UNDERSTANDING THE CIRCADIAN OUTPUT GENE NETWORK USING THE CLOCK-CONTROLLED TRANSCRIPTION FACTOR ADV-1 IN NEUROSPORA CRASSA

A Thesis

by

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ABSTRACT

The circadian clock is an evolutionary conserved time-keeping mechanism that, through the regulation of rhythmic gene expression, coordinates the physiology of an organism with daily environmental cycles. Clock-controlled transcripts are expressed at all possible phases of the circadian cycle; however, we lack a basic understanding of what controls phase. Phase is defined as a reference point on a waveform cycle relative to an environmental cue. To begin to determine the molecular mechanisms controlling circadian output pathways, we identified the direct targets of the core clock component and transcription factor (TF) WHITE-COLLAR COMPLEX (WCC) in *Neurospora crassa* using ChIP-seq and found an overrepresentation of 24 TFs (called first tier TFS) in the roughly 200 direct targets. Among these TFs, ADV-1 was shown to be robustly rhythmic, and was the only TF that when deleted was defective in clock-controlled development. Identification of genome-wide binding sites for the 24 first tier TFs revealed that in addition to the WCC, 13 of the TFs bind to the *adv-1* promoter, and that ADV-1 feeds back to bind to the promoters of these same TFs. The first tier TFs also bind and potentially co-regulate each other, as well as some of the downstream targets of ADV-1. These data suggested that the first tier TFs that bind to the *adv-1* promoter form a network, rather acting independently in linear pathways from the clock to ADV-1. To test this hypothesis, my studies focused on how the TF network controls the period and phase of ADV-1 protein rhythms.

I discovered that deletion of only 4 out of 13 TFs that bind to the *adv-1* promoter in the network altered the period and/or phase of ADV-1 protein rhythms. These data support a network model for circadian phase regulation, and suggest that one role of the network is to compensate for changes in the levels of components of the network, including changes that might occur during stress.

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1. INTRODUCTION

1.1 The circadian clock

The circadian clock is an evolutionarily conserved mechanism that regulates rhythmic gene expression and coordinates behavioral and physiological activities to the 24-hour light-dark environmental cycle(Vitalini, de Paula, Park, & Bell-Pedersen, 2006). Found in organisms that range from plants, bacteria, and mammals, circadian clocks are important for anticipating and adapting to Earth's fluctuating environment(Kondo et al., 1994). Disruption of the circadian clock has been linked to metabolic disorders(Maury, Ramsey, & Bass, 2010; Turek et al., 2005), cancer(Schernhammer et al., 2001), neurodegenerative diseases(Aziz, Anguelova, Marinus, Lammers, & Roos, 2010; Breen et al., 2014; Cermakian, Lamont, Boudreau, & Boivin, 2011), sleep disorders(Dijk & Czeisler, 1994; Weitzman et al., 1981), and cardiovascular disease(Takeda & Maemura, 2011). Furthermore, misalignment of the circadian clock with the light-dark environmental cycle has been shown to have an adverse effect on shift workers. For example, shift workers are less tolerant to glucose, which increases the risks of developing type 2 diabetes(Weitzman et al., 1981). While these and other studies have shown that the clock plays a critical role in human health, how circadian clock conveys signals to regulate gene expression is not full understood in any organism.

1.2 Criteria of circadian rhythms

For a biological rhythm to be considered regulated by the circadian clock and not some other timing mechanism, it must meet three criteria:

i. Circadian rhythms have an endogenous free-running period (FRP) in constant conditions, such as complete darkness (DD), with a period of about 24-hours in the absence of an environmental cue.

ii. Circadian rhythms must be entrainable. This means the rhythm can be reset by an environmental cue, or zeitgeber. The importance of this is to allow the biological clock to entrain to different time zones.

iii. Circadian rhythms are temperature compensated, which means that the free running period is nearly constant over a range of physiological temperatures for the organism(Nakashima & Feldman, 1980). Because external temperatures, and even internal body temperature in homeotherms, varies on a daily basis, biological clocks must overcome the change and maintain rhythmicity or else they would not be able to keep accurate time.

1.3 Components of the circadian clock

The biological clock consists of three fundamental components that allow for a functioning clock: a) the input pathway that keeps the endogenous clock in synchrony with the environmental cycle, b) the core oscillator that keeps track of time, and c) the output pathways that convey the time of day from the oscillator

to regulate rhythmic gene expression and overt rhythmicity(Dunlap & Loros, 2004) (Figure 1). In eukaryotic organisms, the core oscillator is arranged as an interconnected network with two major components, the positive and negative elements, which are essential for driving rhythmic gene expression. The positive-acting elements drive the expression of negative elements in the clock, and the negative elements inhibit the activity of the positive elements. This interaction forms an autoregulatory loop whereby the negative elements inhibit their own expression(Baker, Loros, & Dunlap, 2012). Time-delays in the feedback loop, driven by sequential protein modification and turnover(Darlington et al., 1998; Lee, 2000; Merrow, Garceau, & Dunlap, 1997), are necessary to generate a ~24 hour oscillation(Aronson, Johnson, Loros, & Dunlap, 1994).

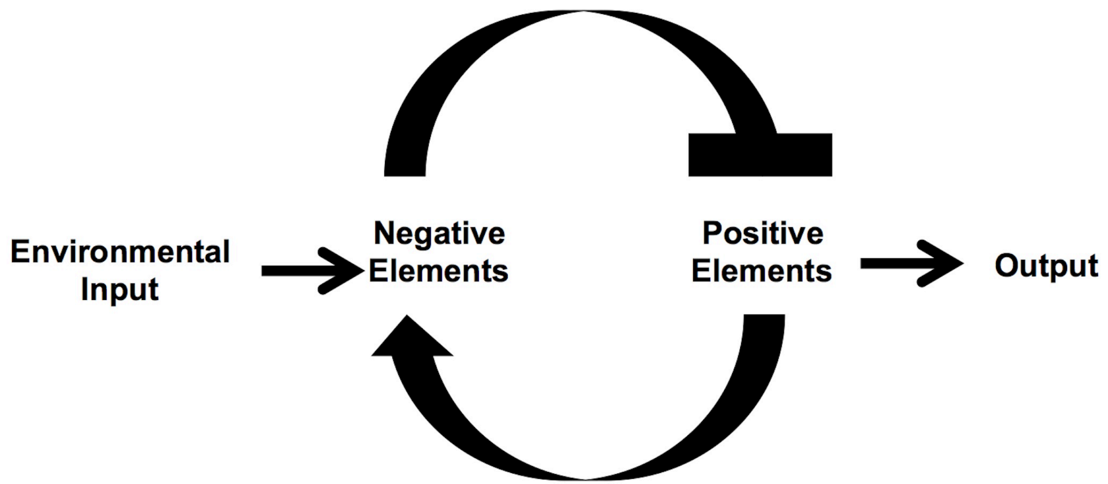

Figure 1. A simple model of the circadian clock system in eukaryotes.

