012; Montenegro-Montero and Larrondo, 2013).

The following section describes our approach for characterizing output pathways mediating rhythmic transcription of bZIP encoding genes in Neurospora.

2.3.4. Determination of the sequence specificity of the Neurospora transcription factors

We reasoned that a genetics approach was not only appropriate, but ideal, to characterize the transcriptional networks that allow rhythms in transcription for bZIP encoding genes, considering the ease with which genetic studies can be performed in Neurospora. As mentioned above, these rhythms are the result of transcriptional networks that are somehow associated with the FWO. Our approach for the study of these networks could be referred to as a "bottoms-up" approach, as we wanted to identify transcriptional regulators that are upstream of the rhythmic bZIP genes in the regulatory cascade emerging from the central oscillator and that are responsible for relaying temporal information (Figure 2.8).

One of our ideas to identify such transcriptional regulators was the following. We would first analyze the promoter region of a selected group of rhythmic bZIPs with the goal of identifying *cis*-regulatory elements associated with certain transcription factors (i.e. sequences matching the motifs generally bound by these TFs). We would then analyze the expression of the

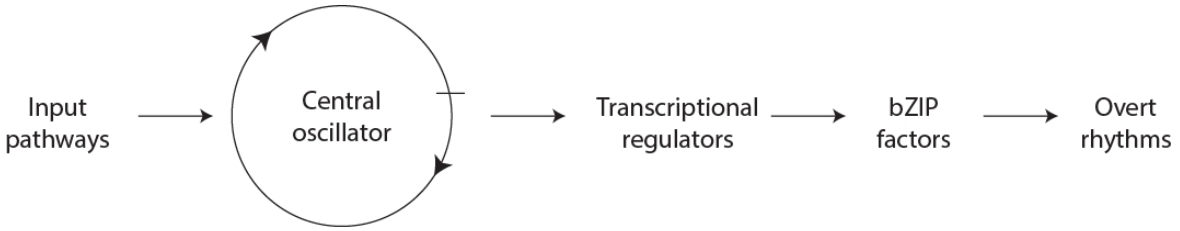


Figure 2.8. Proposed schematic representation of the Neurospora circadian system structure leading to rhythmic bZIP expression. Transcription of several bZIP encoding genes is under circadian control in Neurospora and this rhythmicity depends on a functional oscillator and the transcriptional regulators under its control. These regulators relay the temporal information from the oscillator to the promoter of bZIP encoding genes, whose products can then regulate the expression of its target genes and ultimately, overt rhythms. The identity of these regulators however, is currently unknown.

reporter constructs in strains lacking those transcription factors, to evaluate whether they are relevant for the rhythmic expression of the reporter. This rational approach would help delineate transcriptional networks emerging from the central oscillator and controlling the rhythmic transcription of the bZIP encoding genes of interest.

This approach, while conceptually simple, requires one to know the DNA-binding preference for a large number of TFs. While there is vast information available for organisms like budding yeast (de Boer and Hughes, 2012), there are virtually no experimental DNA-binding data for the TFs in the vast majority of eukaryotes. Accordingly, only a handful of motifs are known for Neurospora TFs (Baum, et al., 1987; Chiang, et al., 1994; Feng, et al., 2000; Fu, et al., 1995; Harrison and Marzluf, 2002; Li and Marzluf, 1996; Peleg and Metzenberg, 1994; Sancar, et al., 2011; Tian, et al., 2007; Tian, et al., 2010). These few motifs have been derived through a variety of methods (EMSA/footprinting, ChIP-seq and promoter analysis of co-regulated genes) and represent only 11 of the predicted 273 TFs in this fungus. This deficit represents a fundamental limitation in our ability to analyze and interpret the function and evolution of DNA sequences, including the implementation of approaches like the one outlined above.

To overcome this limitation, we decided to systematically characterize the DNA-binding preference of Neurospora TFs. This would not only be useful for the implementation of our aforementioned approach, but would also be an important tool for Neurospora functional genomics. In addition, this would be a relevant tool for researchers working on species that are closely related to Neurospora, as it is possible to infer the DNA-binding preference of a TF, if

there is enough similarity in the DNA-binding domain (Weirauch, et al., accepted in *Cell*, available at http://hugheslab.ccbr.utoronto.ca/supplementary-data/CisBP/) (Bernard, et al., 2012).

To accomplish this, and in collaboration with the Hughes Lab (The University of Toronto), we employed protein-binding microarrays (Berger and Bulyk, 2006; Weirauch, et al., 2013), which allow for rapid, high-throughput and unbiased characterization of the sequence specificity of DNA-binding proteins. In protein binding microarrays (PBMs) assays, a tagged version of a DNA-binding protein is 'hybridized' to a microarray that contains ~41.000 35-mer probes, covering all possible 10-mers, followed by the addition of a fluorescently marked antibody against the tag. This reveals the sequences to which the protein of interest has bound and to what degree (See Materials and Methods). As the array contains all possible 10-mers once and only once, all possible 8-mers are present 32 times, allowing for the rapid study of the sequence preference of a DNA-binding protein to all possible 8-mers in different sequence contexts. PBMs allow derivation of position weight matrices (PWMs) as well as IUPAC consensus sequences which can be represented as sequence logos. The PBM methodology results in motifs that are very similar to the ones obtained by more traditional methods (ChIP-seq, promoter analysis of co-expressed genes, etc.), but in a high-throughput manner (see Weirauch, et al, accepted in *Cell*) (Badis, et al., 2008; Berger, et al., 2008; Zhu, et al., 2009).

We set out to clone all predicted TFs in Neurospora to test them in PBMs. We were able to successfully clone 226 of the 273 predicted TF. We then tested all of them in PBM experiments and 134 were deemed successful under our stringent criteria (see Materials and

Methods), including members of all TF families. Not surprisingly, most of the successful experiments correspond to Zn2Cys6 binuclear cluster family members, the most populated family in Neurospora.

Altogether, and organized per family, we obtained motifs for: 86% of the bHLH TFs, 58% of the bZIP TFs, 63% of the C2H2 TFs, 56% of the GATA TFs and 54% of the Zn2Cys6 TFs. Also, we obtained motifs for 39% of the Miscellaneous TFs.

The DNA-binding domains of the 134 DNA-binding proteins for which a motif was obtained, were arranged in a phylogenetic tree and the derived motifs placed at the end of the corresponding branches (Figure 2.9). In addition to these 134, motifs for 11 more Neurospora DNA-binding proteins were obtained based on inference from other organisms and one which has been directly determined via other means was obtained from TRANSFAC (Matys, et al., 2006), bringing the total to 146 Neurospora proteins for which a motif is available (135 directly determined ones). All motifs and related information is accessible at CisBP, http://cisbp.ccbr.utoronto.ca/, with the exception of the information for 16 TFs, which we have excluded from CisBP because we are working on manuscripts that will be including it. These TFs are: *NCU01856*, *NCU03905*, *NCU00329*, *NCU01345*, *NCU00499*, *NCU01154*, *NCU02356*, *NCU08807*, *NCU09315*, *NCU02896*, *NCU02094*, *NCU04731*, *NCU08999*, *NCU04390*, *NCU01629* and *NCU03043*. The information for these TFs is available upon request.

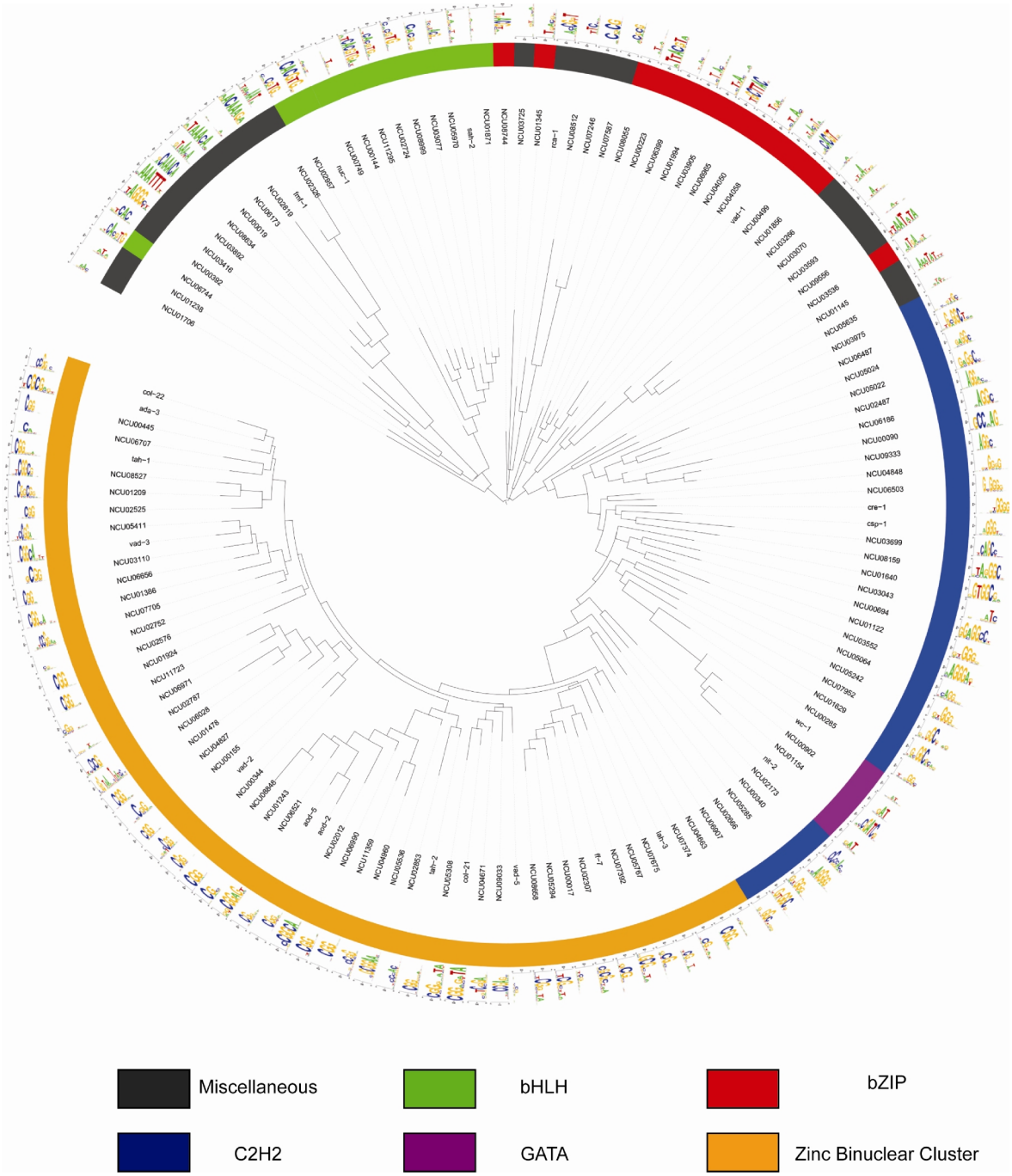


Figure 2.9. Determination of the sequence specificity of Neurospora transcription factors. Neurospora transcription factors were arranged in a phylogenetic tree based on the similarity of their full protein sequences. The corresponding sequence specificity, as determined via protein binding microarrays, is shown at the end of each branch. Motif for *NCU06028* was obtained from TRANSFAC. Only Neurospora transcription factors for which a motif has been directly determined were considered for the analysis. Transcription factors are color-coded based on the family to which they belong.

Our approach, together with our analysis determining motif inference capabilities (see Weirauch, et al., accepted in *Cell*), has resulted in an increase from 4% to over 53% in the coverage of DNA-binding motifs for Neurospora TFs. This places Neurospora as the filamentous fungus with the highest motif coverage, as well as one with the highest number of directly determined motifs among eukaryotes (Figure 2.10).

As has previously been shown for PBM motifs in other systems (Badis, et al., 2008; Berger, et al., 2008; Zhu, et al., 2009), the Neurospora PBM-derived motifs also resemble the ones obtained for the same proteins through other methods, including *in vivo* approaches like ChIP-seq, as shown in Figure 2.11, lending support to the usefulness of these *in vitro* derived motifs.

Considering the wealth of genomics data rapidly emerging from several labs, these motifs can now be integrated in systems biology approaches for the study of a variety of processes in Neurospora. This of course, includes circadian rhythms.

We took these motifs and used them to implement the genetics approach outline above, to characterize transcriptional networks controlling rhythmic bZIP transcription.

2.3.5. Search for upstream regulators controlling rhythmic transcription of selected bZIP genes

As described above, we wanted to take a "bottoms-up" approach to characterize transcriptional networks mediating circadian transcription of bZIP TF encoding genes. We focused on two of

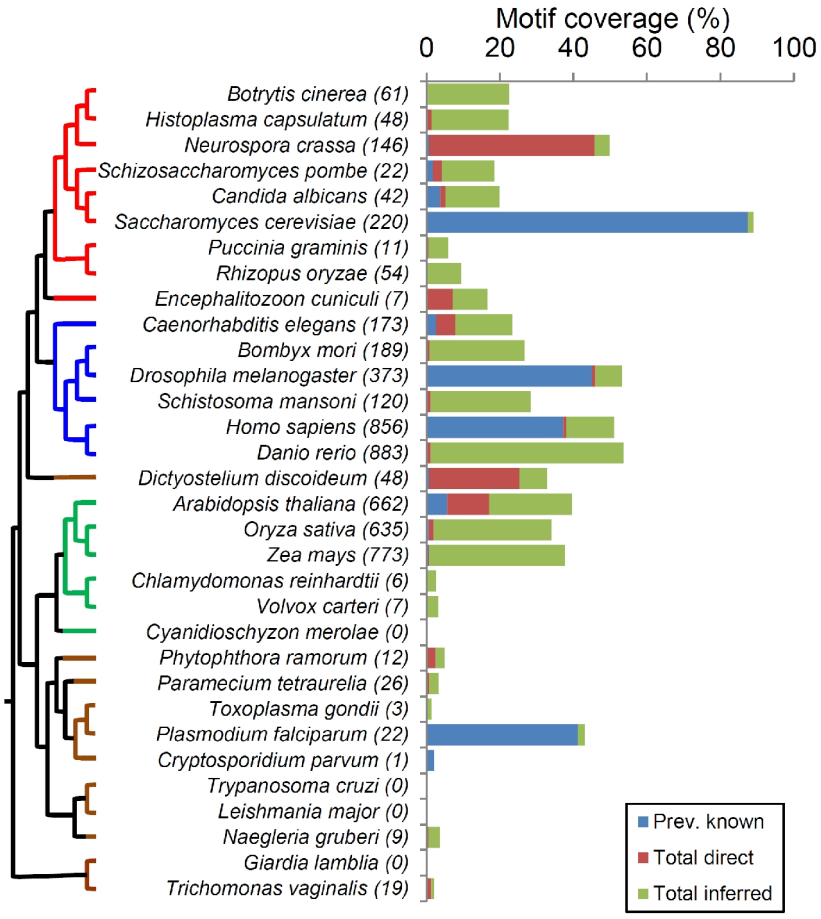


Figure 2.10. Motif coverage by species. The motif coverage (in percentage of total predicted DNA-binding proteins per species) is shown, as listed at CisBP. The number in parenthesis is the number of DNA-binding proteins for which a motif has been obtained. The tree on the left depicts the phylogenetic relationships between organisms as determined by (Baldauf, et al., 2000). Figure is a modified version of the one in Weirauch et al. (accepted in *Cell*).

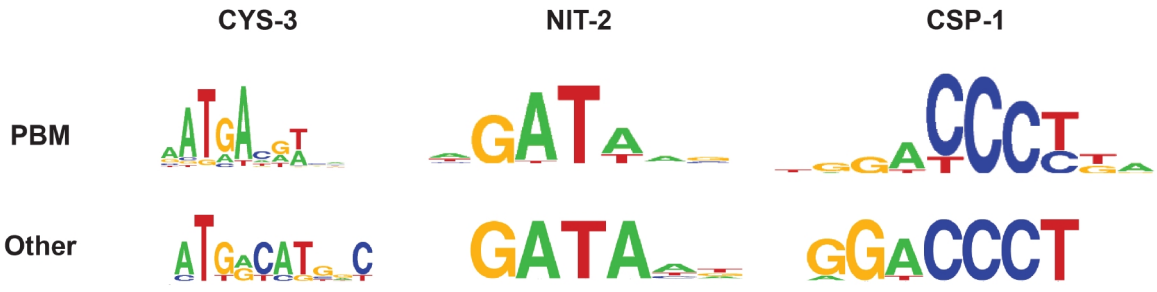


Figure 2.11. Comparison between PBM-derived motifs and motifs obtained through other methods in Neurospora. Comparison is shown for three proteins belonging to three different transcription factor families in Neurospora. CYS-3 belongs to the bZIP family, while NIT-2 and CSP-1 belong to the GATA and C2H2 families, respectively. The previously determined motifs were obtained through different methodologies: the CYS-3 motif was determined via footprinting of two selected promoters (Li and Marzluf, 1996), the NIT-2 motif was determined via EMSA/footprinting of three promoters (Fu and Marzluf, 1990) and the CSP-1 motif was determined via ChIP-seq (Sancar, et al., 2011).

these genes, *NCU01345* and *NCU03905*. The first one, *NCU01345* (also known as *asl-1* or *atf-1*) (Lamb, et al., 2012; Yamashita, et al., 2008), is an ATF/CREB TF involved in osmotic stress responses, closely related to the *S. pombe* bZIP encoding gene *atf1*, required for growth under osmotic stress (Shiozaki and Russell, 1996).

The second one, *NCU03905* (also known as *NcAp-1* or *Nap-1*) (Takahashi, et al., 2010; Tian, et al., 2010), the ortholog of the yeast YAP1 protein, is involved (as its yeast counterpart (Jamieson, 1998)), in oxidative stress responses. We focused on these two TFs because circadian rhythms are known to control stress responses in Neurospora (Montenegro-Montero and Larrondo, 2013) and any insights emerging from these studies could be integrated into current paradigms of output pathways. Both *NcAp-1* and *asl-1* are clock-controlled genes operating in output pathways, as their deletion does not impact the workings of the central clock (Figure 2.12 and (Lamb, et al., 2012)).

As outlined in the previous section, we scanned the promoter regions of these two TF encoding genes for matches to the position weight matrixes (PWMs) obtained via our PBM approach for Neurospora TFs (See Materials and Methods). The goal was to generate a list of TFs that may bind to these promoters and possibly regulate their rhythmic expression. This is the starting point of our genetics approach, the subsequent one being evaluating the expression of these reporters in strains lacking the TFs in that list. This is achieved by crossing the reporter strain with a KO strain for the predicted upstream regulation and evaluating bioluminescence traces in the progeny.

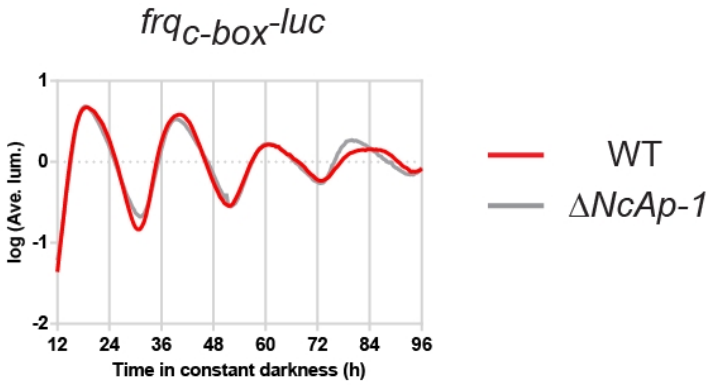


Figure 2.12. Analysis of the status of the central clock in an *NcAp-1* mutant strain. We evaluated the expression of the *C-box* reporter construct in both a WT (red trace) and a Δ*NcAp-1* strain (grey trace) to evaluate whether this gene was part of the oscillator or if it just participates in output pathways. Traces are representative from at least three biological replicates, each with three technical replicates per run.

The list is shown in Table 4. The list includes the predicted upstream regulators for each of the two bZIP TFs, as well as information regarding the availability of KO strains for those proteins. Also, it reports whether a cross was made between the reporter strain and the KO strain.

For most of the predicted upstream regulators, a KO strain is available and a cross was made between this strain and the corresponding reporter one. While the Neurospora Genome Project has generated KOs for most of the protein coding genes in this fungus (Dunlap, et al., 2007), there is still ~ 20% for which no KO is available. Many of these are essential genes or genes required for early ascospore development.

We obtained progeny from these crosses and evaluated reporter activity in the mutant background to test whether the absence of the predicted upstream regulator had any impact on the rhythmic expression of the bZIP in question.

2.3.6. ADA-1 is necessary for proper circadian output

One of the predicted upstream regulators for *NCU03905* (*NcAp-1*), is *a*ll *d*evelopmental *a*ltered-*1* (*ada-1*, *NCU00499*). The KO of this gene has defects in basal hyphal extension, asexual development and sexual development and according to published criteria (Colot, et al., 2006), is referred to as an "all developmental altered" mutant. The mutation of six other genes leads to this *ada* phenotype in Neurospora (*ada-1* through -*7*).

Table 4. Predicted upstream regulators of *asl-1* (*NCU01345*) and *NcAp-1* (*NCU03905*)

NCU01345

Predicted upstream regulator	Reporter crossed to KO	KO strain used
NCU01154	+	FGSC11127
NCU01856	+	xc1376-2
NCU02896	+	FGSC11070
NCU06399	+	FGSC14187
NCU03905	+	FGSC11131
NCU04058	+	FGSC17238
NCU07705	+	FGSC11029
NCU06707	No KO available	
NCU04001	+	FGSC11073
NCU01312	+	FGSC11208
NCU04663	+	FGSC17540
NCU06173	+	FGSC11367
NCU01345	No KO available	
NCU03536	No KO available	
NCU04960		
NCU09333	+	FGSC11395
NCU09387	+	FGSC13451
NCU03352	+	FGSC19465
NCU03489	+	FGSC11095
NCU04671	No KO available	
NCU01478	+	FGSC11002
NCU00019	+	FGSC11437
NCU00289	+	FGSC11085
NCU01386	+	FGSC16373
NCU02173	+	FGSC11440
NCU02787		
NCU03552	No KO available	
NCU03975	No KO available	
NCU05308	+	FGSC18778
NCU05411	+	FGSC11040
NCU03110	+	FGSC11024

NCU03905

Predicted upstream regulator	Reporter crossed to KO	KO strain used
NCU05294	+	FGSC11074
NCU00155	+	FGSC15847
NCU00902	+	FGSC11124
NCU01856	+	xc1376-2
NCU09068	+	FGSC11392
NCU04050	+	FGSC21459
NCU08744	+	FGSC11386
NCU03905	+	FGSC11131
NCU00749	+	FGSC11439
NCU04058	+	FGSC17238
NCU00694	+	FGSC11103
NCU04001	+	FGSC11073
NCU02957	+	FGSC11351
NCU04848	No KO available	
NCU01312	+	FGSC11208
NCU05536		
NCU06173		
NCU07675	+	FGSC15323
NCU01122	+	FGSC11125
NCU02326	+	FGSC12805
NCU05022	+	FGSC17980
NCU09387	+	FGSC13451
NCU03489	+	FGSC11095
NCU05242	+	FGSC11364
NCU07246	No KO available	
NCU07374	+	FGSC11016
NCU09033	No KO available	
NCU00344	No KO available	
NCU00499	+	FGSC11121
NCU01386		
NCU02576	+	FGSC11072
NCU02752	+	FGSC11015
NCU03975	No KO available	
NCU04731	+	FGSC11139
NCU05308	+	FGSC18778
NCU07587	+	FGSC19413
NCU00090	No KO available	
NCU08634	+	FGSC11384
NCU03110	+	FGSC11024

Interestingly, *ada-1* is also a bZIP TF encoding gene (Section 2.3.1) and we found that this gene is also transcribed in a circadian manner (Section 2.3.3). When we evaluated the expression of the *NcAp-1* reporter in the Δ*ada-1* background, we noticed a clear deregulation: reporter traces displayed a low-amplitude, noisy rhythm with a double-peaked waveform (Figure 2.13A). As the rhythm was very noisy, it was difficult to asses whether there was a phase defect and the exact period length, although it appears to be ~1 h shorter than in the WT background.

We reasoned that this phenotype could result either from an alteration of the output pathway leading to rhythmic *NcAp-1* expression, from altered clock function or from a combination of both.

To differentiate between these options, we evaluated the expression of *frequency*, a core clock component, in the Δ*ada-1* background, using a shortened promoter version of the one used in a previously published luciferase transcriptional reporter construct (Gooch, et al., 2008). The new reporter contains only the *clock box* (*C-box*), a *cis* element that is both necessary and sufficient for *frequency* rhythmic expression (Froehlich, et al., 2003) and it is used as a proxy to evaluate the status of the central oscillator (Figure 2.7).

When we evaluated the expression of the $frq_{c\text{-}box}$-*luc* reporter in the Δ*ada-1* strain, we noticed that while rhythmicity was still strong and evident, there was a modest amplitude defect (milder than the one exhibited for the *NcAp-1* reporter) and a slight period defect (~1 hr shorter in the KO), suggesting that while the clock is functional, some parameters are

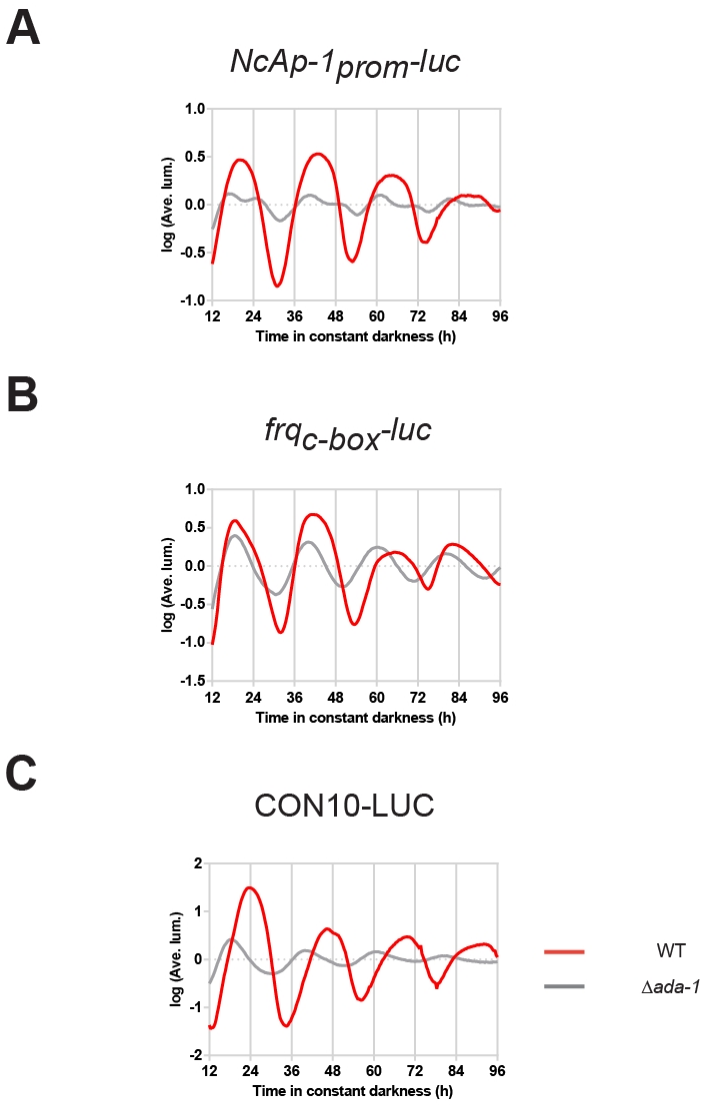


Figure 2.13. ADA-1 is required for proper expression of clock-regulated genes in Neurospora. The expression of three different constructs was evaluated in a WT and Δ*ada-1* strain. We tested the expression of *NcAp-1* in this background (A), because ADA-1 was predicted as an upstream regulator of this gene (Section 2.3.5). The two other constructs, *C-box* (B) and CON-10 (C) were tested to evaluate the status of the core oscillator and a different output gene, respectively, in this strain.

somewhat abnormal in the Δ*ada-1* background (Figure 2.13B).

It is possible however, that the *ada-1* mutation somehow affects the *his-3* locus, where transcriptional reporters are targeted, which could give the impression that *ada-1* affects the rhythmic expression of the genes in question, when in reality it does not. Nevertheless, the fact that the effect of the *ada-1* mutation is not the same for $frq_{C\text{-}box}$-*luc* and *Nc*A*p-1*$_{prom}$-*luc*, despite the fact that they are both at the *his-3* locus, suggested this was not the case or that its contribution was minor.

If the effect of knocking out *ada-1* over *NcAp-1* was simply the result of central clock alteration, we would expect all rhythmic genes to display similar deregulation in this background, that is, similar period and phase defects, echoing the effects over the core oscillator. To test this, we evaluated the expression of another rhythmically expressed output gene, *con-10* (Lauter and Yanofsky, 1993), but using a luciferase translational fusion, designed at the endogenous locus (Larrondo, et al., unpublished results). Besides giving us information regarding the extent of clock control of *ada-1*, the CON10-LUC reporter also allowed us to further explore the idea that the *ada-1* mutation could somehow simply affect the *his-3* locus (where the transcriptional reporters are targeted). The CON10-LUC reporter also exhibited an altered expression pattern in the mutant background, but different from the previous two reporters analyzed: in addition to a shorter period (this time, ~1.7 hr shorter in the mutant) and significantly lower amplitude (parameters that were also altered in the previous reporters), a clear phase defect was present (Figure 2.13C).

The effect of *ada-1* deletion over the expression of *NcAp-1* and CON-10 does not mirror the mild effects over $frq_{C\text{-}box}$-*luc* expression (i.e. the defects are different), suggesting that specific output elements are altered in this mutant, in addition to the minor effects on the clock itself. In other words, our data suggests a prominent role for ADA-1 as a regulator of clock output or clock modulator, rather than as a core clock component. Noteworthy, the defects exhibited by the two output genes analyzed (*NcAp-1* and *con-10*) when *ada-1* is missing, are different, suggesting a role solely in output pathways (Figure 2.13 and Discussion).

As a whole, these results suggest that the *ada-1* mutation affects multiple pathways, some of which may impinge on the rhythmic transcriptional regulation of different genes (most likely indirectly), ultimately leading to defects on the Neurospora circadian system, particularly at the output level.

Two putative ADA-1 binding sites were found in the *NcAp-1* promoter. To test whether these elements are relevant to the rhythmicity of this gene, we performed promoter resection studies and generated reporter constructs that contained either both (p1800) or none (p1600) of these *cis* elements and compared them to the full *NcAp-1* promoter construct (p2600) (Figure 2.14). We observed no difference in the bioluminescence profiles generated from these three constructs, suggesting that *ada-1* regulation over *NcAp-1* expression is exerted indirectly (for example, through regulation of a different TF) or via a non-canonical binding site (or one below our cutoff) in the *NcAp-1* promoter (Figure 2.14C). While these results do not suggest a direct (physical) effect of ADA-1 over *NcAp-1* expression, they do allow us to genetically place ADA-1 as an output regulator working upstream from *NcAp-1* expression.

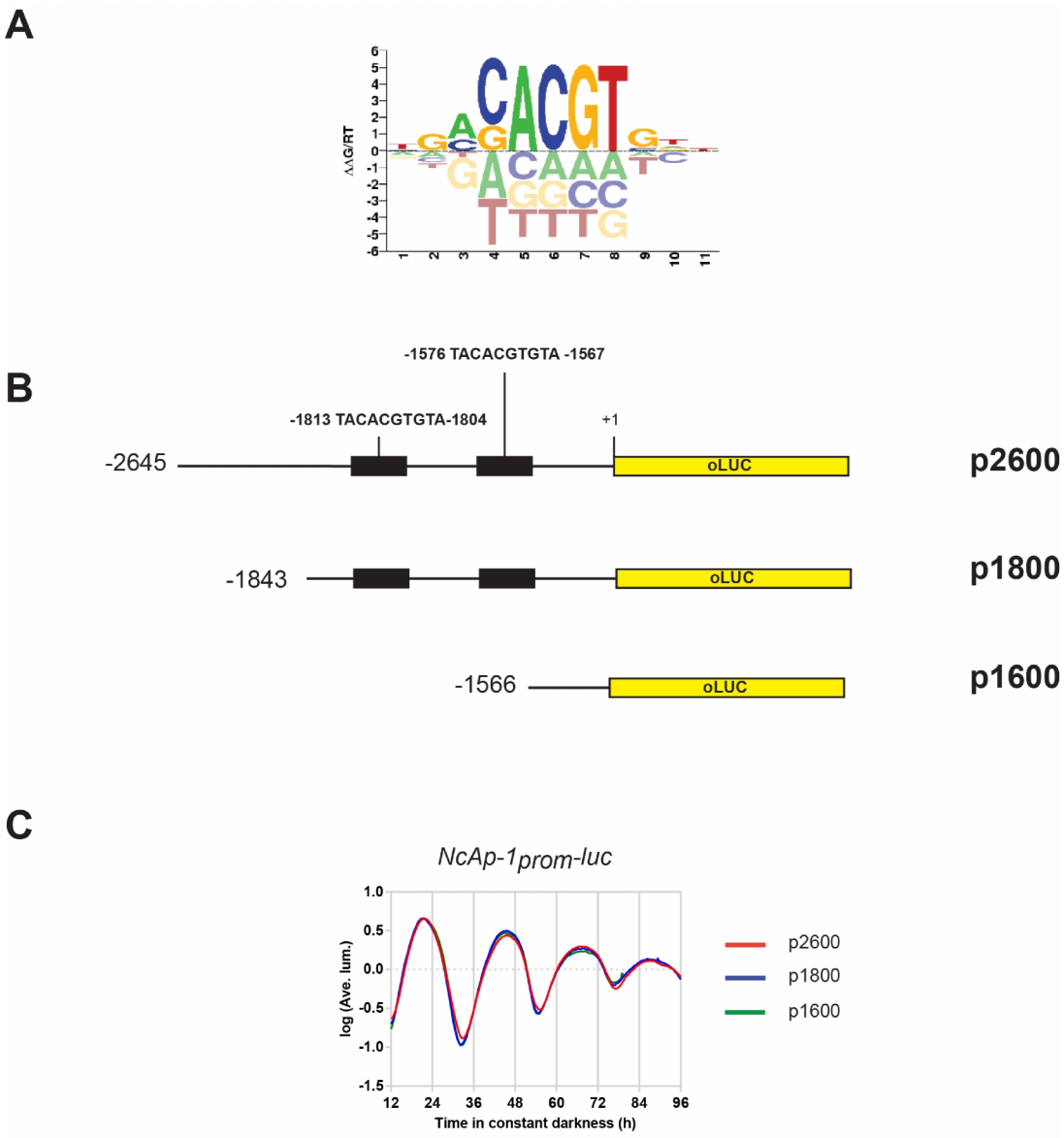


Figure 2.14. Analysis of the role of the putative *ada-1* boxes in the rhythmic activity of the *NcAp-1* promoter. A) The PBM-derived motif for ADA-1. B) Schematic representation of the constructs used to evaluate clock functionality of the putative ADA-1 binding sites identified in the promoter of *NcAp-1*. The p2600 construct is the full promoter used in Figure 2.6. The p1800 is a shortened version of p2600, but containing the two predicted binding sites. The p1600 construct lacks both of them. C) Bioluminescence traces from the three *NcAp-1* promoter constructs. No differences were found between them.