1.4 The model organism *Neurospora crassa*

Neurospora crassa is a filamentous, haploid fungus in the phylum *Ascomycota*. *N. crassa* undergoes both asexual and sexual reproductive cycles, in which the asexual cycle produces conidia and the sexual cycle produces ascospores(Bobrowicz, Pawlak, Correa, Bell-Pedersen, & Ebbole, 2002). N. crassa has been established as a model eukaryotic organism for genetic(Beadle & Tatum, 1941) and circadian(Feldman & Hoyle, 1973; Pittendrigh, Bruce, Rosensweig, & Rubin, 1959) studies. In *N. crassa*, the circadian clock regulates rhythms in asexual spore development (conidiation) (Bell-Pedersen, 2000), hyphal branching(Dunlap & Loros, 2004), growth rate, and gene

expression(Hurley et al., 2014). Circadian rhythms in conidiation were first identified in *Neurospora*(Pittendrigh et al., 1959). Using specialized tubes, called racetubes, rhythms in conidiation can be easily observed in *N. crassa* strains containing the bd^{ras-1} mutation (Figure 2). Racetubes, which contain an agar growth media, are inoculated with fungus at one end of the tube, and incubated in constant light (LL) for 24 h. The tubes are then transferred into DD. This light to dark transition synchronizes all of the cells to dusk(Bell-Pedersen, 2000). The growth front is marked at the time of transfer from LL to DD, and marked every 24 hours following the transition to DD. Neurospora is only sensitive to blue light; therefore, a red safety light to see the growth front is used. Both ends of the racetubes are plugged with cotton to avoid contamination. This leads to CO_2 build up inside the tube, which inhibits conidiation. Strains carrying bdras-1 are used on racetubes because these cells are insensitive to the increased CO_2 levels, yet the mutation does not affect the running of the clock(Belden et al., 2007; Bell-Pedersen, 2000; Sargent, Briggs, & Woodward, 1966). The racetube assay provides a way to measure the period (time between two peaks of conidiation) and phase (time of peak relative to the light to dark transition) of the circadian rhythms of development, and serves as a measure of oscillator activity.

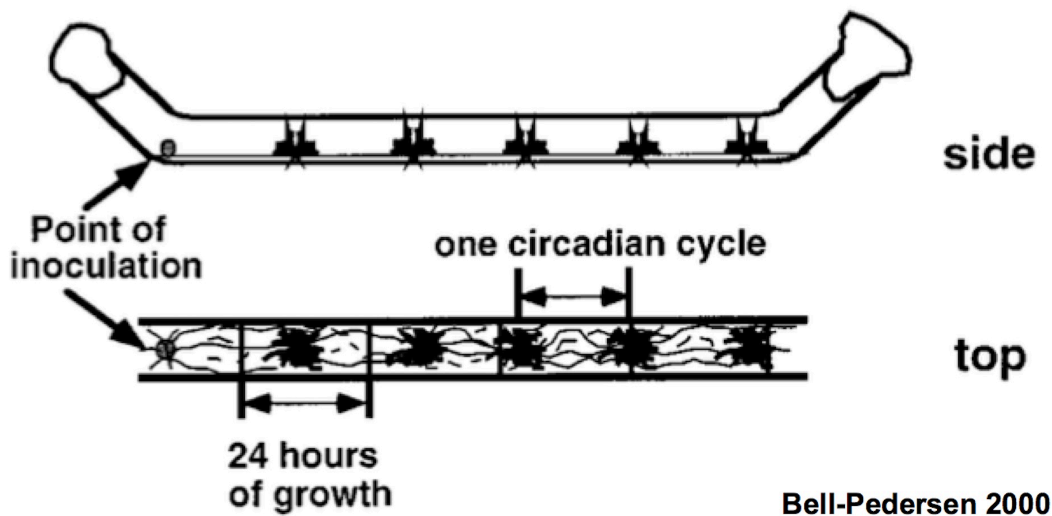

Figure 2. A diagram of a racetube (Bell-Pedersen, 2000). A racetube illustrates the alternation between mycelia and conidia. Racetubes are inoculated at one end, allowed to grow at 30°C in LL for one day, and are then placed in DD at 25°C. The growth front is marked at the time of transfer from LL to DD, and every subsequent 24 hours using a red safety light.

1.5 The *Neurospora crassa* circadian clock

The core oscillator in *N. crassa* is composed of the positive elements, WHITE-COLLAR-1 (WC-1) and WHITE COLLAR-2 (WC-2), and the negative elements, FREQUENCY (FRQ) and FRQ-interacting RNA helicase (FRH) (Figure 3).

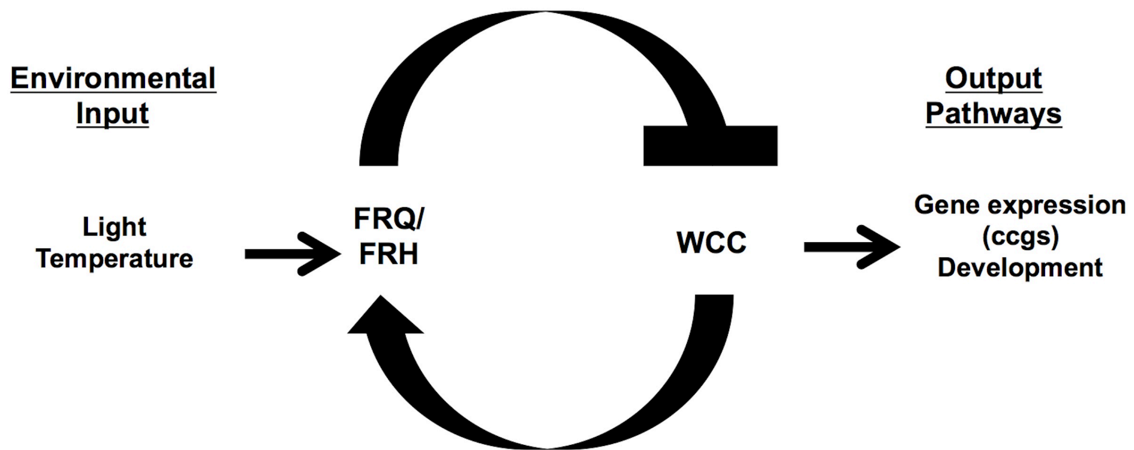

Figure 3. The *N. crassa* circadian clock system.

During the subjective morning, the blue-light photoreceptor WC-1 heterodimerizes to WC-2, forming the WHITE-COLLAR COMPLEX (WCC). The WCC binds to the promoter of *frq* and drives its expression(Froehlich, Liu, Loros, & Dunlap, 2002; He et al., 2002). During the daytime, *frq* mRNA levels increase and FRQ protein accumulates(Aronson et al., 1994; Garceau, Liu, Loros, & Dunlap, 1997). FRQ protein forms a homodimer(Cheng, Yang, Heintzen, & Liu, 2001), and interacts with FRH(Cheng, He, He, Wang, & Liu, 2005). The FRQ/FRH complex promotes the phosphorylation of the WCC leading to WCC inactivation and decreased *frq* transcription(Schafmeier et al., 2005). FRQ protein is progressively phosphorylated by several different kinases(He et al., 2006), and once fully phosphorylated, FRQ protein is degraded via the F-

box/WD-40 repeat-containing protein-1 (FWD-1) pathway(He et al., 2003). Once FRQ protein is fully degraded, the WCC is reactivated by phosphatases and together with new synthesis of the WCC, the molecular cycle restarts the next morning(He & Liu, 2005; He et al., 2005; Schafmeier et al., 2005; Schafmeier, Kaldi, Diernfellner, Mohr, & Brunner, 2006). In constant conditions, this cycle repeats every 22.5 hours(Montenegro-Montero & Larrondo, 2013). However, in 24 h environmental light dark cycles, and/or in temperature cycles, the circadian oscillator is entrained to precisely 24 h(Chang & Nakashima, 1997; Lakin-Thomas, 2006).

1.6 The circadian clock output pathways and regulation of phase

In addition to the clock regulation of development(Dunlap & Loros, 2004), several other processes are regulated by the circadian clock in *N. crassa*, including CO_2 production(Martens & Sargent, 1974), growth rate(Gooch, Freeman, & Lakin-Thomas, 2004), and lipid diacylglycerol metabolism(Lakin-Thomas, Gooch, & Ramsdale, 2001). The first clock-controlled genes (ccgs) were identified in *N. crassa* using subtractive hybridization. In this study, two morning-specific ccgs, *ccg-1* and *ccg-2*, were identified to cycle with a 22.5 h period in mRNA levels in wild-type cells. As expected for a gene regulated by the clock, *ccg-1* and *ccg-2* mRNA levels cycled with a long period in the 29 h period *frq*^{7} mutant strain(Bell-Pedersen, Dunlap, & Loros, 1992; Loros, Denome, & Dunlap, 1989; McNally & Free, 1988). Subsequent to these studies, additional

ccgs were identified using differential screens of time-of-day-specific cDNA libraries(Bell-Pedersen, Shinohara, Loros, & Dunlap, 1996) and microarrays(Dong et al., 2008). Most recently, genome-wide RNA-seq from cultures harvested at different times of the day revealed that up to 40% of the *N. crassa* genome was regulated by the clock(Hurley et al., 2014). Most of the ccgs peaked in expression in the early morning, or early evening; however, individual ccgs peaked at all possible phases of the day.