2.3.7. Analysis of asl-1$_{prom}$-luc expression in mutants of predicted upstream regulators

We followed the same approach as described for *NcAp-1* for the identification of putative upstream regulators involved in the rhythmic expression of *asl-1* (Sections 2.3.5 and 2.3.6). We did not however, find significant alteration of the *asl-1$_{prom}$-luc* expression profile in any of the KO strains of the TFs listed in Table 4. Two major explanations can be proposed. The first one, a biological explanation, concerns the robustness of the network involved in the rhythmic expression of this gene, in the sense that attacks to a single node (that is, evaluating expression in a background lacking a single TF), may not be enough to significantly perturb the network (Kitano, 2004). In this sense, several TFs or major signaling pathways may be involved in regulating the rhythmic expression of this gene and rhythmicity of *asl-1* would persist even in the absence of one of the TFs involved. Each of the participant TFs may play a fine tuning role over the expression of this bZIP encoding gene. The second explanation is more related to technical issues. Due to inherent limitations in the genome-wide computational detection of functional TF binding sites, most of the predicted binding sites that are generated with models for the binding of individual TFs, will have no functional role *in vivo*, despite the strong likelihood that the TF would bind the sequence *in vitro*. This is referred to as the "futility theorem" (Wasserman and Sandelin, 2004). The discrepancy suggests that additional properties specify functional sites *in vivo* and recent evidence supports this notion (White, et al., 2013). Most methods (including the one used in this study) assume that each TF binds independently to its target (instead of, for instance, combinatorially with other TFs) and without any regard to adjacent sequences and putative proximity to other proteins. This contributes to the "futility theorem" and may explain why we did not find any putative

upstream regulator for *asl-1*. More sophisticated methods may be needed to address this issue (See Discussion).

2.3.8. Identification of signaling pathways involved in the rhythmic expression of bZIP encoding genes

Considering the "futility theorem" described above, we wanted to take a more global approach for the identification of elements that regulate the rhythmic expression of *NcAp-1* and *asl-1*.

Mitogen-activated protein kinases (MAPKs) are highly conserved proteins that play important roles in signal transduction in eukaryotic cells (Marshall, 1994). They are part of three-kinase cascade modules integrated into signaling pathways that coordinate responses to environmental changes, mating signals and a variety of other stimuli in fungi. This three-tier module is composed of a MAP kinase kinase kinase (MAPKKK), a MAP kinase kinase (MAPKK) and a MAP kinase (MAPK). These MAPK modules are activated by a variety of signaling mechanisms and through a characteristic phosphorelay system, normally lead to the regulation of TF activity by MAPK-mediated phosphorylation. This, in turn, results in the regulated transcription of specific genes in response to the initial stimulus (Chen and Thorner, 2007; Chen, et al., 2001; Kamei, et al., 2013; Levin and Errede, 1995; Roux and Blenis, 2004).

As MAPK pathways regulate several TFs when activated, we reasoned that we were more likely to observe deregulation of our reporter constructs in strains defective in MAPK proteins, that in single TF KOs. Further, our overall goal is to characterize signaling pathways involved

in the rhythmic transcriptional regulation of the bZIP TF encoding genes, not simply to identify single TFs involved in the relay of time-of-day information from the clock to the rhythmic bZIPs, so we deemed the endeavor of evaluating the expression of the selected bZIP reporters in MAPK-defective backgrounds, worthwhile.

One of the most intensively studied MAPK pathways in fungi is the yeast high-osmolarity glycerol (HOG) pathway, involved in osmotic stress responses (Brewster, et al., 1993; Hohmann, 2009; Saito and Posas, 2012). At the core of this pathway is the Hog1 MAPK, which is the final target of the phosphorelay module and regulates downstream TFs, chromatin-modifying enzymes and other signaling elements, to ultimately regulate gene expression as part of an adaptive response to hyperosmolarity (Capaldi, et al., 2008; Cook and O'Shea, 2012; de Nadal and Posas, 2010; O'Rourke and Herskowitz, 2004).

In Neurospora, this pathway is known as the *o*smotic *s*tress (OS) pathway and the product of the *os-2* gene, the MAPK OS-2, plays the role of Hog1 (Kamei, et al., 2013; Zhang, et al., 2002). Interestingly, the OS pathway has been shown to have clock input and to be involved in the regulation of clock-controlled genes in Neurospora (Vitalini, et al., 2007), which makes it an attractive pathway to interrogate regarding its potential role on the regulation of the rhythmic transcription of bZIP encoding genes.

First, we decided to evaluate whether the absence of *os-2* alters the functioning of the central clock. It has been shown that mutation of the response regulator protein RRG-1, an upstream regulator of the OS pathway (among possibly other pathways), leads to 1 h period shortening

and a delay in conidiation upon transfer to constant darkness in race tube assays (Vitalini, et al., 2007). In fact, the Δ*rrg-1* strain does not form normal conidial bands until the third day in these conditions.

When we analyzed the expression of $frq_{C\text{-}box}$-*luc* in the *os-2* background, we noticed that a clear rhythm was only visible from the third day on (DD 48 onwards) (Figure 2.15A), recapitulating the phenomenon observed in race tubes for the *rrg-1* mutant: that is, while the clock is functional in these OS-defective strains, there is a delay in clock manifestation compared to the WT.

We then evaluated the expression of *asl-1* and *NcAp-1* in strains lacking *os-2*. We observed that the expression profiles of both reporters were drastically altered in the *Δos-2* strain and did not recover after 3 days, as the *C-box* reporter (Figure 2.15B and C). Taken together, these results suggest that OS-2 is part of an output of the clock that regulates rhythmicity of *NcAp-1* and *asl-1*. This is the first evidence of a direct involvement of the OS-2 MAPK in the rhythmicity of a transcription factor or of any other clock-controlled gene in Neurospora, an idea suggested 7 years ago (Watanabe, et al., 2007).

Interestingly, other authors have shown that the OS pathway as a whole (as evidenced via characterization of the *rrg-1* mutant, which is an upstream regulator of the pathway), is required for the rhythmic mRNA accumulation of the clock-controlled gene *ccg-1* (Vitalini, et al., 2007). The authors reported that *rrg-1* is required for proper rhythms of a subset of clock-controlled genes, including *ccg-1,* and that the OS-2 MAPK is phosphorylated rhythmically.

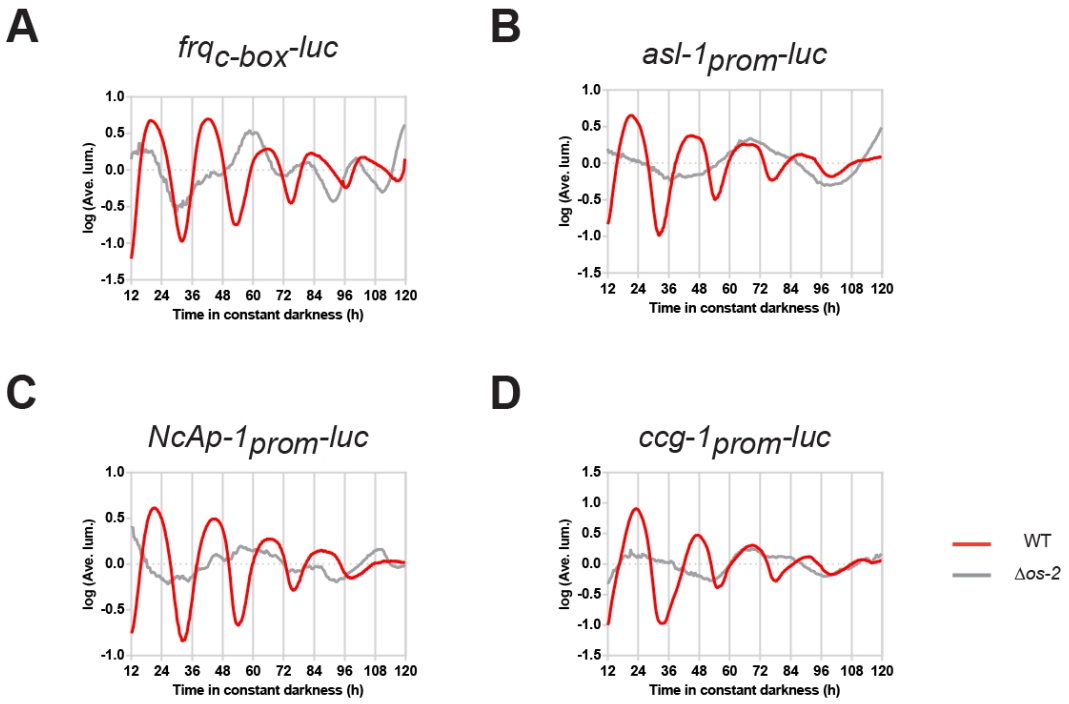


Figure 2.15. Analysis of the role of the MAPK OS-2 over the rhythmic expression of different clock-controlled genes. The status of the central clock (A) and the expression profile of different clock-controlled genes (B, C and D), was analyzed in a WT and a Δ*os-2* strain. A clear rhythm in the *C-box* construct is observed only after 48 h in constant darkness, while no circadian rhythm is evident for the three other constructs at any time. More hours were analyzed in these strains (120 h) compared to other analyses on this Thesis, to reveal the recovered rhythm in the *C-box* construct.

The authors showed that both the rhythm in OS-2 phosphorylation and the rhythms in the mRNA accumulation of *ccg-1* are lost in the *rrg-1* mutant, so they concluded that this "correlation between the levels of phospho-OS-2 and *ccg-1* mRNA (…) indicate that the OS pathway functions as an output pathway that connects the FRQ/WCC oscillator to the rhythmic expression of *ccg-1*".

To directly evaluate the role of OS-2 in the rhythmicity of the *ccg-1* gene, rather than rely on correlations, we decided to evaluate the expression of *ccg-1* in the Δ*os-2* strain. We generated a transcriptional fusion to luciferase, using the 631 bp region upstream of the *ccg-1* coding sequence as promoter. This region has been shown to be sufficient for *ccg-1* rhythmic expression (Lindgren, 1994) and we were able to reproduce these results using luciferase reporters (Figure 2.15D). When we evaluated the bioluminescence profile of the *ccg-1*$_{prom}$*-luc* reporter in the Δ*os-2* background, we observed that rhythmicity was lost in this mutant (Figure 2.15D), suggesting that OS-2 regulates transcriptional rhythms of *ccg-1* and that lost of these transcriptional rhythms are at least partly responsible for the loss in the rhythms of the mRNA levels of *ccg-1* observed in the *rrg-1* mutant (Vitalini, et al., 2007).

2.3.9. ASL-1 is required for proper activation of the ccg-1 promoter upon osmotic stress, but is dispensable for its rhythmicity

In *S. cerevisiae*, the Hog1 MAPK signaling cascade is activated upon osmotic stress, resulting in the rapid phosphorylation of Hog1 and its translocation into the nucleus, where it can stimulate the expression of its target genes (Saito and Posas, 2012 and references therein).

Various transcriptional regulators have been shown to regulate the expression of subsets of the Hog1 target genes in yeast (Nadal and Posas, 2008) and this list includes Sko1, a bZIP TF of the ATF/CREB family (Garcia-Gimeno and Struhl, 2000; Nehlin, et al., 1992; Proft, et al., 2005; Proft and Serrano, 1999; Rep, et al., 2001; Vincent and Struhl, 1992). Sko1, as other TFs working downstream of Hog1, is phosphorylated by this MAPK upon osmotic stress (de Nadal, et al., 2003; Proft, et al., 2001). Similarly, ATF/CREB TFs in *S. pombe* (Shiozaki and Russell, 1996; Wilkinson, et al., 1996) and in mammalian cells (Gupta, et al., 1995; van Dam, et al., 1995) have been shown to be targets of stress-activated MAP kinases and to play important regulatory roles in response to different kinds of stressful conditions (Tibbles and Woodgett, 1999; Wilkinson and Millar, 1998).

Interestingly, the Neurospora ortholog of Sko1 is ASL-1, which we have herein shown to be rhythmic and regulated by the OS pathway, specifically, by the stress- and clock-activated OS-2 MAPK (the Neurospora ortholog of Hog1). As its yeast counterpart, ASL-1 has been shown to be necessary for osmotic induction of several genes in Neurospora, including *ccg-1* (Yamashita, et al., 2008). *In vitro* data suggests direct interaction between ASL-1 and the *ccg-1* promoter (Yamashita, et al., 2008). The *ccg-1* gene has also been shown to require OS-2 for proper up-regulation under osmotic stress (Watanabe, et al., 2007).

While the OS pathway has been shown to be required for the rhythmicity of *ccg-1* (this work and (Vitalini, et al., 2007)), the TF(s) mediating the rhythmic expression of this gene is/are unknown. Considering that ASL-1 and OS-2 are both required for *ccg-1* up-regulation upon osmotic stress (Watanabe, et al., 2007; Yamashita, et al., 2008), that OS-2 is necessary for

transcriptional rhythms of this gene (this work), the fact that we have shown *asl-1* to be rhythmically expressed and that OS-2 is also necessary for transcriptional rhythms of *asl-1*, we hypothesized that ASL-1 may be involved in regulating *ccg-1* transcriptional rhythms.

As an *asl-1* KO strain cannot be crossed (an *asl-1* KO strain exhibits an *as*cospore *l*ethal phenotype, hence the name), we couldn't directly test our hypothesis using our reporter constructs in a Δ*asl-1* strain. Instead, we took a different approach, making use of our PBM data.

We reasoned that if ASL-1 is in fact directly involved in *ccg-1* transcriptional rhythms and taking into account that it can bind the *ccg-1* promoter *in vitro* (Yamashita, et al., 2008), then this promoter should harbor ASL-1 binding sites.

We scanned the Neurospora *ccg-1* promoter (the one used in the reporter construct described in the previous section, which we have shown to be sufficient for rhythmic expression, Figure 2.15D) using the PBM-derived ASL-1 PWM. We identified two putative ASL-1 binding sites, between -584, -575 and -305, -296 (Figure 2.16). The site between -584,-575 has been suggested as a putative ASL-1 binding site in the *ccg-1* promoter (Yamashita, et al., 2008) and both sites have been suggested as binding site for ATF/CREB TFs (Lindgren, 1994), supporting our approach.

We decided to test our hypothesis about the involvement of ASL-1 in *ccg-1* transcriptional rhythms by evaluating the role of the predicted ASL-1 binding elements in this gene's promoter activity.

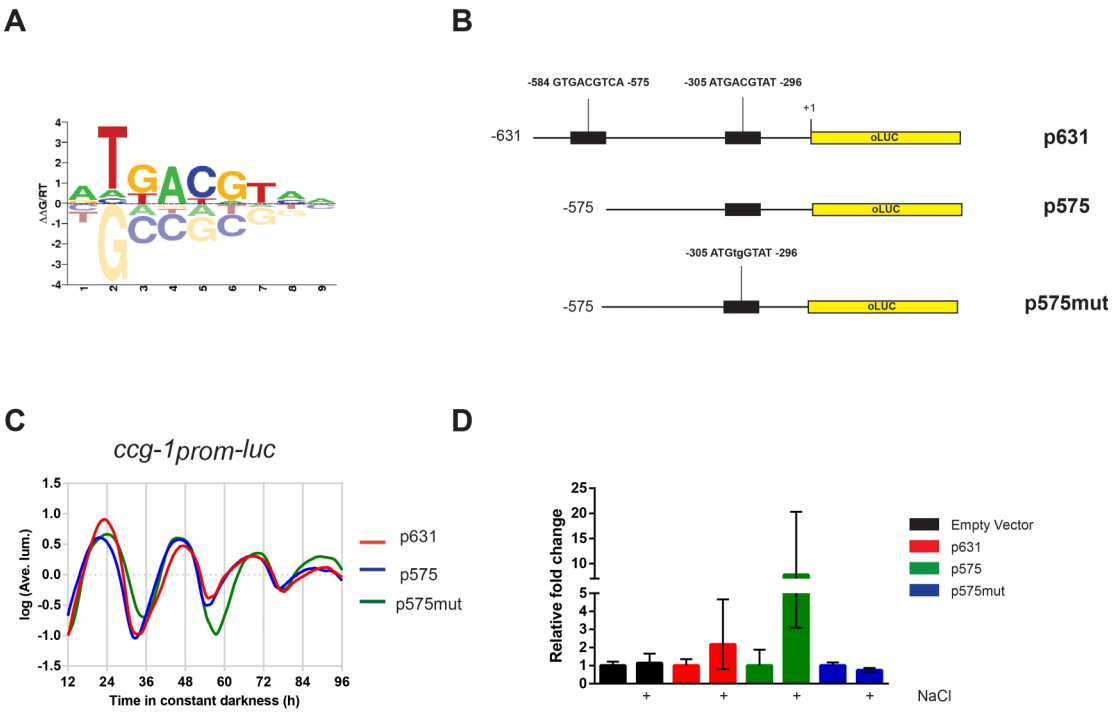


Figure 2.16. Analysis of putative ASL-1 binding sites in the *ccg-1* promoter. A) The PBM-derived motif for ASL-1. B) In a *ccg-1* promoter region capable of supporting rhythmic activity (p631), two putative ASL-1 binding sites were found. Constructs generated for studying these boxes are shown. The p575 construct is missing one of these boxes, while the p575mut lacks both, through a combination of promoter resection and box mutation. C) Bioluminescence profiles of the three constructs in constant darkness. No significant differences were observed. D) Strains harboring each of the three constructs and an empty vector, were subjected to osmotic stress (1 M NaCl pulse for 1 h) and RT-qPCR was performed to quantify luciferase mRNA levels. Error bars represent mean expression +/- 95 % confidence intervals (n=3).