Circadian phase, a reference point on a waveform cycle relative to a specific reference point, such as an environmental signal, can be affected by period and amplitude of the oscillator(Bordyugov et al., 2015; C. Sancar, Sancar, Ha, Cesbron, & Brunner, 2015). For example, in mice, the suprachiasmatic nucleus (SCN) is the master pacemaker that synchronizes peripheral oscillators, such as oscillators in the liver, kidney, heart, and pancreas, to regulate daily rhythms. The SCN largely depends on photic cues that are projected to the SCN from the retinohypothalamic tract (RHT) by neurotransmitters, such as glutamate. The neurotransmitter *gamma*-Aminobutyric acid (GABA) also plays an important role in the SCN. Activating GABA inhibits phase shifts caused by light, while glutamate increases phase shifts in response to light. Thus, phase shifts in mice seem to be regulated partly by the interactions between GABA and glutamate neurotransmitters, which are inhibitory and excitatory neurotransmitters, respectively(Mintz, Jasnow, Gillespie, Huhman, & Albers, 2002). The phase of rhythmic transcript accumulation can also be regulated by

the time of food intake. Feeding mice during the rest period (daytime) causes a misalignment between the SCN and the peripheral clocks. Eating during the wrong time upregulates the peroxisome proliferator-activated alpha (PPARα) and glucagon receptors, while these receptors are not detected in the SCN. PPARα is involved in lipid metabolism, and glucagon receptors regulate blood glucose levels. Upregulation of PPAR and glucagon receptors does not affect the SCN, but is does the affect the peripheral clocks. The phase of transcripts in both the liver and pancreas is affected by food intake at the wrong time. These transcripts include *Bmal1*, *Rev-Erbα*, insulin receptors, and glucose transporters, SREBP1, and genes that are crucial for lipogenesis(Mukherji et al., 2015).

The clock component and transcription factor complex BMAL1/CLOCK bind to many targets in the mammalian genome to generate a temporal profile of gene expression. By looking at BMAL1 binding from ChIP-seq and pre-mRNA accumulation by real-time RT-PCR, three discoveries were made about ccgs: a) for ccgs expressed at day, such as *Rev-Erbα/β*, *Dbp*, *Dec2*, and *Tef*, the binding of BMAL1/CLOCK is followed by pre-mRNA expression, b) other ccgs such as *Per1*, *Per2*, and *Cry* are bound by BMAL1, but pre-mRNA levels are delayed by about 4 hrs, and c) *Cry1*, *Rorγ*, and *E4bp4* are also bound by BMAL1, but pre-mRNA levels are delayed by 12 h. These data indicated that there are other unknown regulators are controlling phase of rhythmic gene expression(Rey et al., 2011).

In *Arabidopsis thaliana*, circadian phase is controlled by temperature and light cues, but temperatures greater than 10°C are dominant over light cues(Michael et al., 2008). Phase in *A. thaliana* is regulated by two main molecular oscillator loops, the evening loop and morning loop(Harmer & Kay, 2005; Kaldi, Gonzalez, & Brunner, 2006; Michael et al., 2008). Both loops are connected by the clock component TIMING OF CAB EXPRESSION 1 (TOC1). The morning loops consists of two transcription factors, CIRCADIAN CLOCK ASSOCIATED 1 (CCA1) AND LATE ELONGATED HYPOCOTYL (LHY)(Schaffer et al., 1998; Wang & Tobin, 1998), and pseudoresponse regulators (PRR) 5, 7, and 9(Matsushika, Makino, Kojima, & Mizuno, 2000). PRR5, PRR7, and PRR9 genes are activated by CCA1 and LHY during the day(Farre & Kay, 2007), and CCA1 and LHY are in turn repressed by PRR5, PRR7, PRR9(Nakamichi et al., 2010). Both CCA1 and LHY genes are activated by TOC1(McClung & Gutierrez, 2010). The morning loop interlocks with the evening loop through TOC1(Greenham & McClung, 2015). The evening loop is composed of TOC1, GIGANTEA(Fowler et al., 1999), EARLY FLOWERING 3 and 4 (ELF)(Nusinow et al., 2011), and LUX ARRYTHMO (LUX)(Nusinow et al., 2011). TOC1 activates genes encoding ELF 3, ELF4, LUX, and GI, all of which have an evening element (EE) motif in their promoter, and are expressed during with an evening-specific phase. ZEITLUPE (ZTL), a photoreceptor, is stabilized by GI and controls the degradation of TOC1(Kim et al., 2007). Together, the

complexity of the evening and morning loops regulate up to 50% of the transcriptome that cycles with morning or evening phases(Michael et al., 2008).

In *Drosophila*, the phase of the circadian locomotor motor behavior is regulated by miR-124 during constant DD. Knocking out miR-124 (miR-124^{KO}) causes a phase advance of 5 h in locomotor behavior in constant DD; however, the phase advance caused by miR-124^{KO} in DD is partially corrected in light/dark cycles, indicating that light is a critical for miR-124^{KO}. miR-124^{KO} did not affect the amplitude or phase of PERIOD (PER), a core oscillator clock component, in any of the three circadian neurons, small ventral neurons, dorsal later neurons, and dorsal neurons. Because miR-124^{KO} does not affect the phase of the clock neurons, these data suggests that miR-124 works downstream of the circadian pacemaker(Zhang, Lamba, Guo, & Emery, 2016). The mechanism of action by miR-124 has yet to be identified.

In *N. crassa*, a fully functional clock is important for downstream processes. For example, proper phasing of the output pathways requires a fully functional WC-1(Kaldi et al., 2006). *wc-1* has three promoters, P_{dist}, P_{prox}, and P_{int}. P_{dist} is located upstream of the transcription initiation site, and P_{prox} is found downstream of the P_{dist} site. P_{int} is located within the *wc-1* ORF and has an unknown function. To test how the P_{dist} and P_{prox} start sites affect *wc-1* expression, both promoter start sites and each individual promoter region was placed in front of firefly luciferase at an exogenous location. In addition, the truncated promoters, attached to the native *wc-1* ORF, were placed in an

exogenous locus in a *wc-1* deletion strain. P_{prox} seems to provide basal levels of WC-1, while P_{dist} supplies low levels of WC-1. Expressing WC-1 with either P_{prox} or P_{dist} yields rhythmic WC-1 protein levels; however, compared to wild-type cells, the phase of conidiation rhythm is delayed or advanced in P_{prox} or P_{dist}, respectively. These data suggests that proper expression of *wc-1* is important for the proper phase of rhythmic conidiation(Kaldi et al., 2006).