As shown in Figure 2.16B, we generated two additional *ccg-1* promoter constructs. Besides the p631 one, which we have shown can support rhythms in bioluminescence (Figure 2.15D), in agreement with (Lindgren, 1994), we generated one that lacked the most upstream of the two putative ASL-1 binding sites (construct p575) and another that lacked both sites, which resulted from mutating the remaining ASL-1 binding site in p575 (hence the name of the construct, p575mut) (See Materials and Methods). This avoided the potential technical problem of working with a promoter that was too small and that may be missing minimal promoter binding sites, which could give the false impression of deregulation.

When we analyzed the expression of these constructs, we observed no significant difference in any of the circadian parameters considered (period, phase, amplitude) between them (Figure 2.16C). This suggests that ASL-1 is not (or at least not directly) involved in the transcriptional rhythms of *ccg-1*. Alternatively, it may do so by binding to non-canonical sites (which we could have missed in our scanning).

To test whether the identified ASL-1 binding sites are relevant for *ccg-1* expression, we decided to perform osmotic stress studies, as we know that *ccg-1* is induced under conditions that activate the OS pathway, in an ASL-1-dependent manner (Yamashita, et al., 2008). Strains harboring p631, p575 and p575mut were subjected to osmotic stress studies and the expression of the luciferase transcript was quantified by RT-qPCR.

As shown in Figure 2.16D, p631 and p575 are able to support osmotic stress induction of the reporter, while p575mut does not, similar to a no-promoter control. While differences were

only statistically significant for the p575 construct ($p < 0.05$), there was a clear up-regulating trend in p631 under osmotic stress compared to the control. We noticed that induction was higher for the p575 construct than for p631. We think that these results reflect some issues with the p631 construct, rather than bone fide biological differences (see Discussion). In any case, the data clearly shows that at least the -305,-296 site is functional and is involved in the proper activation of the *ccg-1* promoter upon osmotic stress, consistent with it being an ASL-1 binding site.

Our data suggests that ASL-1 is not directly involved in the transcriptional rhythms of *ccg-1*, but it is, consistent with previous reports (Yamashita, et al., 2008), required for its proper up-regulation in response to osmotic stress, although this would require the ASL-1 binding site between -305 and -296, which was not the one suggested by Yamashita et al. (2008) at -584,-575. These authors missed detection of the other box which we were only able to find via the PBM-derived motif obtained in this study.

We considered a second strategy to evaluate the role of ASL-1 in *ccg-1* transcriptional rhythms. We have found the deletion of OS-2 alters both *asl-1* and *ccg-1* transcriptional rhythms (Figure 2.15). We reasoned that if there is a "circadian hierarchical arrangement" in the pathway, such that OS-2 is responsible for *asl-1* rhythms, which in turn regulates *ccg-1* rhythms, then maybe the transcriptional misregulation of *asl-1* in the Δ*os-2* mutant is responsible for the lost of rhythmicity in *ccg-1* transcription in that mutant background. In other words, maybe the mere deregulation of *asl-1* is responsible for *ccg-1* misexpression in the Δ*os-2* strain.

To test this, we replaced the promoter of the rhythmic and photoinducible gene *asl-1* (this work and (Chen, et al., 2009), respectively), with that of the non-rhythmic and non-photoinducible gene *sod-1*, encoding *superoxide dismutase-1* (Aronson, et al., 1994; Chen, et al., 2009; Linden and Macino, 1997). Our reasoning was that by replacing the endogenous *asl-1* promoter with that of another gene, its own *cis* regulatory elements would be lost and consequently, the normal expression of *asl-1* would be altered, allowing us to test whether mere deregulation of *asl-1* was enough for the loss of *ccg-1* rhythmicity.

When we analyzed expression of the *ccg-1*$_{prom}$*-luc* reporter, in both WT and *sod-1*$_{p}$*-asl-1* strains, we observed no significant differences (Figure 2.17A). Similarly, and as expected for a TF functioning in output pathways, no differences were observed for a *frq*$_{C\text{-}box}$*-luc* reporter (Figure 2.17B). As the *asl-1* ortholog in *S. pombe* (*atf1*) has been shown to bind its own promoter (Eshaghi, et al., 2010), we also tested whether the expression of *asl-1*$_{prom}$*-luc* was altered in the *sod-1*$_{p}$*-asl-1* strain. It may be worth remembering that the *asl-1*$_{prom}$*-luc* construct is at the *his-3* locus, while the promoter replacement was performed at the endogenous locus. As with the other two constructs, no alterations in the rhythmic expression profile of *asl-1*$_{prom}$*-luc* were observed (Figure 2.17C).

Altogether, our results suggest that *asl-1* and its proper transcriptional regulation, play no obvious role in the rhythmic transcriptional control of *ccg-1*.

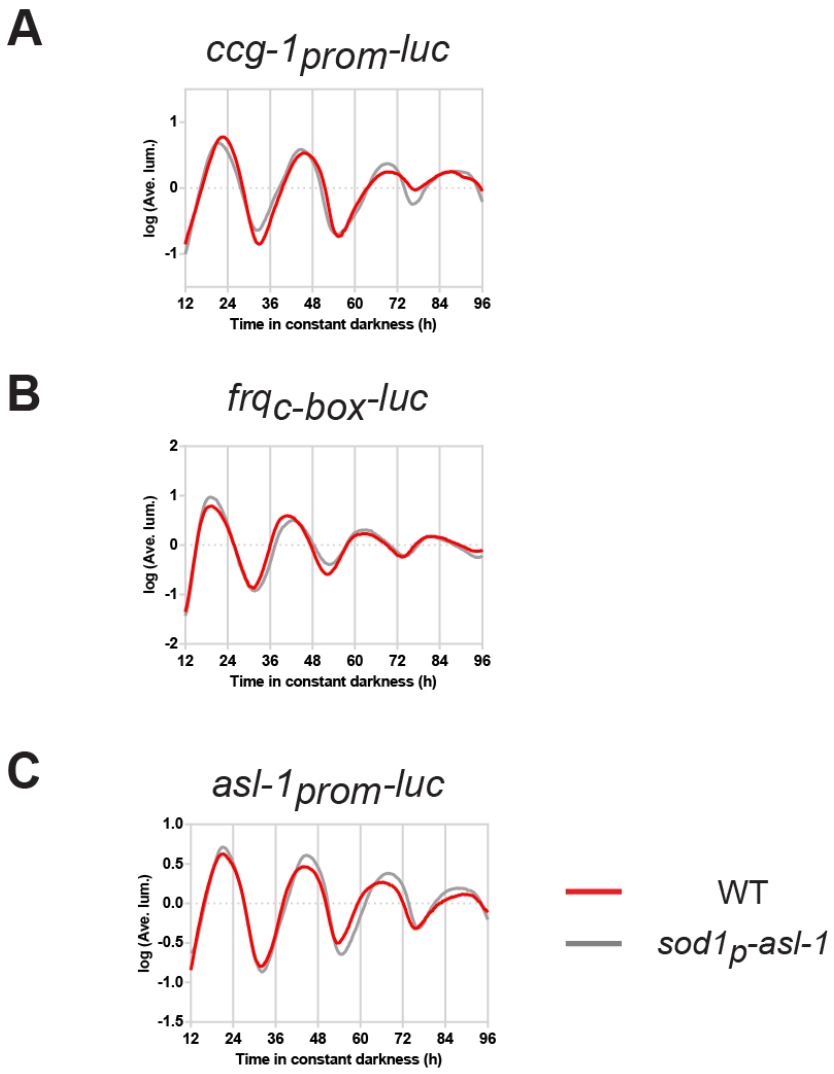


Figure 2.17. Effects of *asl-1* promoter replacement over the rhythmic expression of *ccg-1*. A) The endogenous promoter of *asl-1* was replaced by the promoter of the non-rhythmic and non-photoinducible *sod-1* gene and the expression of *ccg-1* was evaluated. No defects were observed. Similarly, no alterations in rhythmic profiles were observed for the *C-box* construct (B) or the *asl-1*$_{prom}$*-luc* reporter (C).

2.3.10. An unmapped mutation affecting asl-1 expression links the circadian clock to cell fusion pathways

In Section 2.3.7 we described our attempt to find TF KO strains in which the expression of *asl-1* is altered. We did not find one based on our approach and discussed possible reasons behind it. While we could not associate a particular TF to the rhythmic regulation of *asl-1*, we did find a mutant strain in which the expression of this gene was altered. This seemingly contradictory statement is explained below.

One of the predicted upstream regulators of *asl-1* is *a*ll *d*evelopmental *a*ltered-3, or *ada-3* (*NCU02896*, Table 4), a predicted Zinc cluster TF. As mentioned before, mutants of this class display defects in basal hyphal extension, asexual development and sexual development, hence its name (Colot, et al., 2006). More recently, this mutant was reported to exhibit defects in hyphal cell fusion (Fu, et al., 2011), an important process in the Neurospora life cycle (Fleissner, et al., 2008; Glass and Fleissner, 2006; Simonin, et al., 2012).

Cell fusion events ("anastomosis"), occur during all stages of the filamentous fungal life cycle and are essential for the vegetative and sexual development of members of this group (Glass and Fleissner, 2006). In Neurospora, cell fusion takes place between germinating conidia through specialized thin hyphae called conidial anastomosis tubes (CATs), which allows for colony establishment. Additionally, hyphal fusion also ocurrs within a mature colony, resulting in an interconnected hyphal network, which presumably facilitates communication and nutrient distribution between the cells. Lastly, as described in Chapter 1, cell fusion is

instrumental for fertilization, in the sexual phase of the Neurospora life cycle (reviewed in Fleissner, et al., 2008). Although many fusion mutants have been identified (Aldabbous, et al., 2010; Fleissner, et al., 2009; Fu, et al., 2011; Wilson and Dempsey, 1999; Xiang, et al., 2002), including mutants in MAPK components (Pandey, et al., 2004), a detailed understanding of the molecular basis of cell fusion is far from complete (Glass and Fleissner, 2006; Read, et al., 2009).

The aforementioned *ada-3* mutant has been shown to display typical characteristics of cell fusion mutants (Aldabbous, et al., 2010): short aerial hyphae over the entire slant surface, resulting in a "flat, carpet-like conidiation pattern" (Figure 2.18B), failure to produce CATs and defects in sexual development. These traits made Fu et al. (2011) classify *ada-3* as a gene required for normal cell fusion.

As a predicted upstream regulator of *asl-1*, we evaluated the expression of the *asl-1* reporter in the Δ*ada-3* strain obtained from the Fungal Genetics Stock Center (strain FGSC #11070). As shown in Figure 2.18, we observed a strong defect in the rhythmic expression of *asl-1* in this background. However, while evaluating additional progeny from the cross between FGSC#11070 and the *asl-1* reporter strain, to further study this interesting result, we made an striking observation: the growth phenotype displayed by the FGSC #11070 strain, did not always co-segregate with the hygromycin resistance cassette (the cassette used to generate the KO strains, see Materials and Methods). In other words, we identified strains that while hygromycin-resistant and lacking the *ada-3* gene, did not exhibit the "all developmental altered" phenotype.

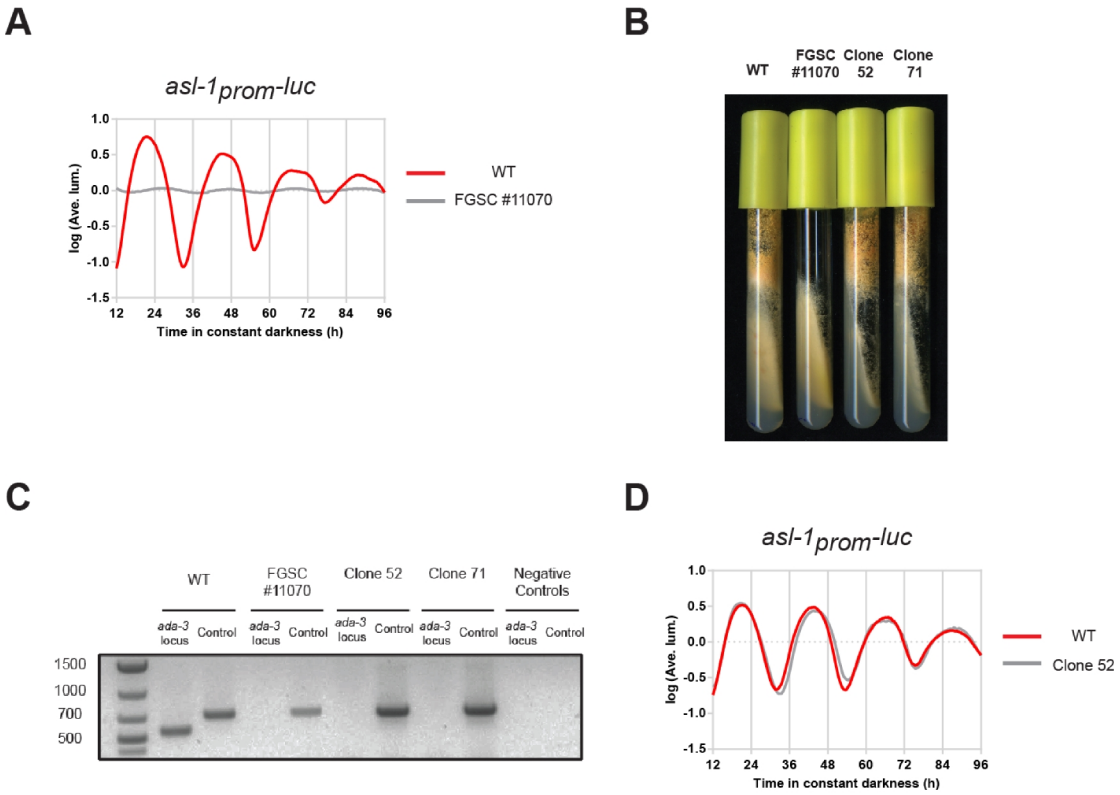


Figure 2.18. An unmapped mutation affects the rhythmic expression profile of *asl-1*. As ADA-3 was predicted as an upstream regulator of *asl-1*, we evaluated the expression of *asl-1* in the deposited KO strain for this gene, FGSC #11070 (A). The rhythmic profile of *asl-1* was dramatically altered in this background. B) The growth phenotype of the FGSC #11070 is unrelated to the absence of *ada-3*. Two clones, which lack this gene (C), exhibit WT growth in slants. D) The expression profile of *asl-1* was evaluated in one of these strains (Clone 52). Normal *asl-1* expression was observed, contrasting the situation observed in the FGSC #11070 background.

We characterized two of these strains, clone 52 and clone 71. As evidenced in Figure 2.18B, clones 52 and 71 display WT growth phenotypes in slants, as opposed to FGSC #11070, which displays the typical phenotype of a cell fusion-defective mutant, in agreement with (Fu, et al., 2011). To test whether clones 52 and 71 indeed lack the *ada-3* gene, we genotyped these strains and found that these two clones were indeed Δ*ada-3* strains, just as FGSC #11070 (Figure 2.18C). These results show that the reported cell fusion phenotype of the FGSC #11070 strain, is not the result of *ada-3* mutation, as clones 52 and 71, which lack this gene, display WT growth on slants, and that a secondary mutation is responsible for the phenotype.

We contacted the authors from the Fu et al. (2011) study and they re-evaluated their data and supported our conclusion: the hygromycin resistance cassette does not co-segregate with the cell fusion phenotype, hence, the product of the *ada-3* gene is not involved in this process (Steven Free, personal communication).

As the phenotype was not co-segregating with the absence of the *ada-3* gene, we re-evaluated our reporter data: we had to test whether the deregulation observed for the expression of *asl-1* was due to the lack of *ada-3* or the secondary mutation.

We evaluated the expression of *asl-1* in a "clean" *Δada-3*, that is, an *ada-3*-deficient strain that displays a WT growth phenotype. We observed that the expression of *asl-1* was normal in this strain, suggesting that the originally observed misregulation of *asl-1* in the FGSC #11070 strain was due to the secondary mutation and not to the lack of *ada-3* (Figure 2.18D). The

secondary mutation then, leads to both a cell fusion phenotype and deregulation of *asl-1*. Our data suggests that the mutation segregates as a single recombination unit (data not shown).

We then evaluated whether the defect over *asl-1* expression caused by this secondary mutation was affecting the central clock rather than an output pathway. We analyzed the expression of the $frq_{C\text{-}box}$-*luc* reporter in both the FGSC #11070 and the clean *ada-3* background. No major impact (compared to the one over *asl-1* expression) was observed on the *C-box* reporter in any of the backgrounds (Figure 2.19), suggesting that the secondary mutation is mainly affecting an output of the clock that regulates rhythms in *asl-1* expression and that, as expected based on Figure 2.18, the clock does not require *ada-3* for proper function. A slight reduction in amplitude was observed for the *C-box* reporter in the FGSC #11070 background, though.