The core-clock component WCC complex is not the only component needed for proper regulation of phase in downstream processes. The WCC controls downstream processes through the regulation several TFs(Smith et al., 2010). One of these TFs is CSP-1, which is encoded by the gene *conidial separation-1*. CSP-1 is a clock-controlled TF that peaks in the morning and acts mainly as a repressor. As predicted based on repressor activity of CSP-1, genes directly controlled by CSP-1 peak in the evening, antiphase to the phase of CSP-1 peak levels(G. Sancar et al., 2011). This data suggested the hypothesis that ccgs are being regulated via a hierarchal pathway. In this hierarchal pathway first tier TFs are directly activated by the WCC. Those TFs that function as activators would control ccgs that peak in the morning, the time of day in which the WCC is active. First tier TFs that function as repressors would bind and repress the promoters of ccgs during the day but not at night, allowing this class of ccgs to be expressed during the night(G. Sancar et al., 2011). The ccgs that are regulated by the first tier TFs may encode second tier TFs that function similarly, but with a delay in their peak activity. These linear pathways would be

predicted to lead to a cascade of gene regulation to allow ccgs to peak at all possible times of the day. However, recent evidence suggests that linear hierarchical pathways may not be sufficient to describe circadian output. ChIP-seq of WC-2 revealed that WCC binds to the promoter of about 200 genes, including 24 genes encoding TFs that were highly enriched(Smith et al., 2010). ChIP-seq data of these first tier TFs indicated that they form interconnected feed forward and feedback loops, suggesting that the TFs form a network, rather than functioning in a flat hierarchy.

1.7 Understanding output transcription factor network surrounding the first tier TF ADV-1

To test the hypothesis that the first tier TFs form a network and that this network functions to regulate the phase of ccg expression, I have focused my attention on one of the first tier TFs, ADV-1. ADV-1, which stands for Arrested Development Protein-1. The WCC binds to the promoter of *adv-1* and drives early morning-specific expression of *adv-1* mRNA and protein(Smith et al., 2010). Furthermore, rhythmic conidiation was abolished in strains containing a knockout of *adv-1* (*Δadv-1*), indicating that the TF ADV-1 links the circadian clock to asexual reproduction(Smith et al., 2010). Interestingly, deletion of the other first tier TFs did not alter rhythmic conidiation. *Δadv-1* cells are unable to generate protoperithecia, which is required for sexual reproduction(Dekhang et al., 2017). Cell to cell fusion is the process by which germinating conidia fuse

together, and it is important for protoperithecia formation. Consistent with the sexual development phenotype, ADV-1 regulates several genes involved in cell fusion genes, including *prm-1*, *ham-9*, *lfd-1*, and *ham-6*(Dekhang et al., 2017).

Gene Ontology (GO) analysis of genes that are bound by ADV-1 revealed that ADV-1 targets are highly enriched for metabolic processes, including sugar, nitrogen, and sulfur metabolism(Dekhang et al., 2017). In addition, analysis of genes regulated by ADV-1, identified through RNA-seq from wild-type versus *Δadv-1* cells, supported that ADV-1 regulates key metabolic processes, including carbohydrate metabolism, amino acid metabolism, polysaccharide metabolism, and lipid, fatty acid, and isoprenoid metabolism(Dekhang et al., 2017). Preliminary data shows that ADV-1 also regulates *fructose-1, 6-bisphosphatase 1* (*fbp-1*). *fbp-1* encodes for an enzyme that is involved in gluconeogenesis, and a defect is associated with metabolic issues(Noguchi et al., 2007). In Δ*adv-1* cells, *fbp-1* mRNA rhythmicity and levels are dampened when compared to wild-type cells (Rigzin Dekhang, unpublished data). Together, the role of ADV-1 in controlling metabolism, its direct regulation by the clock, and its involvement in circadian output pathways, led me to focus my efforts on using ADV-1 to test the hypothesis that the first tier TFs form a network and that this network functions to regulate the phase of ccg expression. In mammals, the clock regulates rhythms in enzymes that breakdown and metabolize food products(Mukherji et al., 2015; Salgado-Delgado et al., 2013; Stokkan, Yamazaki, Tei, Sakaki, & Menaker, 2001). These enzymes peak in

levels and activity during the day under control of the circadian clock, the time of day when we would normally be active and eating. However, shift workers eat at night when these enzymes are less active, leading to storage of food energy as fat and predisposing shift workers to metabolic disorders. The control of rhythmic metabolism by ADV-1 may provide a good model for understanding clock control of the phase metabolism, which may lead to new ideas for therapies for circadian clock-associated metabolic disease.

1.8 The TF network surrounding ADV-1

The WCC binds to the promoters of genes encoding 24 TFs that in turn bind to downstream genes(Smith et al., 2010), but how these TFs generate a temporal array of rhythmic gene expression is not known. ChIP-seq analysis of the first tier TFS revealed that several of these TFs (13), including the WCC, bind to the *adv-1* promoter and that 11 TFs bind to the promoters of other first tier TFs that, in turn, bind directly to the *adv-1* promoter. Based on ChIP-seq data, these 13 TFs also potentially regulate each other, and 6 TFs potentially feedback to the clock. To understand this network, my study focuses on using the ChIP-seq and RNA-seq data that I helped to generate to determine how individual TFs in the network alter ADV-1 protein rhythms, and how they might affect the clock. The ADV-1 data are being used to develop and train a computational model of the first tier TF network, which will allow predictions for how perturbations of any part of the network will alter the phase of downstream ccgs. Model training also

requires a comprehensive view of what genes are regulated by ADV-1. Thus, I have worked to generate rhythmic RNA-seq libraries from wild-type and *Δadv-1* cells. These data will provide the platform to identify ADV-1 target genes and determine if ADV-1 influences their peak phase of RNA abundance. The long-term goal is to identify ccgs that encode key metabolic genes that are controlled by the network, and predict based on the model, what changes would be needed to alter the phase of the desired ccg.

2. MATERIALS AND METHODS

2.1 Strains and culture maintenance

All *Neurospora crassa* strains including *wild-type 74-OR23* (FGSC 2489), *Δadv-1* (FGSC 11042)*, Δwc-1* (FGSC 11712), *Δwc-2* (FGSC 11124), *Δsah-1* (FGSC 11132), *Δcsp-2* (FGSC 13563), *Δfkh-1* (FGSC 11437), *Δznf-21* (FGSC 11357), *Δvos-1* (FGSC 13536), *Δclr-1 (*FGSC 11029), *Δcsp-1* (FGSC 11348), *Δsub-1* (FGSC 11127), *Δtah3* (FGSC 11076), and *Δncu09615* (FGSC 19000), were obtained from the Fungal Genetics Stock Center (FGSC, Kansas State University, Manhattan, KS; http://www.fgsc.net). The TF knockout strains were generated by replacing the endogenous gene with a hygromycin resistant cassette in *Δmus-51::bar* and *Δmus-52::bar* in *N. crassa* as previously described*(Colot et al., 2006)*. Wild-type strains were grown in Vogel's minimal medium (1x Vogel's and 2% glucose). Strains resistant to hygromycin were grown in Vogel's minimal medium that contained 200ug/mL of hygromycin B, *Streptomyces* sp. (Calbiochem, Darmstadt, Germany). Strains with resistance to cyclosporin A were grown in Vogel's minimal medium that contained 5ug/mL of cyclosporin A (Sigma-Aldrich, St. Louis, MO).

To determine which transcripts are rhythmic in *Δadv-1* cells by RNA-seq, a 48 h timecourse with a 2 h resolution with wild-type (DBP 984) and *Δadv-1* (DBP 917) were grown in DD and harvested every 2 hrs over a 48 hr period. Conidia from wild-type and *Δadv-1* were inoculated into 20mL of 1x Vogel's, 2% glucose, and 0.5% arginine in a petri dish and incubated at 25°C in LL until a

mycelial mat was formed. 125mL Erlenmeyer flasks containing 75mL of 1x Vogel's, 2% glucose, and 0.5% arginine were inoculated with disk-shaped mycelial mats cut with a cork borer (size 1). Each flask received 1 plug. The flasks were transferred to LL at 25°C for 24 h with shaking (100 rpm). The flasks were then transferred one at a time every 2 h on to DD 25°C. After all the flasks were transferred into DD, the mycelia was harvested, frozen in liquid nitrogen and stored at -80°C.

ChIP-seq data for the 24 clock-controlled TFs was used to generate the computational model for the ADV-1 network. I took part in a collaborative effort in which the TFs were tagged with a V5 epitope and recombined into the endogenous locus and used for ChIP-seq with V5 antibody following light treatment(Dekhang et al., 2017) [Azzizi et al, in preparation]. I helped prepare the individual cultures with tagged TFs and isolated ChIP DNA for sequencing. The tagged TFs were induced to the highest abundance using light as clock-controlled TFs are generally induced by light(Smith et al., 2010). A loop full of conidia for NCU08159, V5 (DBP1603), NCU01871, V5 (DBP 1556), NCU03273, V5 (DBP 1644), NCU09068, V5 (DBP 1626), NCU03184, V5 (DBP 1642), and NCU09829, V5 (DBP 1643) was inoculated in a sterile petri dish containing 20mL of 1x Vogel's and 2% glucose. The petri dishes were placed at 30°C in LL until a mycelial mat grew. Once the mycelial mat grew, disk-shaped plugs were cut using a cork borer (size 1). One plug was then inoculated in 65 ml of media that consisted of 1x Vogel's and 2% glucose. The flasks were placed at 30°C LL

for 24 hrs with shaking (100 rpm) to allow the cultures to grow. After 24 hrs, the cultures were transferred into the dark at 25°C for 24 hrs with shaking (100 rpm). The cultures were then exposed to light for 0 (DD) 15, 30, and 60 min of light. Protein was extracted and the levels of protein by western blots. The cultures were prepared for ChIP DNA isolation and sequenced as previously described(Dekhang et al., 2017).

2.2 Generation of translational and transcriptional luciferase constructs

To determine the effect of loss of first-tier TFs on ADV-1 rhythmicity, ADV-1 protein levels were examined in strains deleted for individual TFs. To accomplish this, a ADV-1::LUC (DBP 1356) reporter stain was generated. The *adv-1* ORF to fused with a codon optimized luciferase(Gooch et al., 2008) and a 896 bp of the *adv-1* 3'flank using recombinational cloning in yeast, similar to the procedure used to generate TF knockouts in *N. crassa* (Colot et al., 2006; Gooch et al., 2008). To determine the effects of deletion of the first tier TFs on the clock, a FRQ::LUC (DBP 1563) reporter was used(Larrondo, Loros, & Dunlap, 2012). This construct contains 1 kb of the *frq* ORF, 1.734 kb of the codon optimized luciferase, 200 base pairs of the *frq* 3' flank, 1.12 kb of the phosphinothricin resistance gene *bar(Larrondo, Colot, Baker, Loros, & Dunlap, 2009)*, and 1 kb of *frq* 3' sequences(Larrondo et al., 2012). To examine FRQ protein levels in TF knockout strains, a strain (DBP 1563) containing the FRQ::LUCIFERASE translational fusion construct was crossed with individual TF knockouts using

standard procedures(Davis & de Serres, 1970). To determine the effect of the deletion of ADV-1 on rhythms of MITOGEN-ACTIVATED PROTEIN KINASE-1 (MAK-1), a MAK-1::LUC construct previously generated was used(Bennett, Beremand, Thomas, & Bell-Pedersen, 2013). The MAK-1::LUC construct, generated by 3-way PCR, consists of the *mak-1* orf, an optimized codon luciferase(Gooch et al., 2008) and a 970 bp of the *mak-1* 3'flank. The full construct was then transformed into wild-type cells in *N. crassa* using standard procedures(Lamb, Vickery, & Bell-Pedersen, 2013) and targeted to the native locus by homologous recombination(Bennett et al., 2013).

To determine if the TF CSP-1 is affecting the rhythmicity of ADV-1::LUC protein directly of indirectly, the CSP-1 binding site was deleted using 2-way PCR. A 3.5kb fragment containing a 36bp deletion of the CSP-1 bind site was generated using Phusion taq (Table 1), and the product was then purified by gel extraction using the QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany). The purified was then transformed into *N. crassa* cells in a strain containing the ADV-1::LUC translational construct. For the transformation cells containing the ADV-1::LUC translational construct were grown in 500 ml Erlenmeyer flasks with 1x Vogel's, 2% glucose, and 1.5% agar for 8 days in constant LL at 30°C. Conidia were washed in ice-cold 1 M sorbitol (Sigma-Aldrich, St. Louis, MO) 5x times. About 50ul of clean conidia, 10ul (1mg) of purified DNA, and 1ul (200ng) of a plasmid containing a *bar* cassette for selection were added into a microcentrifuge tube. The conidia and DNA were gently mixed with a pipette,

and the mixture was then transferred into a electroporation cuvettes (VWR, Radnor, PA). Cuvettes were electroporated (600 ohms, 25 microFarads, 1.5 kVolts) one at a time as previously described(Lamb et al., 2013). The mixture in the cuvettes was then transferred into 9 ml of 1x Vogel's recovery solution and gently shaken for 2 h. After the recovery stage, the conidia with the recovery solution were mixed with pre-warmed 2x top agar, which consisted of 2% agar, 2M sorbitol, 1x Vogel's, and 250 ug/ml glufosinate ammonium (G59690; Toronto Research Chemicals). The mixture was poured onto a sterile 150 x 15 mm petri dish containing 80 ml of 1.5% agar, 1x Vogel's, and 1x glufosinate (BASTA). The petri dishes were placed at 30°C in constant LL until colonies grew and were ready to be picked. Colonies were picked and grown in media containing 1x Vogel's, 2% glucose, and 1.5% agar.

2.3 Sexual crosses

To examine how the upstream network affects of ADV-1 and FRQ protein, a ADV-1::LUC (DBP 1356) and FRQ::LUC (DBP 1563) translational fusion construct were crossed to the 13 single TF knockout strains using standard techniques(Davis & de Serres, 1970). Briefly, conidia was inoculated in a 1.5 ml microcentrifuge tube (USA Scientific, Orlando, FL) that contained 300 ml of sterile water. 5 ul of the conidial suspension of the strains to be crossed were inoculated at opposite ends on a small piece of Whatman paper (GE Healthcare Life Sciences, Pittsburgh, PA) that was laid on top of the synthetic crossing

media. Synthetic crossing media consists of 1x Westergaard's salts(Westergaard & Mitchell, 1947), 0.1% sucrose, 0.1 mg/ml biotin and 0.15% agar. The plates were wrapped with parafilm and places in LL at 25°C until the release of ascospores from mature perithecia towards a light (http://www.fgsc.net). Ascospores that shot onto the lid were collected by adding sterile water and placing them into an eppendorf tube. A strain containing the MAK-1::LUC translational construct (DBP 1328) was crossed to a Δ*adv-1* strain (DBP 1572) to determine how deleting ADV-1 affects MAK-1::LUC protein rhythms.