As the unmapped, secondary mutation results in cell fusion phenotype, we wanted to evaluate whether normal hyphal cell fusion (or the genes required for it) were needed for proper rhythms in *asl-1* expression. To test this, we evaluated rhythms in bioluminescence of the $asl\text{-}1_{prom}$-*luc* reporter in three additional mutants that display defects in this process: a) *soft*. This is a classic and well characterized mutation that leads to cell fusion defects. This mutant is allelic to *ham-1*, the first hyphal fusion mutant reported in Neurospora (Wilson and Dempsey, 1999) and accordingly, introduction of a WT copy of the *ham-1* gene (*NCU02794*) complements the *soft* phenotype (Fleissner, et al., 2005). We therefore used FGSC #11292, the Δ*ham-1* strain, for this experiment; b) *ham-7* (FGSC #13775). The Free group reported that the Δ*ham-7* strain exhibits a cell fusion phenotype (Fu, et al., 2011). This mutant can be complemented by introduction of a WT copy of the *ham-7* gene (*NCU00881*), suggesting that the phenotype is

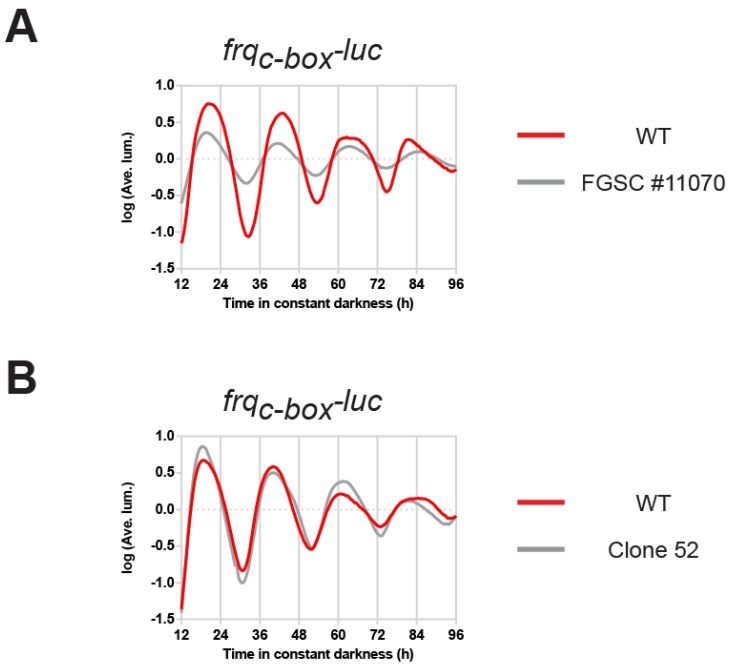


Figure 2.19. The unmapped mutation in FGSC #11070 has no dramatic impact on the central clock. We examined the status of the central clock via analysis of the *C-box* reporter in both the FGSC #11070 (A) and Clone 52 strains (B). While the unmapped mutation leads to strong deregulation of the *asl-1* promoter, it has no significant impact on the *C-box* reporter. B) Deletion of *ada-3* has no impact on the workings of the clock.

indeed a result of *ham-7* inactivation; c) *acw-4* (FGSC #12957). This mutant also displays cell fusion defects, however the phenotype is not reverted by introduction of a WT copy of the corresponding gene (*NCU09263*) (Maddi, et al., 2012). In this strain, an unmapped mutation would be responsible for the cell fusion phenotype, just as in FGSC #11070.

As mentioned, all of these mutants display cell fusion phenotypes and as shown in Figure 2.20A-C, *asl-1* expression is significantly altered in all of them, including amplitude and phase defects. This supports the idea that defects in cell fusion regulatory pathways, somehow affect the rhythmic expression of *asl-1*.

We also tested the *C-box* reporter in the *so/ham-1* background and noticed only a modest reduction in amplitude in an otherwise normally working clock (Figure 2.20D), similar to the situation in the FGSC #11070 strain (Figure 2.19A). Finally, we tested another output gene, *NcAp-1*, and observed its rhythmic expression profile was also altered in the *so/ham-1* mutant (Figure 2.21A). This gene, as *asl-1*, also displayed an altered expression profile in the FGSC #11070 strain (Figure 2.21B), but did not require *ada-3* for normal rhythmicity, as evidenced using the clean *ada-3* mutant (Figure 2.21C).

Taken as a whole, these results suggest that the cell fusion regulatory pathway is part of the output of the circadian clock in Neurospora and that it regulates a subset of clock-controlled genes.

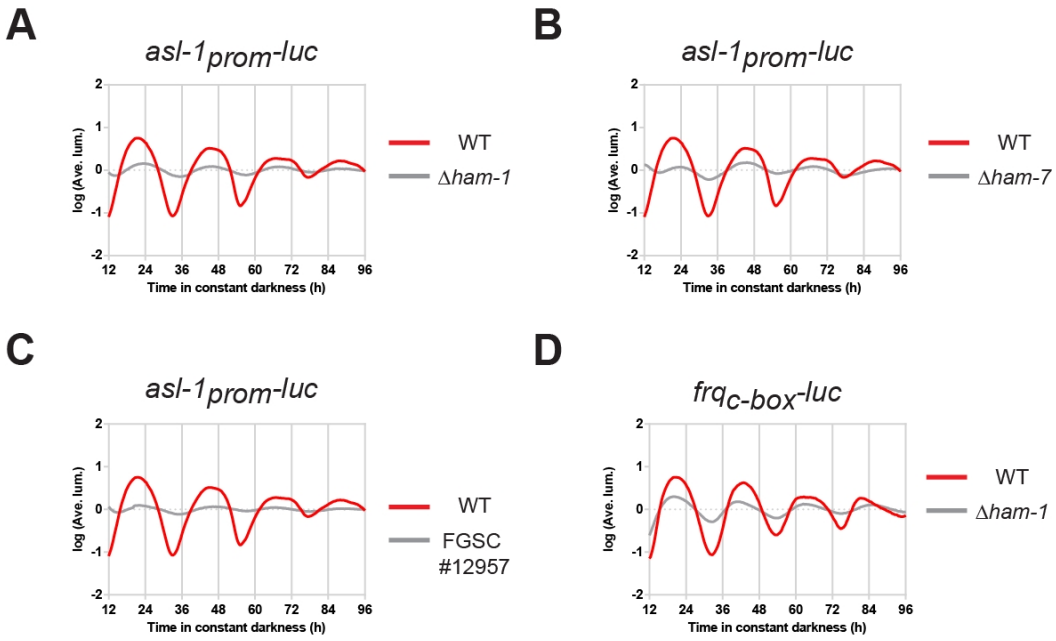


Figure 2.20. Strains defective in cell fusion exhibit altered rhythms in *asl-1* expression. The expression of *asl-1* was analyzed in three different strains, all displaying cell fusion defects. A mutant in the well described *ham-1* gene (A), a mutant in a newly identified gene involved in cell fusion, *ham-7* (B) and a strain in which a secondary mutation is responsible for the cell fusion phenotype (C), were all used to evaluate rhythms in *asl-1* expression. The expression is altered in all the strains tested. D) The status of the clock was evaluated in the Δ*ham-1* strain. A modest reduction in amplitude was observed, similar to the one in FGSC #11070.

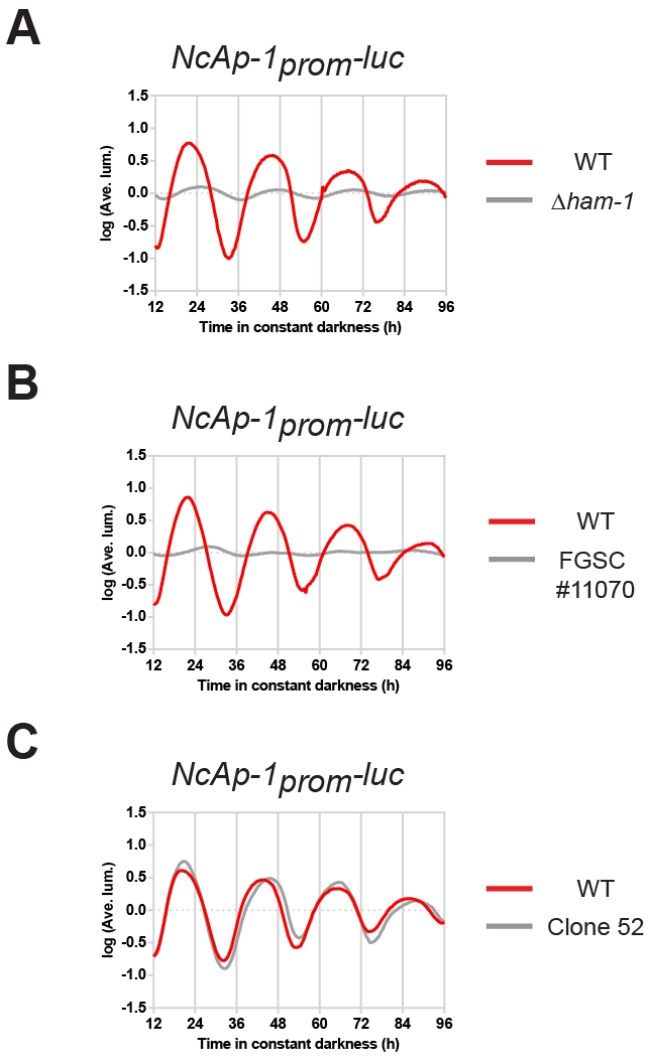


Figure 2.21. The rhythmic expression profile of *NcAp-1* is altered in the *ham-1* mutant. A) The expression of the output gene *NcAp-1* was evaluated in a WT and a Δ*ham-1* strain. The rhythmic profile of *NcAp-1* is altered in this mutant background. B) Analysis of the expression of *NcAp-1* in the FGSC #11070 strains shows that this gene, just as *asl-1*, is also deregulated in this background. C) Analysis of the *NcAp-1* reporter in Clone 52 shows that ADA-3 is dispensable for rhythms in *NcAp-1* transcription.

2.3.11. PBM data leads to the identification of the first TF required for growth under osmotic stress in Neurospora

As we mentioned previously, the motifs obtained for the Neurospora DNA-binding proteins (Section 2.3.4), will undoubtedly be relevant for characterizing transcriptional regulatory networks underlying a variety of processes in Neurospora.

We hypothesized that these motifs could be used to rationally search for and identify TFs associated with particular processes in Neurospora. Our reasoning was the following: if a particular TF motif is enriched in the promoters of genes associated with a particular Gene Ontology (GO) category, then that TF may be involved in that particular process.

As a proof of principle, we chose osmotic stress, particularly because no TF has been shown to be required for growth under this stress in Neurospora. This contrasts with the situation in other fungal species, where TFs have been reported to be necessary for proper growth under hyperosmolarity (de Nadal, et al., 2003; Peterbauer, et al., 2002; Rep, et al., 1999; Shiozaki and Russell, 1996; Van Nguyen, et al., 2013).

We scanned the promoter region of all Neurospora genes using all of our PBM-derived PWMs, so that each PWM gets a list of putative target genes. We then overlapped the genes in every GO category with these target gene lists to identify significant associations (See Materials and Methods). We identified 18 TFs for which GO categories associated with osmotic stress were overrepresented among its putative targets (Table 5). Interestingly, and

Table 5. Transcription factors predicted to be involved in osmotic stress responses in Neurospora

Gene Name	KO strain used
NCU01154	FGSC11126
NCU06399	FGSC14187
NCU08744	FGSC11386
NCU08807	FGSC10372
NCU03905	FGSC11131
NCU06173	FGSC11366
NCU06799	FGSC11001
NCU07675	FGSC15323
NCU01122	FGSC11125
NCU01345	DBP1314
NCU04960	FGSC17631
NCU09315	FGSC11448
NCU05064	FGSC18573
NCU07374	FGSC11016
NCU06186	FGSC11370
NCU00289	FGSC11085
NCU02356	FGSC11711
NCU03110	FGSC11024

lending support to our method, we found ASL-1 in that list (*NCU01345*). As mentioned previously, ASL-1 has been shown to regulate gene expression in response to osmotic stress in Neurospora (Lamb, et al., 2012; Yamashita, et al., 2008), similar to the role of its orthologs in other fungi (Balazs, et al., 2010; Proft, et al., 2005; Wilkinson, et al., 1996). While important for the up-regulation of various genes under high osmolarity conditions, this TF is not required for normal growth under these conditions in Neurospora (Figure 2.22), as has been similarly found by Yamashita et al. (2008) and Lamb et al. (2012). Such dispensability for normal growth under osmotic stress, resembles the situation of *asl-1* orthologs in other fungi, like *A. nidulans* and *B. cinerea* (Hagiwara, et al., 2008; Temme, et al., 2012), but differs from the scenario in *S. pombe* and *F. graminearum*, in which the corresponding ortholog is indeed required for normal growth under hyperosmolarity (Shiozaki and Russell, 1996; Van Nguyen, et al., 2013). Such heterogeneous role for *asl-1* orthologs in different fungal species, is well documented (Temme, et al., 2012; Van Nguyen, et al., 2013, and references therein).

We tested all 18 of the TF KO strains for osmotic stress sensitivity using both salt stress (1M NaCl) and osmotic stress (2M sorbitol). We identified one strain that exhibited growth defects under these conditions, the KO strain for the *NCU09315* gene, known as *nuc-1* (Figure 2.23A). This mutant, as the mutant for the OS-2 MAPK, which is known to be osmotic sensitive (Zhang, et al., 2002), displays a severe growth defect when grown under these stressful conditions. This is the first TF shown to be necessary for growth under osmotic stress conditions in Neurospora.

NUC-1 is a bHLH TF involved in the upregulation of genes in response to phosphate

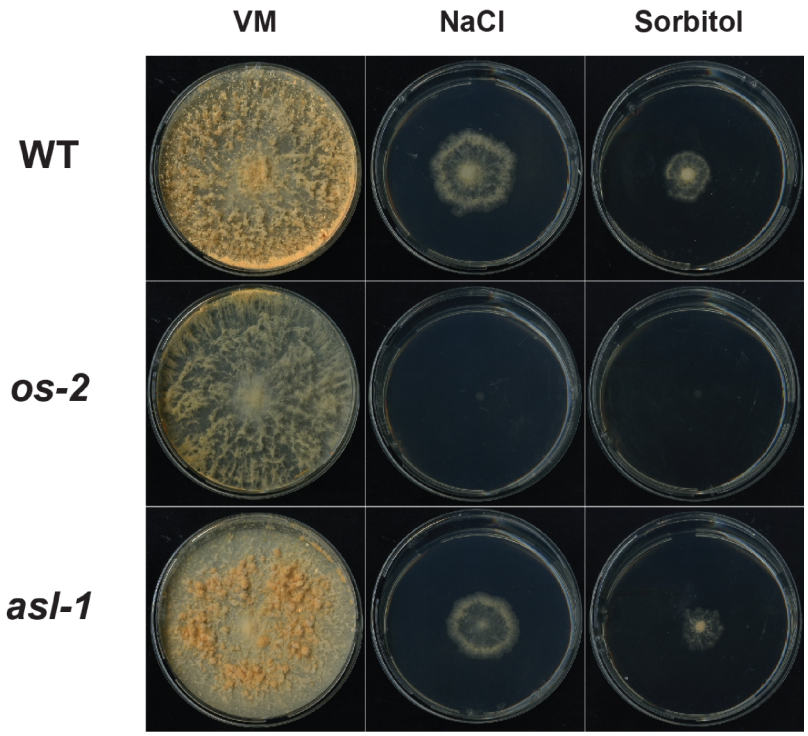


Figure 2.22. ASL-1 is not required for growth under osmotic stress conditions in Neurospora. Conidia from WT, Δ*os-2* and Δ*asl-1* strains was inoculated on VM, VM + 1M NaCl and VM + 2M sorbitol and grown for 3 days in constant darkness at 34 °C. Plates were left for 1 day in constant light at 25 °C before imaging.

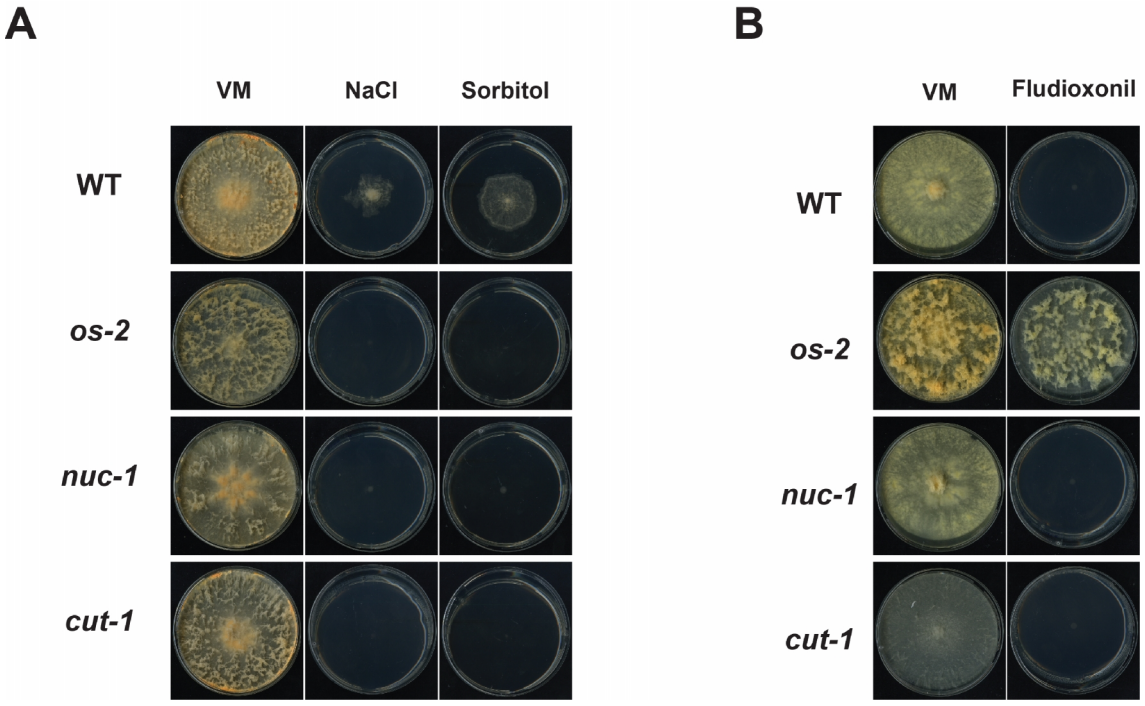


Figure 2.23. The bHLH transcription factor NUC-1 is required for normal growth under osmotic stress in Neurospora. A) Conidia from the strains listed in the Figure were inoculated on VM, VM + 1M NaCl and VM + 2M sorbitol and grown for 3 days in constant darkness at 34 °C. Plates were left for 1 day in constant light at 25 °C before imaging. B) Conidia from the strains listed in the Figure were inoculated on VM and VM + 10 µg/mL fludioxonil and grown for 4 days in constant light at 25 °C before imaging.

deprivation in Neurospora (Kang and Metzenberg, 1990; Peleg, et al., 1996a). This gene is related to PHO4, a TF that together with PHO2, controls the expression of genes involved in phosphate acquisition in yeast (Lenburg and O'Shea, 1996; Winderickx, et al., 2003). In Neurospora, upon phosphate shortage, a hierarchical regulatory network involving the products of at least 3 other genes (Metzenberg and Chia, 1979), leads to the up-regulation of genes encoding phophatases, phosphate transporters and many other genes necessary to achieve phosphate homeostasis (Gras, et al., 2013; Gras, et al., 2007).