2.4 Screening progeny from crosses (luciferase assays and PCR)

Spores from crosses were placed in 300 ml of sterile water in a 1.5 ml microcentrifuge tube (USA Scientific, Orlando, FL). Four drops of 10 ul of the spores suspended in water were spread out with a loop in a sterile petri dish containing 4% agar. An individual spore is picked up with a scalpel using a stereo microscope (Leica Microsystems, Buffalo Grove, IL) and inoculated onto minimal media (1x Vogel's, 2% glucose, and 1.5% agar). The spores were then heat activated in a 65°C water bath for 1 h (Goodrich-Tanrikulu, Howe, Stafford, & Nelson, 1998) and germinated at 30°C in LL. A loop full of conidia was inoculated in 1 ml of 1x Vogel's and 2% glucose and grown for 24 hrs in LL. Plugs from the mycelial mats were cut using a cork borer (size 3), and one plug was inoculated into one well of a 96-well plate containing 150 ul of 1x Vogel's

and 25 uM of luciferase (LUNCA-300; Gold Biotechnology, St Lousi, MO). The plugs were allowed to grow for 5 hrs at 30°C in LL. Luciferase activity was measured using a TopCount NXT Microplate Scintilation and Luminescence Counter (PerkinElmer Life Sciences, Boston, MA). Strains with luciferase activity were kept for further screening by diagnostic PCR. For PCR, DNA was extracted from the mycelial mats using DNA extraction buffer (100 mM Tris-HCl, 50 mM EDTA, and 1% SDS), following the Gentra Puregene (Qiagen, Hilden, Germany) protocol with some modifications. Mycelial mats were placed in a microcentrifuge tube with 600 ul of DNA extraction buffer, 3 ul of Proteinase K (Sigma-Aldrich, St. Louis, MO), and incubated for 1 hr at 65°C. 3 ul of RNase A (Sigma-Aldrich, St. Louis, MO) was added to the samples and incubated at 37°C for 1 hr. After incubation, 200 ul of 7.5 M ammonium acetate (Sigma-Aldrich, St. Louis, MO) and 500 ul of chloroform (Avantor, Center Valley, PA) was added to the samples. The samples were then vortexed for 1 min and centrifuged at 14000 rpm for 3 min. The supernatant, approximately 650 ul, was transferred to a new microcentrifuge containing 600 ul of isopropanol (Sigma-Aldrich, St. Louis, MO). After mixing the samples by inversion, the samples were left at room temperature for 20 min. The samples were then centrifuged for 3 min at 14000 rpm. Pellets were washed with 70% ethanol and then suspended in sterile water. PCR was carried out to amplify the hygromycin cassette outside the region of homology of the knock out strains (Table 1). Strains that had luciferase activity

and were validated to be knockouts by PCR were stored and examined in rhythmic assays.

2.5 Rhythmic luciferase analysis

To rhythms between for ADV-1::LUC, FRQ::LUC, and MAK-1::LUC rhythmic assays were done as previously described but with some modifications(Dekhang et al., 2017). A loop full of conidia was inoculated in sterile water and measured with a spectrophotometer with an O.D.$_{420}$ 1=5x10^6conidia/mL. The conidia per mL was adjusted to 1x10^5conidia/mL, and 5uL were loaded into a 96-well microtitre plate. The in 96-well microtitre plate contained 150uL which consisted of 1x Vogel's, 0.01% glucose, 0.03% arginine, 0.1M quinic acid, 1.5% agar, and 25uM firefly luciferin (LUNCA-300; Gold Biotechnology, St Louis, MO); the pH was adjusted to pH 6. The 96-well microtitre plate was placed at 30°C in constant light for 24-hours to synchronize the cells. After 24-hours, the plate was transferred to constant dark at 25°C where rhythmic assays were done using the multi-mode detection EnVision Instrument (PerkinElmer Life Sciences, Boston, MA), which takes recordings every 90 minutes for 7 days. The raw data was extracted and analyzed using a circadian software called BioDare2 (http:///biodare2.edu.ac.uk)(Zielinski, Moore, Troup, Halliday, & Millar, 2014). The first 12-hours are not included in the analyses to exclude artifacts from transferring the plate to the EnVision. BioDare2 analyzed the rhythms to provide period length and phase for the rhythmic data. The rhythmic data was

normalized to the mean and plotted using PRISM (La Jolla, CA). A Student t-test analysis was done in PRISM to test if the period length and phase of the rhythmic data was significant when comparing translational constructs in wild-type cells versus cells with a deletion of a TF.

2.6 Protein extraction and western blots

To compare FRQ protein from the 48 hr timecourses in *wild-type* and *Δadv-1* cells, protein was extracted from the timecourse samples. Total protein extraction to detect FRQ protein in the 48-hour timecourse with 2-hour resolution was done as it has been described(Garceau et al., 1997) with some modifications. The extraction buffer consisted of 5mM HEPES pH7.5, 137mM KCL, 1mM EDTA, 10% glycerol, 1mM PMSF, 1x aprotinin (Sigma-Aldrich, St. Louis, MO), 1x leupeptin hemisulfate salt (Sigma-Aldrich, St. Louis, MO), and 1x pepstatin A (Sigma-Aldrich, St. Louis, MO). Protein extracts (100ug) were separated on a 10% SDS-PAGE and transferred to an Immobilon-P PVDF transfer membrane with 0.45um pore size (Sigma-Aldrich, St. Louis, MO). Membranes were blocked overnight at 4°C in 7.5% non-fat milk with 1x TBS and 0.1% Tween-20 (1x TBST). Mouse monoclonal anti-FRQ primary antibody (Supernatant from clone 3G11-1B10-E2, from M. Brunner’s laboratory, Heidelberg) at a concentration of 1:200 was used to detect FRQ. Transfer membranes were incubated with the primary anti-body in fresh 7.5% non-fat milk with 1x TBST at 4°C overnight. After the incubation, membranes were washed

6x for 10 minutes with 1x TBST. Membranes were then incubated overnight at 4°C with goat anti-mouse IgG HRP secondary antibody (Bio-Rad, Hercules, CA) at a concentration of 1:10,000 in 7.5% non-fat milk with 1x TBST. Membranes were washed 6x for 10 minutes after the incubation, detection was done by chemiluminescence using the SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Scientific, Waltham, MA).

To determine differential gene expression in wild-type versus *Δadv-1* cells, total RNA was also extracted for the 48 hr timecourses with 2-hour resolution with *wild-type* and *Δadv-1* was done as previously described(BellPedersen, Dunlap, & Loros, 1996). The 48 hr timecourses will be used to identify rhythmic and arrhythmic transcripts in *Δadv-1* cells by comparing to *wild-type* cells.

2.7 RNA-seq preparation and analysis

To perform RNA followed by sequencing (RNA-seq) on the light induction samples, Poly(A) mRNA preparation, cDNA synthesis and sequencing library preparation was done as previously published(Wu et al., 2014). For the 48 hr timecourses with 2 hr resolution, the RNA was prepared as described, with some modifications(Wu et al., 2014). Conventional RNA-seq uses oligo-dT to prime to the cDNA followed by the addition of the adaptors for sequencing; therefore, this leads to the loss of information about the original strand that was transcribed(Levin et al., 2010). Knowledge of which strand was first transcribed

is useful to identify antisense transcripts, other noncoding RNAs, and set boundaries for gene that are too close or overlapping(Levin et al., 2010). Instead of conventional RNA-seq, strand specific RNA-seq was done for the 48-hour timecourses using the SENSE Total RNA-seq Library Prep Kit (Lexogen, Inc., Greenland, NH). Strand-specific RNA-seq used in study to prepare the cDNA requires synthesizing the second strand in the presence of dUTP instead of Oligo-dT, which will allow of the differentiation between the first and second strand(Levin et al., 2010). The second strand containing dUTP is subsequently degraded in the sequencing library, which will be useful to identify the orientation of the transcripts. Sequencing of cDNA is done on Illumina HiSequation 2000, and the reads were processed using Illumina pipeline RTA 1.13.48 as previously shown(Wu et al., 2014).

Table 1. Primers used to validate TF knockouts.