Fludioxonil is a widely used phenylpyrrole fungicide derived from the antibiotic pyrrolnitrin (Gehmann, et al., 1990). This drug is proposed to act through abnormal hyper-activation of the Hog1/OS pathway leading to the up-regulation of genes involved in the osmotic stress response. The OS-2 MAPK in Neurospora and its orthologs in other fungi are phosphorylated in response to fludioxonil (Kojima, et al., 2004; Noguchi, et al., 2007) and this drug stimulates glycerol accumulation (an osmolyte) in Neurospora and other fungi in an OS pathway-dependent manner (Fujimura, et al., 2000; Kojima, et al., 2006; Ochiai, et al., 2002; Zhang, et al., 2002).

In fungi, several osmotic-sensitive mutants have been found to also be resistant to fludioxonil, a finding that uncovered a relationship between osmoregulation and phenylpyrrole fungicide resistance (Fujimura, et al., 2000; Jespers, et al., 1993; Kojima, et al., 2004; Moriwaki, et al., 2006; Zhang, et al., 2002).

It was generally assumed that abnormal accumulation of glycerol in the presence of this drug was solely responsible for its antifungal effects, leading to conidial swelling and lysis (Zhang, et al., 2002). The Neurospora *os-2* mutant for instance, which is defective in the OS pathway, fails to accumulate glycerol after fludioxonil treatment, which would explain its resistance to the drug (Zhang et al., 2002) (Figure 2.23B). Other mutants defective in different components of the OS pathway also exhibit this resistance phenotype (Fujimura et al., 2000). A Neurospora mutant strain called *cut* however, which harbors a premature stop codon in the *cut-1* gene (which encodes for a protein of unknown function) and which does not accumulate glycerol in response to fludioxonil, is nevertheless sensitive to this drug (Fujimura, et al., 2000; Youssar, et al., 2005) (Figure 2.23B). In addition, this mutant is sensitive to osmotic stress (Mays, 1969), presumably due to its inability to produce this osmoprotectant (Figure 2.23A). Hence, while fludioxonil can activate the Hog1/OS pathway and may exert some of its effects via abnormal glycerol accumulation, it possibly also targets other signaling pathways, which would contribute to its toxicity in a way that is independent of glycerol content (Fujimura, et al., 2000; Kojima, et al., 2006; Kojima, et al., 2004).

We evaluated growth of the Δ*nuc-1* strain in the presence of fludioxonil to test whether its sensitivity to osmotic stress was related to defects in the OS pathway. Similar to both the WT and the KO of the *cut-1* gene, the *nuc-1* mutant is sensitive to fludioxonil (Figure 2.23B). On the other hand and as expected, the Δ*os-2* strain is resistant to this drug. These results suggest that the osmosensitive phenotype of the Δ*nuc-1* strain is unrelated to defects in the OS pathway and that NUC-1 may participate in other signaling pathways that are required for proper growth under osmotic stress, similar to the product of the *cut-1* gene.

Further studies will help elucidate the role of NUC-1 is osmotic stress resistance. At present, we are performing several experiments aimed at characterizing the role of NUC-1 and phosphate signaling in osmotic stress responses in Neurospora (see Discussion).

2.4. Discussion

Output pathways are the least characterized aspect of circadian systems. This "unbalance" in knowledge among the different elements of circadian systems (input, pacemaker, output), is even more pronounced in Neurospora. In this fungus, a hierarchical arrangement of TFs has been proposed as the main regulator involved in setting the different phases of clock-controlled gene expression (Lakin-Thomas, et al., 2011; Montenegro-Montero and Larrondo, 2013). The identification of such Neurospora TFs that would mediate rhythms in gene expression or that are themselves rhythmically expressed -suggestive of a role in the Neurospora circadian system- is on its infancy. Further, despite decades as a model organism, little is known about transcriptional regulatory networks in Neurospora and the limited information available concerns only a handful of TFs. The vast majority of TFs in this organism then, remains largely uncharacterized.

In this Chapter, we report a complete revision of the list of putative TF in the Neurospora genome, with a focus of the eukaryotic-specific bZIP family, together with the identification of members of the bZIP family that display circadian rhythms in their transcription. We further report the determination of the sequence specificity of over half of the Neurospora transcription factors (and 63% of the predicted bZIP proteins) and how this information can lead to characterization of circadian transcriptional regulatory networks and to the identification of novel transcription factors involved in diverse processes in Neurospora.

Our analysis of the Neurospora genome identified 273 putative DNA-binding proteins, including 24 bZIP TFs. Previous estimates for this family of transcriptional regulators in Neurospora have ranged from 9 to 17 (Borkovich, et al., 2004; Tian, et al., 2010). The differences most likely arise due to the search method used and its strictness. On the one hand, we performed a typical Pfam-based search, similar to what was reported by Tian et al. (2010). They wrote, "(…) the bZIP member prediction was performed by hidden Markov model (HMM) searches; the bZIP TF HMM model (PF00170.10) was downloaded from Pfam (http://pfam.sanger. ac.uk/). The sequence was defined as a DNA-binding motif if the HMM search P value was $< 1 \times 10^{4}$". First, surely they must have meant 1×10^{-4}. Second, they did not report the software used for the HMM search. To test their approach, we used the well known and widely used HMMER software (Eddy, 2009), which we used for our own scanning, with the cutoff selected by Tian et al. (2010). Even under these stringent conditions, we found that 17 bZIP proteins could be identified. The reason behind their identification of only 9, is thus unclear.

We used a more lenient cutoff as the one reported by Tian et al. (2010) (See Materials and Methods), because ultimately, we planned to test the predicted proteins for their DNA-binding capabilities via PBMs, which would provide strong evidence of a functional TF. It is then possible, that our list includes false positives. In this regard however, three of the eight bZIP proteins that we identified, which had not been listed as TFs in any previous report, indeed display sequence-specific DNA-binding activity. It should be noted that this does not mean that the other five do not exhibit such a trait: it just means that we could not determine one via our *in vitro* approach. PBM failures may be due to any of several causes, including poor

expression in *in vitro* transcription reactions, protein misfolding and requirement for cofactors/dimerization partners. Alternatively, these proteins may require particular conditions to bind DNA *in vitro* that were not met in our experimental setting (e.g. specific buffer conditions), so further studies are required to determine whether they are bona fide TFs. Interestingly, a TF (encoded by *NCU00329*) that did not meet our cutoff, but that we nevertheless included in our global list, indeed exhibited sequence-specific DNA-binding properties, so even though our cutoff was lenient, it is still possible that some TFs were missed, which is a much bigger problem for the other two lists than for ours. Regarding comparison to Borkovich et al. (2004), their search method was not reported, so we cannot discuss the differences. Borkovich et al. (2004) however, listed some co-repressors that are known to lack sequence-specific DNA-binding activity as TFs, so their criteria for listing DNA-binding proteins was inherently different to the one we used in this study, as our method was based on scanning for domains that resembled known sequence-specific TF domains.

Finally, a report was published very recently listing predicted TFs from a variety of fungi (Todd, et al., 2014). Their method was similar to the one used in this study and when the same cutoffs are used, the results obtained are identical, further supporting our method.

We can confidently state that none of the lists discussed are complete, which probably reflects limitations of the methods used for the automated annotation of TFs. On the other hand, and also owing to such limitations, it is possible that all of these lists include false positives.

Our phylogenetic analysis of bZIP proteins among evolutionary informative fungal species, led to the identification of putative recent gene duplications among euascomycete fungi (although absent from Neurospora) and support previous claims regarding the timing of major bZIP family expansion in fungi, although more sophisticated methods may be required to evaluate this in depth. It would be interesting to evaluate the functional relationship of the identified gene pairs, to study the evolutionary regulatory fate of these duplication events.

As a research group, we are interested in characterizing circadian output pathways in Neurospora and in this work we decided to focus on the bZIP family of TFs. We reasoned that we could identify bZIP proteins involved in the Neurospora circadian system by identifying those with rhythmic expression patterns. To do this, we generated transcriptional reporter constructs by fusing the promoter region of all predicted bZIP TF encoding genes to the luciferase gene. Luciferase-based reporters have been successfully used in all clock model systems, providing sensitive and high-resolution studies of rhythmic promoter activity (Gooch, et al., 2008; Kondo, et al., 1993; Michael and McClung, 2003; Millar, et al., 1995; Stempfl, et al., 2002; Welsh, et al., 2005). We identified 17 bZIP encoding genes exhibiting rhythmic transcriptional activity. Interestingly, some of these genes have been associated with stress responses, an aspect that is known to have clock input in Neurospora (Vitalini, et al., 2006). For instance, *NCU01345* (*asl-1*), which we described extensively in this Chapter, is involved in osmotic stress responses (Lamb, et al., 2012; Yamashita, et al., 2008) and *NCU03905* (*NcAp-1*), which we also characterized in this study, participates in oxidative stress responses (Tian, et al., 2010). Further, *NCU01856*, the Neurospora homolog of the yeast *HAC1* gene, is also rhythmic and is involved in the unfolded protein response (Montenegro-Montero et al.,

manuscript in preparation). Interestingly, this pathway has been shown to have clock input in mammals (Cretenet, et al., 2010). Most of the other bZIP encoding genes, either rhythmic or not, have not been characterized in Neurospora, which opens an exciting area of research, particularly as some have been found to display various growth phenotypes (Colot, et al., 2006).

How does the high proportion of rhythmic bZIP encoding genes found in Neurospora compare to the situation in other organisms? A survey of rhythmic mRNA levels of bZIP genes in *Arabidopsis thaliana*, found that ~26% of the bZIPs that were found to be expressed in at least one time point in the database queried, could be scored as rhythmic (Hanano, et al., 2008). It is possible that many of these also have rhythmic transcription profiles and importantly, many of the ones that do not oscillate at the mRNA level, may anyway exhibit rhythms in transcription, as has been reported in other systems (Koike, et al., 2012; Le Martelot, et al., 2012; Menet, et al., 2012). So, even though only about 26% of them exhibit rhythms in mRNA levels, many more could have rhythmic transcriptional patterns. In order to compare our results with those obtained using somewhat similar approaches (that is, studying transcription rather than using mature mRNA levels as proxy), we analyzed the data from (Rodriguez, et al., 2013) and (Menet, et al., 2012), which used Nascent-seq to evaluate rhythmic transcription genome-wide in fly heads and mouse liver, respectively. We obtained a list of the predicted bZIP TFs from Drosophila and mouse from CisBP and compared it to the list of genes that exhibited rhythmic transcription in the aforementioned studies. From the 25 bZIP proteins predicted in the Drosophila genome, only 8% exhibited rhythms in transcription. Additionally, we found that about 9% of the mouse predicted bZIP TFs were found to be rhythmically transcribed in

mouse liver. These comparatively low numbers, when contrasted to the ones observed in Neurospora, beg however, a few comments. These two studies were not only underpowered for cycling detection, but also used very conservative criteria for scoring rhythmic genes, so the actual numbers may be higher. Also, these papers only reported rhythms in a particular and specific tissue. Further, and more importantly, even though Nascent-seq and luciferase reporters are both used to study transcriptional regulation, some differences are expected. For some genes, for instance, the amplitude at the RNA level, which is what Nascent-seq evaluates, may be at the limit of detection and the algorithms may not be appropriate for their detection as "rhythmic". Together, all these factors can influence the number of genes that are classified as "rhythmic" (Doherty and Kay, 2010). Moreover, the limited time resolution of RNA-based approaches could be another limiting factor, which is overcome using luciferase. Both the extended time frames for study and the sensitivity that comes with luciferase reporters, may explain the higher proportion of genes which display rhythmic transcriptional regulation in our system (discussed in Montenegro and Larrondo, 2013; Michael and McClung, 2003). In any case, as these are different systems, unless such Nascent-seq approaches are performed in Neurospora, the differences found are purely anecdotic.

When a gene is found to have clock input under a particular condition, the question that immediately follows is whether it is part of the clock mechanism itself or part of output pathways: the rhythmic bZIP proteins that we studied in detail in this study, appear to act mainly on output pathways. In Neurospora and in all clock model systems, the different actors involved in output pathways are largely unknown. Considering the ease with which genetic approaches can be implemented in Neurospora, we decided to study how the clock signal was

being transmitted to the promoter of the rhythmic bZIP encoding genes identified, using a genetics-based strategy. The implementation of the rational approach herein described however, required knowledge of the sequence preference of a large number of Neurospora TFs. We therefore set out to characterize the sequence specificity of all Neurospora TFs using protein binding microarrays, which allow for rapid, high-throughput and unbiased characterization of the sequence specificity of DNA-binding proteins (Berger and Bulyk, 2006; Weirauch, et al., 2013). This is the first study aimed at studying DNA-binding specificities on a global scale in filamentous fungi, the largest group within the fungal kingdom. We determined the sequence preference of over half of the predicted Neurospora DNA-binding proteins, obtaining motifs for members of all identified TF families. For some families, the coverage was outstanding. For instance, we obtained motifs for 86% of the predicted bHLH TF in Neurospora. All this information placed Neurospora as the filamentous fungus with the highest motif coverage, as well as one with the highest number of directly determined motifs among eukaryotes (Weirauch, et al., accepted in *Cell*). We anticipate that this dataset will be an invaluable resource for functional genomics and analysis of gene regulation not only in Neurospora, but in related species and we are currently collaborating with several groups to use these motifs in systems biology approaches aimed at characterizing fungal regulatory networks (Bertolini, et al., manuscript in preparation).

We employed these motifs to characterize the transcriptional networks underlying the rhythmicity of two bZIP encoding genes, *NcAp-1* and *asl-1*. We found that the product of the *ada-1* gene, incidentally also a bZIP encoding gene, is a player involved in circadian output pathways in Neurospora, leading to the rhythmic transcription of the bZIP encoding gene

NcAp-1, involved in oxidative stress responses. Additionally, the normal rhythmic expression of *con-10*, a rhythmic gene associated with asexual development, also requires ADA-1 (Figure 2.13C). Moreover, preliminary data shows that the *ada-1* deletion also affects the normal rhythmicity of an NcAP-1-LUC translational fusion (expressed from the endogenous locus, not shown) and the defect exhibited is different from the one displayed by the CON-10 reporter, suggesting that ADA-1 may control various output pathways. Our data also supports a minor role for *ada-1* in the workings of the central clock, as evidence by a ~1 hr defect and a modest amplitude reduction in the expression of the *C-box* reporter in the Δ*ada-1* strain. Recent experiments however, suggest that *ada-1* may mainly, if not exclusively, only regulate output pathways, as evidence by the analysis of a FRQ-LUC translational fusion. This fusion is made at the *frq* endogenous locus and allows for the monitoring of FRQ dynamics (Larrondo, et al., 2012). While we continue to observe a very modest amplitude reduction, no significant period, phase or waveform defect was found for the FRQ-LUC reporter in the *ada-1* mutant strain (Figure 2.24). We are currently analyzing more clones, but this suggests that while *ada-1* deletion may somehow impact *frq* transcription, no major defect is observed at the protein level. We conclude then, particularly by comparing the mild effects of *ada-1* deletion over *frq* expression and the stronger one over the expression of the output genes analyzed (*NcAp-1* and *con-10*), that *ada-1* is mainly involved in output pathways, with no significant impact on the central clock. Alternatively, the modest effect on *frq* transcription and FRQ levels observed in Δ*ada-1*, could somehow get amplified through the signaling pathways involved in output and lead to the observed deregulation in the rhythmicity of *NcAp-1* and *con-10*. This would mean that the defects observed for these two genes arise originally from defects on the clock. This is unlikely, considering that the effect over the two genes is

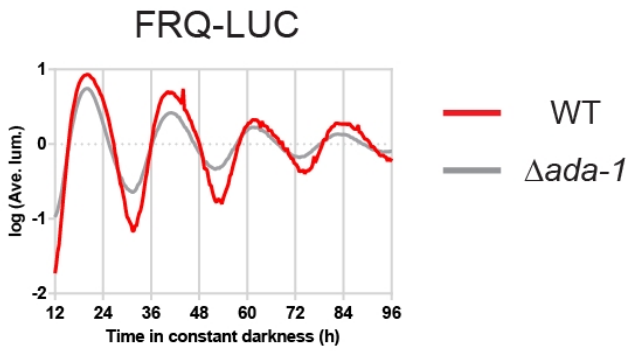


Figure 2.24. Deletion of *ada-1* does not impact FRQ expression. The expression of a FRQ-LUC translational fusion, expressed from the *frq* endogenous locus, was evaluated in a WT and a Δ*ada-1* strain by monitoring bioluminescence in constant darkness. No significant difference was found in the bioluminescence traces between the genetic backgrounds tested.

different. Further studies on the role of ADA-1 in the Neurospora circadian system are definitely warranted to clarify its position on the clock network. Interestingly, a putative binding site for ADA-1 is present in the *wc-2* promoter region, which merits further evaluation (not shown).