Validation of TF knockouts	Forward/Reverse primer	Primer sequence
ΔVOS-1	Forward	5' TACCAGGTACACGCCTCGCA 3'
	Reverse	5' AGTGTGTTGCCGGTGGACAG 3'
ΔSUB-1	Forward	5' CAGGACGGGAGGTAATCCCAGA 3'
	Reverse	5' CCATTATTAAACCAGCCTAGCCGCG 3'
ΔNCU09615	Forward	5' GCGAACTGGATGGTGCCTGA 3'
	Reverse	5' GTACGTGCGGAACATTGAACT 3'
ΔTAH3	Forward	5' AAGAGCGCAGGAGATCCTTAC 3'
	Reverse	5' CTTTTCGCTTCATTCACTTCG 3'
ΔCSP-1	Forward	5' ATATCCCATCCACATCAGACGG 3'
	Reverse	5' CACCCTTCGATATACACGGCAT 3'
ΔNCU03184	Forward	5' TCAAAGCTTGGGTCTGAAAGA 3'
	Reverse	5' CCATGTGAAACGGCGTACATC 3'
ΔCLR-1	Forward	5' ACTGCACGCACTGCTTACCTAA 3'
	Reverse	5' CATAGTCAGGGCCATGGTTTA 3'
ΔCSP-2	Forward	5' CGACCCAAATCGACCATCCAATT 3'
	Reverse	5' AGGAACTGTGCCCGTAGGTAT 3'
ΔFKH-1	Forward	5' CCAAACATTGGTCTCGGTAGA 3'
	Reverse	5' GAAACACAAAGACCCTGGACA 3'
ΔSAH-1	Forward	5' AACCGAAGCCTAAAGTTCGTC 3'
	Reverse	5' TGATCAGCGGAGTTGTAAAGG 3'
ΔWC-1	Forward	5' CGTTCGATAGACGCAACGTCAC 3'
	Reverse	5' GTTGCGTGCCTTCCAGGGACC 3'
ΔWC-2	Forward	5' AAAGCAGATGAAGCCGATGA 3'
	Reverse	5' CGACAGCGTCTCCGACCTGATG 3'
Primers to delete CSP-1 binding site from the ADV-1::LUC translational construct		
Piece 1F	Forward	5' TTACGAAGAGGAACTGCAACG
Piece 1R	Reverse	5'GGAGAGTCGAGTTGGTACGTACTTCGGAAAAAGAG
Piece 2F	Forward	5'AAGTACGTACCAACTCGACTCTCCCCGAAACTA
Piece 2R	Reverse	5' TCATCAACGGATATCGTCCTC
Primers to sequence CSP-1binding site deletion from ADV-1::LUC translational construct		
	Forward	5' GATTTTCAAGAGGGCCGATA
	Reverse	5' GTCAAGTCGTTCGACAAGCA

3. RESULTS

3.1 The effect of the first-tier TF network on ADV-1 protein rhythmicity

ChIP-seq using WC-2 antibodies identified about 200 targets of the WCC across the genome following a short light pulse to activate WCC binding(Smith et al., 2010). 24 TFs were significantly enriched among these targets. In collaborative studies that I participated in, the TFs were tagged with a V5 epitope and recombined into the endogenous locus and used for ChIP-seq with V5 antibody following light treatment(Dekhang et al., 2017) [Azzizi et al, in preparation]. The time of exposure to light for ChIP-seq was determined empirically by examining the levels of protein following light treatment of cells for 0 (DD), 15, 30, 60, 120 min of light by western blot (Azzizi et al, in preparation). The TFs that I helped prepare for ChIP-seq were NCU08159, NCU01871, NCU03273, NCU09068, NCU03184, and NCU09829 and preparation was done as previously described(Dekhang et al., 2017). To determine the impact of the first tier TFs on gene expression, RNA-seq in TF deletion strains grown in the dark or exposed to light for the appropriate time to induce high levels of the TF in WT cells, was compared to WT RNA-seq. This work is still in progress, but we currently have RNA-seq data for 10 of the TFs. Analysis of the binding sites for the TFS revealed that 6 clock-regulated TFs, including the WCC, bind to the regulatory regions of *adv-1*. The potential TF network surrounding ADV-1 compromises 13 TFs that not only bind to the *adv-1* locus, but also potentially regulate each other. In addition, some of the TFs within the network appear to feedback to

regulate their own transcription. In order to determine how the ADV-1 network is affecting ADV-1 protein, I examined the rhythmicity of a ADV-1::LUC translational construct (Figure 4).

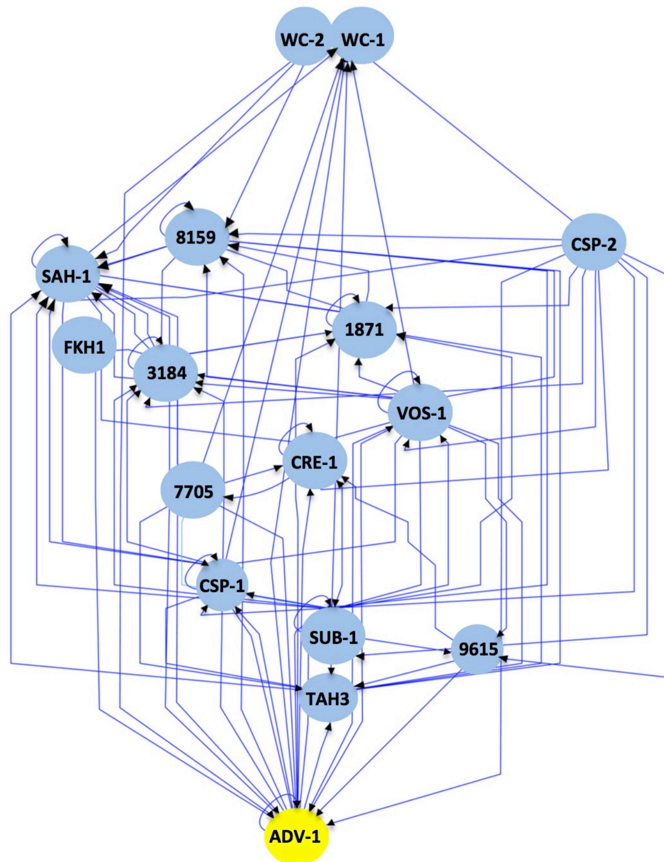

Figure 4. ADV-1 upstream network. The network shows key TFs and complexity of feed forward and feedback loops.

To determine if these TFs affect ADV-1 protein rhythmicity, and to begin to distinguish linear pathway regulation by specific TFs versus regulation through a

network, I examined the affect of single TF knockouts on ADV-1 protein rhythms using an ADV-1::LUC translational fusion construct (Figure 5).

Figure 5. ADV-1::LUC translational fusion construct.

csp-1 (*conidial separation-1*; *ncu02713*) encodes for the CSP-1, a transcription repressor(G. Sancar et al., 2011), and is a component in the ADV-1 network. CSP-1 binds to the promoter of *adv-1* (Figure 6B), and *adv-1* mRNA is expressed at a higher abundance in *Δcsp-1* cells in comparison to wild-type cells according to RNA-seq from a light-induced experiment (Figure 6A). Thus, CSP-1 seems to repress *adv-1* transcript levels in wild-type cells. Deleting *csp-1* causes a phenotype in which the conidia clump together and fail to separate(Springer & Yanofsky, 1989). Further, CSP-1 generally controls genes that play a role in metabolism, such as gene involved in regulating ergosterol synthesis and fatty acid desaturases(G. Sancar et al., 2011). To examine ADV-1 protein rhythmicity, *Δcsp-1* was crossed with a strain containing the ADV-1::LUC translational fusion construct. In *Δcsp-1* cells, ADV-1::LUC protein has a significant phase delay of 7.7±0.5 hrs in comparison to wild-type cells, which have a phase of 1.3±0.2 hrs. The period of the rhythm is not affected (Figure 6C and Table 2).