As a whole, we were able to shed light on the possible role of bZIP proteins in the Neurospora circadian system: we identified several members of this family with clock input and we further found one of such bZIP proteins, ADA-1, to have significant roles in output pathways.

Besides *NcAp-1*, we also used *asl-1* as "bait" in our genetics approach. Our strategy however, did not lead to the identification of a TF involved in mediating rhythms in *asl-1* expression, which as mentioned in Section 2.3.7, may arise from network robustness or simply due to the "futility theorem", an excessive amount of false positive predictions of TF binding, due to using the simple (and popular) strategy of single binding site prediction using PWM scanning (Wasserman and Sandelin, 2004). This is an unavoidable consequence of using short and often degenerate sequences for scanning for binding sites, such as the typical DNA-binding sequence motifs derived for eukaryotic TFs (Fickett and Wasserman, 2000). Despite this, direct PWM scanning has the advantage of being simple and has been shown to result in meaningful results in compact genomes (Badis, et al., 2008), so testing it in the relatively small Neurospora genome was warranted. In addition, direct PWM scanning can serve as a starting point for generating a preliminary list of candidate binding sites that can later be refined using additional information (see below), further supporting its choice.

While we decided on a typically used cutoff score for the prediction of upstream regulators for *asl-1* (85% of the maximum possible score), this might have excluded real regulators of this gene which may bind with lower affinity: it is always a compromise between sensitivity and specificity. Lowering the threshold, would greatly increase the number of predicted binding sites in the promoter of interest, consequently increasing false positive calls. Prediction of TF binding sites is a complex computational problem which requires a multi-faceted approach (Aerts, 2012). The integration of different datasets can help improve the predictive power of these methods. One element that can be used to improve these predictions, particularly to reduce false positives when lowering the threshold, is sequence conservation in regulatory regions. Using related species, one could search for conserved sequences in the promoter region of orthologous genes. The working assumption would be that functionally important elements are under sequence constraint (that is, they exhibit slower rates of sequence change than background) and would be conserved between the orthologous promoters. This approach is known as "phylogenetic footprinting" (Duret and Bucher, 1997; Gumucio, et al., 1992; Tagle, et al., 1988) and has been proven useful in different species for the identification of functional TF binding sites (Cliften, et al., 2003; Glenwinkel, et al., 2014; Hong, et al., 2003; Lenhard, et al., 2003). Functional elements would stand out as "footprints" of sequence conservation among the background sequence variation of the nonfunctional regions. Considering the vast number of fungal genomes available, such a comparative genomics approach, which relies on conservation of functional binding sites between species, together with the large number of motifs reported in this study, can be of great help in the identification of relevant binding sites for Neurospora TFs.

Additionally, integration of gene expression signature data, motif clustering, ChIP-seq and DNA accessibility data, together with the PWMs herein reported, can greatly improve the predictive power of the computational methods, reducing false positive calls (Aerts, 2012). Computational approaches using such data for the genome-wide identification of functional TF binding sites are available (Aerts, 2012; Hannenhalli, 2008) and we are currently evaluating them using our PBM data to further characterize circadian transcriptional networks in Neurospora.

Finally, it should be mentioned that we only included predictions for the TFs for which we had motifs, so we only tested upstream regulators among about half of all predicted TFs in the Neurospora genome. It is possible then, that TFs for which we do not yet have sequence preference information, regulate the rhythmic expression of *asl-1*. Also, the fact that there is a number of TFs for which no KO is yet available, limits our approach. Altogether, technical limitations are the most likely culprit in the failure of the identification of a particular TF involved in regulating rhythms in *asl-1* expression and efforts are being made to improve predictions and have a clearer view of circadian transcriptional networks regulating rhythmicity of bZIP encoding genes.

We did find however, a mutant strain in which the rhythmic expression of *asl-1* was altered. Initially, our data suggested that ADA-3, a Zn cluster TF, was involved in output pathways regulating *asl-1* expression, but a thorough genetic analysis suggested that a secondary mutation in the strain harboring the *ada-3* deletion, but not the *ada-3* deletion itself, was responsible for both the growth phenotype exhibited by this strain and the misregulation of

asl-1. Our segregation results suggest that alteration of a single gene (or of additional genes, but laying very close to each other in the chromosome, so that they segregate as a single unit) is responsible for both phenotypes (not shown). This highlights the power of Neurospora for classical genetic analyses. These results, together with additional experiments, led us to conclude that cell fusion pathways may be immersed in the Neurospora circadian system, particularly output pathways, as different mutants with altered cell fusion also result in altered *asl-1* rhythmic profiles, with little to no impact on the central clock. To identify the genetic alteration leading to both the cell fusion phenotype and misregulation of *asl-1* expression, we conducted whole genome sequencing of the strains (Montenegro-Montero, et al., manuscript in preparation). Our preliminary results, based on high-throughput sequencing data, suggest that FGSC #11070 has ~30 kb deletion in linkage group I (the same *ada-3* is in) and that this deletion may be responsible for the growth phenotype reported in Colot et al. (2006) and Fu et al. (2011). The fact that this deletion and *ada-3* are in the same linkage group, may explain why Fu et al. (2011) originally failed to observe that hygromycin resistance (resulting from the cassette at the *ada-3* locus in FGSC #11070) and the "all developmental altered" phenotype do not always co-segregate (as they are close together, recombination events would be infrequent). Interestingly, the particular region deleted in FGSC #11070, harbors the *ham-1* gene in a WT strain. As mentioned previously, this gene has been shown to be necessary for normal cell fusion (Fleissner, et al., 2005; Wilson and Dempsey, 1999) and we herein report it to be necessary for proper *asl-1* transcriptional rhythms. This suggests that both the cell fusion phenotype and misregulation of *asl-1* in FGSC #11070 may be due to the absence of *ham-1*. At present, we are conducting experiments to see if introduction of a WT copy of *ham-1* reverts both of these phenotypes. As cell fusion phenotypes are present in several of the strains

of the Neurospora knockout collection, with only few of them co-segregating with hygromycin resistance or being reverted by the introduction of a WT copy of the corresponding knocked out gene (Fu, et al., 2011), it is possible that just as FGSC #11070, many of them lack *ham-1*, which would highlight the relevance of our findings to the Neurospora community. This is a hypothesis we are currently pursing. In agreement with the aforementioned idea, we have evidence that FGSC #12957, a mutant strain that exhibits cell fusion defects that do not co-segregate with hygromycin resistance (Maddi, et al., 2012), indeed lacks the *ham-1* gene, in addition to the intentionally deleted gene, *acw-4* (not shown). Interestingly, a high rate of spontaneous mutations giving rise to mutants with cell fusion defects (resembling *soft*), has previously been noted (Kafer, 1982).

Besides individual TFs, we evaluated the role of complex signaling pathways in controlling rhythms in bZIP gene expression. We found that the MAPK pathway involved in osmotic stress responses, the OS pathway, particularly the MAPK OS-2, is required for rhythms in *asl-1* and *NcAp-1* expression. Interestingly, the orthologs of these bZIP proteins have been shown to be regulated by the corresponding osmotic-associated MAPK pathway in yeast species (Saito and Posas, 2012; Wilkinson and Millar, 1998) and the proper expression of the *asl-1* homolog in *F. graminearum*, depends on the OS-2 MAPK (Van Nguyen, et al., 2013). The OS pathway has previously been found to have clock input in Neurospora and to control rhythms in clock-controlled genes (Vitalini, et al., 2007), but no TF has previously been reported to be controlled by this pathway in this fungus in the context of circadian rhythms. We further show that rhythms in *frq* transcription are altered in the Δ*os-2* strain, but that rhythmicity is restored after ~ 3 days. No restoration however, is observed for the bZIP encoding genes *NcAp-1* and

asl-1. The reason behind the delay in rhythm generation in the Δ*os-2* strain is unknown, but mutations in regulators of the OS pathway has been shown to lead to a delay in the appearance of conidial banding in race tube assays (Vitalini, et al., 2007), reminiscent of the effect seen for *frq* transcription in the absence of OS-2. At present, we are evaluating the impact of direct *os-2* ablation on rhythms in asexual development.

It has previously been shown that rhythmicity at the mRNA level of *ccg-1* requires the OS pathway, as evidence by mutating an upstream regulator of the pathway, the *rrg-1* gene (Vitalini, et al., 2007). We now show that the OS pathway, via OS-2, control rhythms in transcription of the clock-controlled gene *ccg-1* and we propose that loss of these transcriptional rhythms in the absence of OS-2 are at least partly responsible for the loss in rhythmicity of the mRNA levels of *ccg-1* observed when the pathway is altered (i.e. in the Δ*rrg-1* strain).

We explored that idea that ASL-1 was required for *ccg-1* transcriptional rhythms, but our cumulative evidence suggested this was not the case. While this work was in preparation, a report from the Bell-Pedersen lab argued that *asl-1* was indeed required for normal *ccg-1* expression and further, that *asl-1* expression (as assessed via its mRNA levels) is not controlled by the clock (Lamb, et al., 2012). How can these results be explained, in light of the ones reported in this study? Let us analyze these results separately. Regarding rhythmicity, our data clearly shows rhythms in transcription of *asl-1*, using very strict criteria (see Materials and Methods). It could be argued however, that the gene is rhythmically transcribed, but that levels of the corresponding mRNA do not oscillate daily, as has been shown for several genes

in other systems. Our preliminary data however, using quantitative PCR with carefully selected reference genes (Montenegro-Montero, et al., in preparation) shows clear daily rhythms in *asl-1* mRNA levels, which altogether suggest to us that this gene indeed has clock input at various levels. Additionally, an ASL-1-LUC translational fusion, also results in rhythmic bioluminescence profiles (Figure 2.25). The data of Lamb et al. (2012) on the other hand, suggests that *asl-1* has no clock input, as evidence by evaluating *asl-1* mRNA levels in circadian time courses. Their data however, is non-quantitative: they used Northern Blots assays using ethidium bromide-stained gels as loading controls, which they argue is "standard in the field". Such normalization strategy is not accurate and unnecessarily assumes that the mRNA/ribosomal RNA ratio is invariant between the samples, an assumption that can be wrong (Hansen, et al., 2001; Solanas, et al., 2001; Spanakis, 1993). Further, it provides no measure regarding the consistency of messenger RNA loading in each lane. Additionally, the reporting of the results in Lamb et al. (2012) is inconsistent: in some figures they show two ribosomal bands, and in others, just one, which makes it difficult to follow their normalization criteria.

Second, the experimental setting used by Lamb et al. (2012) is different from ours. Considering that our transcriptional reporter assays were studied on solid maltose media, fungal growth for the RT-qPCR experiments was also performed in such solid media. The experiments by Lamb et al. (2012) on the other hand, were performed in liquid Vogel's media. Media conditions may have hindered their ability to detect rhythms for *asl-1* at the mRNA level, as this gene may have different inputs under different growth conditions (discussed in Montenegro-Montero and Larrondo, 2013). As a whole, we stand by our results that *asl-1*

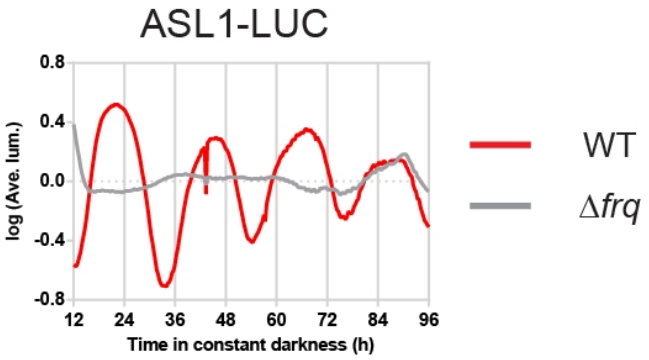


Figure 2.25. ASL-1 levels oscillate with a circadian rhythm. A translational fusion between the *asl-1* coding gene and luciferase was engineered at the *asl-1* endogenous locus and its expression was evaluated by monitoring bioluminescence in a WT and Δ*frq* strain under constant darkness.

indeed has clock input and differences with the data reported by Lamb et al. (2012) may derive from technical issues.

Regarding the role of ASL-1 in regulating *ccg-1* rhythms, Lamb et al. (2012), again using their non-quantitative Northern Blot approach, report that while rhythms in *ccg-1* mRNA levels are still evident in the Δ*asl-1* strain, the amplitude appears to be reduced compared to a WT strain. We observed no difference in amplitude in *ccg-1* transcriptional rhythms when removing ASL-1 binding sites from its promoter, so the effect may either be post-transcriptional and indirect over *ccg-1* mRNA levels. Alternatively, there may still be ASL-1 binding sites in the *ccg-1* promoter that we missed and that may explain the WT nature of its rhythmic transcription in all our constructs. We consider the latter unlikely, but the yeast homolog of ASL-1, Sko1, has been shown to bind to two slightly different motifs, both *in vitro* and *in vivo* (Gordan, et al., 2011) and it is possible that ASL-1 may do as well and may bind different regions in the *ccg-1* promoter that we did not identified using the primary motif. We are currently evaluating our PBM data to see if such a secondary motif is bound by the Neurospora protein and if so, if that motif is present in the promoter of *ccg-1*. In any case, we provided evidence that motifs identified via the PBM-derived ASL-1 motif are functional, as shown with the osmotic stress experiments in Figure 2.16.

One thing should be mentioned about these osmotic stress experiments. We noticed that the p575 construct led to higher *luciferase* mRNA levels upon osmotic stress than that p631 construct. Even though it could be proposed that a repressor present in p631, but absent from p575, could be responsible for these results, we think this most likely reflects some issues with

the p631 construct. When we analyzed rhythms from this construct from primary transformants, we observed that while rhythms were evident, results were significantly more variable than those obtained using p575. Interestingly, when we homokaryonized this strain by crossing, this variability disappeared. We are repeating the experiments using homokaryon strains for all constructs, which we hope will help to clarify the original p631 results.

To further evaluate the role of ASL-1 in regulating rhythms in *ccg-1* expression, we exchanged its rhythmic and light-induced promoter with that of *sod-1*, a gene that lacks both of these traits. This was under the assumption that there is a "circadian hierarchical arrangement" in the pathway, such that OS-2 is responsible for *asl-1* rhythms, which in turn regulates *ccg-1* rhythms. We observed no difference in the rhythmicity of *ccg-1* transcription in such strain, again suggesting that ASL-1, or at least its endogenous transcriptional regulation, is not required for such rhythms. It is conceivable however, that the promoter exchange strategy was not enough to significantly alter ASL-1 levels to the point of impacting downstream targets. We are currently conducting experiments to evaluate this hypothesis. Additionally, ASL-1 may not need to be rhythmically expressed to control rhythms in *ccg-1* levels. Additional clock input at other levels may be enough to maintain its proper role in regulating rhythms in *ccg-1* expression, i.e. at the post-transcriptional and post-translational levels. In yeast, Sko1 is known to be regulated by Hog1 via phosphorylation (Proft, et al., 2001). The Neurospora Hog1 homolog, OS-2, is rhythmically activated (Vitalini, et al., 2007) and conceivably, it may regulate the rhythmic activity of its targets, one of which may be ASL-1, via phosphorylation. In this setting, regardless of its transcriptional rhythms, ASL-1 activity would still be rhythmic (modulated by OS-2-dependent phosphorylation) and could

normally regulate rhythms in *ccg-1* transcription. Rhythmicity at multiple levels is thought to contribute to system robustness (discussed in Montenegro and Larrondo, 2013) and *asl-1* may be subjected to such control.

In order to evaluate whether the reported reduced amplitude of *ccg-1* mRNAs is a result of transcriptional or post-transcriptional deregulation in the Δ*asl-1* strain, we are currently performing experiments to evaluate the expression of the *ccg-1* transcriptional reporter in this strain. As mentioned in Section 2.3, this cannot be done via crossing the reporter strain with the *asl-1* KO strain, as homokaryon strains harboring this mutation do not germinate. We are employing a different strategy that consists in transforming the *ccg-1*$_{prom}$*-luc* construct directly into the *asl-1* strain and monitoring bioluminescence levels. This approach however, relies on using the same Δ*asl-1* strain as Lamb et al. (2012), which they did not test by re-introducing a WT copy of *asl-1*. This means that it is possible that the rhythm defect they observe for a number of *ccgs*, including *ccg-1*, may be due to a secondary mutation, as we have described above.

In any case, again, the differences between the results obtained for the role of ASL-1 over *ccg-1* rhythms, may also be due to the experimental setting used: in solid maltose media, loss of ASL-1 regulation over *ccg-1* may simply not lead to defects in *ccg-1* expression, while it may do so in liquid Vogel's media (as in Lamb, et al., 2012), making both results valid. By performing this whole suite of experiments under the same conditions, we expect to get a clearer view of ASL-1 regulation of *ccg-1* rhythms. In yeast, TFs other than Sko1 have been shown to be under Hog1 control (Nadal and Posas, 2008) and the corresponding Neurospora

orthologs are interesting candidates to test as upstream regulators of *ccg-1* rhythms, particularly because we have shown that loss of the Hog1/OS-2 MAPK leads to arrhythmic *ccg-1* transcription.

In addition to the OS pathway, we evaluated another MAPK signaling pathway in Neurospora for its role in the rhythmic expression of bZIP encoding genes. We examined the expression of *NcAp-1* and *asl-1* in strains lacking the MAPK MAK-1, predicted to be involved in cell wall integrity and maintenance, as its yeast counterpart Slt2 (Chen and Thorner, 2007), besides other processes (Park, et al., 2008). Indeed, Δ*mak-1* strains exhibit cell wall defects, in addition to short aerial hyphae, reduced asexual spore formation, female sterility and cell fusion defects (reviewed in Kamei, et al., 2013).

We observed that transcriptional rhythms of both *NcAp-1* and *asl-1* were severely affected in the Δ*mak-1* strain (Figure 2.26). As customary in our approach, we proceeded to evaluate the expression of the *C-box* reporter in this strain and observed that rhythmicity was also dramatically impaired (Figure 2.26C). While this work was in preparation, Bennett et al. (2013), reported that similar to OS-2, MAK-1 is also rhythmically phosphorylated in Neurospora and that while it is required for the proper rhythmicity of a few clock-controlled genes (including *ccg-1*), it is dispensable for proper clock function: their Western Blot assays showed WT daily oscillations of FRQ protein levels in the Δ*mak-1* strain. This latter evidence suggested to us that the effect that we were observing with our luciferase reporters could be due to misregulation of the *his-3* locus, where the reporter constructs are targeted, in the

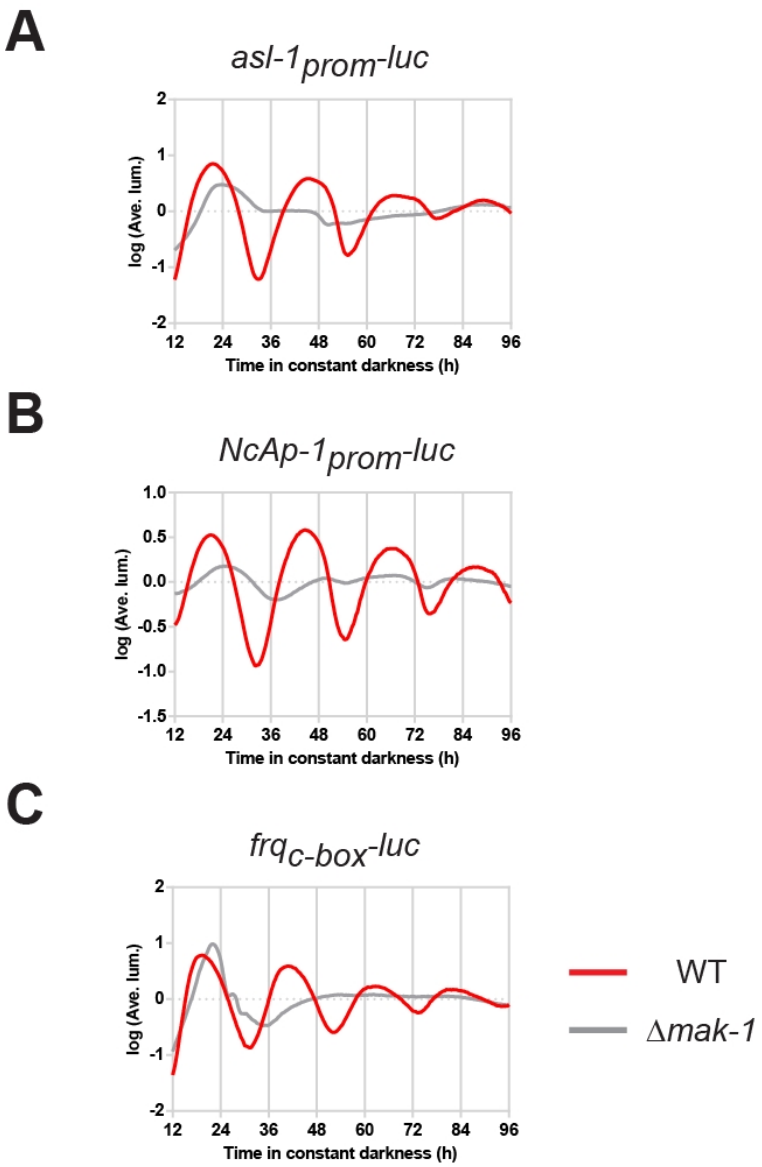


Figure 2.26. Analysis of bioluminescence profiles of different constructs at the *his-3* locus in the Δ*mak-1* strain. Strains harboring the reporter constructs for A) *NcAp-1*, B) *asl-1* and the C) *C-box* control construct, were crossed to a Δ*mak-1* strain and bioluminescence was evaluated in both WT and *mak-1* progeny. All three constructs exhibited deregulation in the absence of MAK-1.

Δ*mak-1* strain. Consistent with this idea, mRNA levels of the *his-3* gene are deregulated in the *mak-1* mutant (Bennett, et al., 2013). We concluded then, that the *his-3* locus was not appropriate to evaluate transcriptional reporter constructs in the Δ*mak-1* background. At present, we are testing the expression of *NcAp-1* and *asl-1* translational fusions (which are expressed from the endogenous loci) and luciferase transcriptional reporters, but targeted at another locus, in this mutant to see whether this MAPK plays a role in their rhythmic expression patterns. Interestingly, we have observed that ASL-1 is required for normal growth under cell wall stress conditions in Neurospora, suggesting that it could be a downstream target of the MAK-1 pathway (Figure 2.27A). Notably, this is, to our knowledge, the first transcription factor shown to be required for proper growth under this type of stress in Neurospora. Interestingly, on reviewing the literature, we found that *asl-1* orthologs in the yeast *Candida albicans* and the filamentous fungus *Botrytis cinerea*, have been shown to exhibit some cell wall defects (Rauceo, et al., 2008; Temme, et al., 2012) and that Sko1 mutants in *S. cerevisiae* are sensitive to caffeine (Garcia-Gimeno and Struhl, 2000). As we have shown that this TF is required for cell wall stress in Neurospora and that it is deregulated in the absence of OS-2, we tested whether this MAPK was required for cell wall stress. As shown in Figure 2.27B, a strain lacking this MAPK exhibits a phenotype that differs from that of an *asl-1* mutant: the *os-2* mutant can efficiently grow under caffeine and Congo red, but not under SDS, which is probably the most indirect cell wall stress agent used. Similar results were obtained when using Δ*os-4*, a strain deficient in a different member of the OS pathway (not shown). As a whole, this suggests that the requirement for ASL-1 in cell wall stress responses is OS-pathway-independent, consistent with results from other fungal species (Rauceo, et al., 2008).

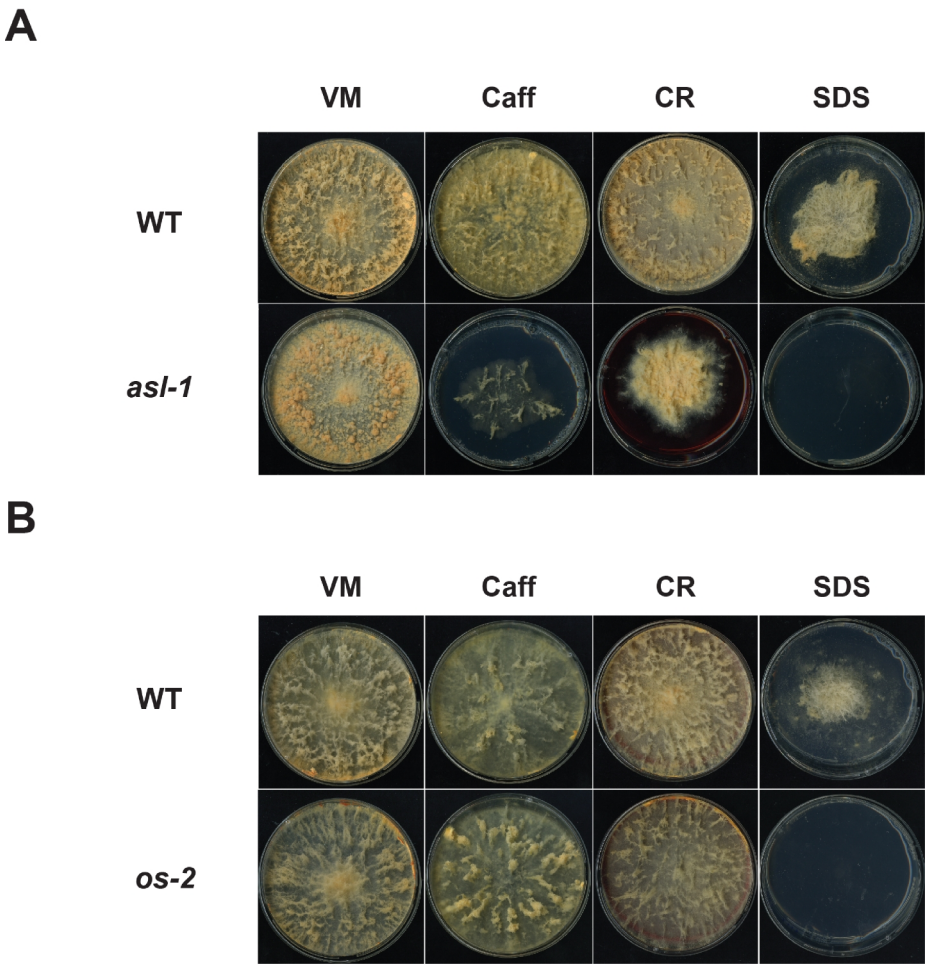


Figure 2.27. The bZIP transcription factor ASL-1 is required for proper growth under cell wall stress in Neurospora. Conidia from 7 day old slants from both WT (328-4) and *asl-1* strains (A) and WT (FGSC #2489) and *os-2* strains (B) were inoculated on solid VM and VM supplemented with caffeine (5 mM), Congo Red (200 µg/mL) and SDS (0.01 % w/v) and grown for 3 days in constant darkness at 34 °C. Plates were left for 1 day in constant light at 25 °C before imaging. The compounds tested are all well known cell wall stressing agents. Caffeine has been shown to activate the cell wall integrity (CWI) pathway in *S. cerevisiae* and yeast strains defective in components of the CWI pathway, are sensitive to caffeine (Levin, 2011). Congo red interferes with cell wall assembly by binding to chitin (Roncero and Duran, 1985) and SDS, on the other hand, probably leads to cell wall stress indirectly, by affecting membrane stability (Arias, et al., 2011).

At present, we have evidence that the rhythmic expression profile of *con-10* (as assessed using a translational fusion reporter construct) is altered in the *mak-1* mutant, which is also a novel finding (Figure 2.28). This was not an obvious result, as several clock-controlled genes are not altered in the Δ*mak-1* strain (Bennett, et al., 2013).

Even though emphasis has been placed throughout this Chapter exclusively on transcriptional regulation as the master regulatory step for rhythms in clock-controlled gene expression, clock control could in principle take place at any other stage of gene expression, namely, mRNA processing, export, stability and subcellular localization, as well as at the translational and post-translational levels. All of these steps have been shown to exhibit clock input in different systems, including Neurospora (Garbarino-Pico and Green, 2007; Kojima, et al., 2011; Mehra, et al., 2009; Staiger and Koster, 2011; Wang, et al., 2013; Zhang, et al., 2011) and they may play a widespread relevant role in output pathways in this system as has been shown in others (Lim and Allada, 2013; Partch, et al., 2013).

In this regard, it is possible that additional bZIP encoding genes (besides the rhythmic ones found in this study) may display rhythms at the mRNA level, notwithstanding flat transcriptional rhythms. This of course, could also happen the other way around, that is, that bZIP encoding genes found to exhibit rhythms at the transcriptional level, may have flat daily mRNA levels. In any case, any combination of these states may result in rhythms at the protein level (Kojima, et al., 2012; Reddy, et al., 2006). Global studies as the ones described in this Chapter, focusing on a single step of gene expression, allow keeping research focused on a single mechanism and identify the extent to which each step controls clock-regulated gene

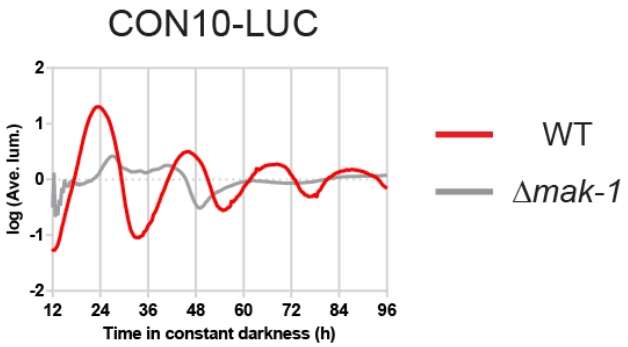


Figure 2.28. MAK-1 is required for rhythms in CON-10. A translational fusion between the *con-10* coding gene and luciferase was engineered at the *con-10* endogenous locus and its expression was evaluated by monitoring bioluminescence in a WT and a Δ*mak-1* strain under constant darkness.

expression. The integration of transcriptional, RNA and proteomic circadian data, will surely unveil the many mechanisms that the clock uses globally to control biological processes in Neurospora (Montenegro-Montero and Larrondo, 2013).

As mentioned throughout Section 2.3, we anticipate that the information on the DNA-binding preference of a large number of Neurospora TF reported here (available at CisBP, http://cisbp.ccbr.utoronto.ca), will be an invaluable resource and knowledge base for functional genomics and analysis of gene regulation (Weirauch, et al., accepted in *Cell*).

In addition to the motifs themselves, the CisBP database also contains web-based interfaces to tools for scanning DNA sequences for putative motifs and for the reporting of TFs with motifs similar to a given motif, among others. The latter is useful to assign a TF to motifs derived for instance, from gene co-expression analyses. For example, as mentioned in Section 2.1, an 8 nucleotide element, very similar to the core of the ACE, a *cis*-regulatory element that has been shown to be both necessary and sufficient for *ccg-2* rhythmicity (Bell-Pedersen, et al., 1992), was identified in the promoter of several rhythmic genes (Correa, et al., 2003). This information however, has not led to any significant advancement in characterizing output pathways, because little information is available on sequence binding specificity for Neurospora TFs and it has not been able to be matched to the motif bound by any Neurospora TF. We analyzed the motif and used CisBP to see if it matched the sequence preference of any characterized TF. Interestingly, the sequence was a significant match to the motif bound by the Neurospora C2H2 TF encoding gene *NCU00090* and its corresponding yeast homolog, RIM101. This then, allows proposing the hypothesis that the TF encoded by this gene in

Neurospora, may play a role in output pathways, an idea that can now be experimentally explored and which would not have been able to be proposed without the data generated in this study.

As a proof of principle for the usefulness of this dataset for the mapping of transcriptional networks, we used it to identify the first TF in Neurospora required for growth under osmotic stress, NUC-1.

This TF, involved in transcriptional responses to phosphate shortage in Neurospora, was identified as required for the osmotic stress response solely by the presence of its motif among the promoter regions of genes involved in osmotic stress. This same approach could be used for any other phenotype of interest with a related Gene Ontology category, which could open the door to addressing the transcriptional regulation of a variety of processes in Neurospora and related fungi.

The mechanism underlying the osmotic sensitivity in the Δ*nuc-1* strain is unclear. An ionic imbalance in the presence of large amounts of salt (NaCl), such that would somehow alter phosphate transport, could be surmised, but our data shows that the growth defect is also present when non-ionic osmotic stress is used (sorbitol), suggesting true osmotic sensitivity in this mutant. To gain insights into the role of NUC-1 in osmotic stress responses, we have performed total RNA sequencing (RNA-seq) experiments under osmotic stress conditions, for both WT and *nuc-1* strains and this is currently under analysis. Interestingly, *pho2* and *pho4*, two genes that are up-regulated under phosphate shortage (Gras, et al., 2013) and that appear

to be direct targets of NUC-1 in Neurospora (Peleg and Metzenberg, 1994), were not induced in the WT strain under our osmotic stress conditions (as determined via RNA-seq), suggesting that our protocol does not lead to problems in phosphate availability. Further, a pathway regulator-encoding gene, *nuc-2*, whose mRNA increases several fold in response to low phosphate (Peleg, et al., 1996b), was also not induced in our RNA-seq data (not shown), suggesting that the osmotic sensitive phenotype exhibited by the *nuc-1* mutant is unrelated to reduced phosphate availability. At present, we are evaluating the growth phenotype of other mutants in the phosphate pathway to see if they are also osmotic-sensitive, including upstream regulators and phosphate transporter mutants. This will help clarify whether the pathway as a whole is required for growth under osmotic stress or if this is a phosphate pathway-independent role of NUC-1. These ongoing experiments will help shed light on the role of NUC-1 and the phosphate signaling pathway on osmotic stress responses in Neurospora.

In this Chapter we have addressed the most neglected aspects of circadian systems, i.e. output pathways and identified regulatory circuits and specific TFs involved in it in the clock model system *N. crassa*, focusing on the eukaryotic-specific bZIP TF family. We also focused on the characterization of TFs in general in this fungus, by determining the sequence preference of over half of its predicted TFs, information that will undoubtedly be useful for the study of gene regulatory networks. The work presented in this study will be an important addition to the field of circadian biology and the study of transcriptional regulatory networks in fungi.

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CONCLUSIONS

The work described in this Thesis represents a significant advancement in the characterization of transcriptional regulatory networks not only in Neurospora, but in related species, among which there are plant and animal pathogens, together with fungi with biotechnological applications. We anticipate that the information herein reported on the sequence preference of Neurospora transcription factors will be an invaluable resource for functional genomics and will help in systems biology approaches aimed at studying gene regulation in fungi.

Output pathways are the most neglected aspect of circadian systems and we set out to study them in a model eukaryote, by evaluating the role of the eukaryotic-specific family of bZIP transcription factors in the Neurospora circadian system. We report not only that a significant number of them exhibit clock input, but that they can play a relevant role in output pathways. Additionally, through the power of Neurospora genetics, we unveiled a novel clock-associated process, cell fusion, and showed it to be part of output pathways that are important for the rhythmic expression of bZIP encoding genes. Further characterization of the role of this process -so relevant for fungal biology- in output pathways, will surely result in interesting discoveries.

The integration of the data reported in this Thesis, with the vast amount of high throughput data emerging from different laboratories, will undoubtedly help in the study of a variety of processes in Neurospora, a system in which significant discoveries have been made over its long standing history as a research model organism and that continues to provide a wellspring of knowledge for eukaryotic biology.