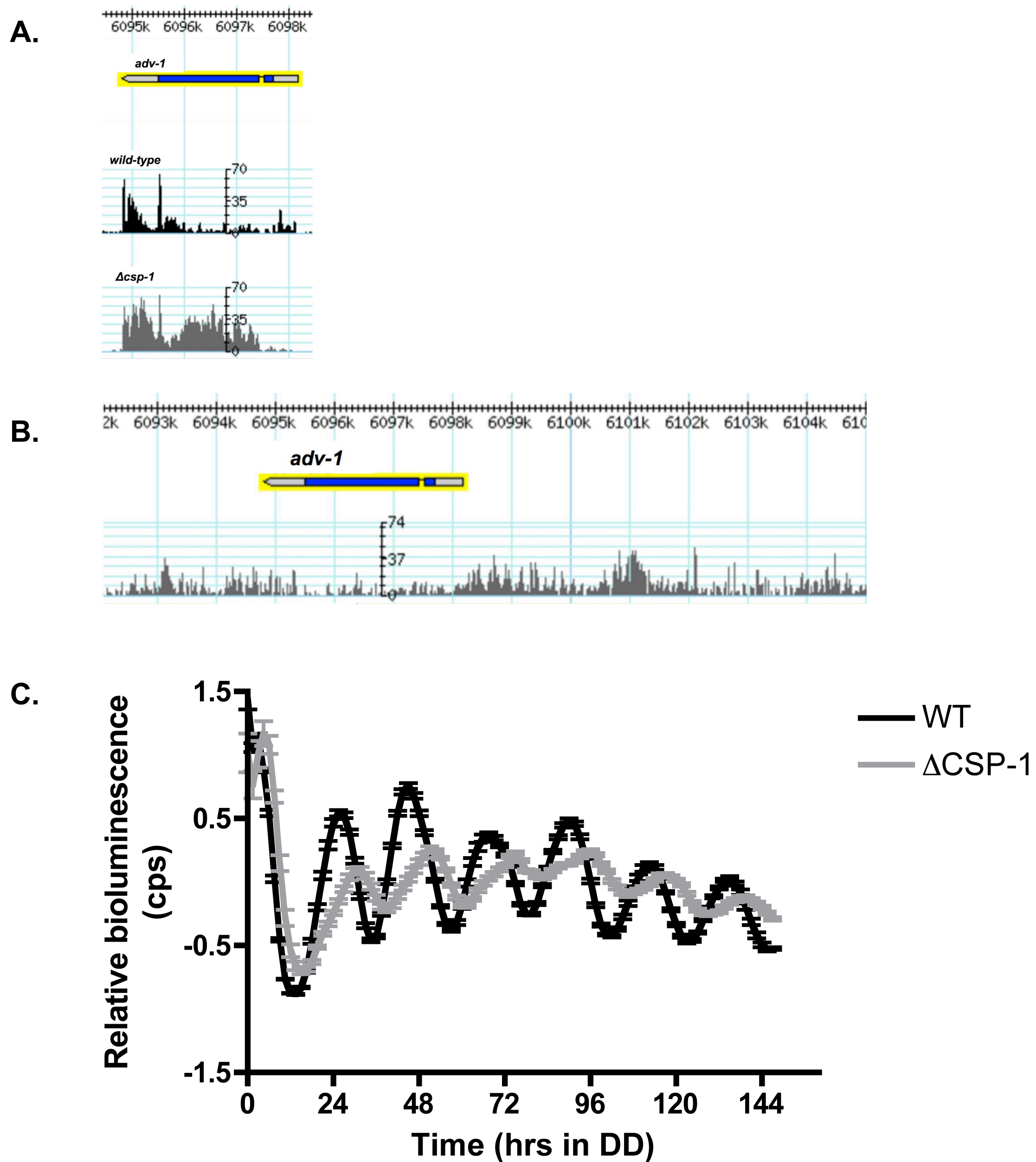

Figure 6. The effect of ΔCSP-1 on ADV-1::LUC. A.) RNA-seq data showing *adv-1* transcript levels in wild-type and *Δcsp-1* cells. B.) ChIP-seq showing the binding of CSP-1 at the *adv-1* promoter. C.) ADV-1::LUC protein rhythms have a significant phase delay of 7.7±0.5 hrs in *Δcsp-1* cells. (n=30 p<0.001).

To determine if CSP-1 affects the rhythmicity of ADV-1::LUC protein by binding directly to the *adv-1* promoter or indirectly via another TF, a CSP-1 binding site was deleted (Figure 7A) because it was enriched by ChIP-PCR (data by Jennifer Jung not shown), indicating that CSP-1 binds to the promoter of *adv-1*. However, deletion of the CSP-1 binding site did not affect the rhythmicity of ADV-1::LUC protein (Figure 7B). This outcome might be explained by the fact that there are a two other CSP-1 binding sites on regulatory regions of *adv-1*, one at the 5' region and another located at the 3' region (Figure 7A). Furthermore, it might also be possible that the other TFs in the network are compensating for the loss of one binding.

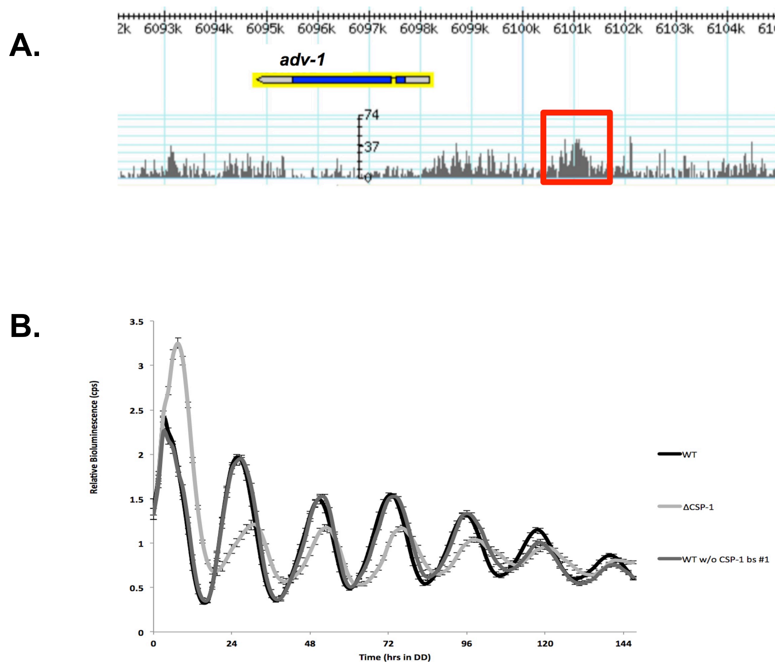

Figure 7. The effect of deleting a CSP-1 binding site on ADV-1::LUC. A.) ChIP-seq data showing the CSP-1 binding that was deleted. B) ADV-1::LUC protein rhythmicity was not affected when the CSP-1 binding site was deleted.

sub-1 (*submerged protoperithecia-1; ncu04179*) encodes the TF SUB-1 and is important for the proper formation of protoperithecia(Colot et al., 2006; Schafmeier & Diernfellner, 2011). SUB-1 also binds to *adv-1* at the promoter region (Figure 8B), and RNA-seq shows that *adv-1* mRNA is misregulated in *Δsub-1* when compared to wild-type cells (Figure 8A). In *Δsub-1* cells, *adv-1* transcript levels are higher than in wild-type cells, suggesting that SUB-1, like CSP-1, represses *adv-1* transcript levels. Published data suggests that SUB-1 functions downstream of the WCC to regulate early (15 min) and late (30 min) light-induced genes(Chen, Ringelberg, Gross, Dunlap, & Loros, 2009), but this cannot be done without the WCC(C. Sancar, Ha, et al., 2015). Upon a light signal, the WCC binds to the promoter of *sub-1*, and SUB-1 then activates early (15 min) light-induced genes. However, how SUB-1 activates late (30 min) light-induced genes is not known, but RNA-seq of *sub-1* versus wild-type cells show that without SUB-1, late light-induced genes are no longer expressed(Chen et al., 2009). In addition, data suggests that SUB-1 is interacting with a putative TF, FEMALE FERTILITY-7 (FF7), to regulate both light-induced and non-light-induced genes(C. Sancar, Ha, et al., 2015). In *Δsub-1* cells, the period of ADV-1::LUC ~1.5 h shorter period than in the wild-type cells, but unlike CSP-1, has no effect on phase (Figure 8C and Table 2).

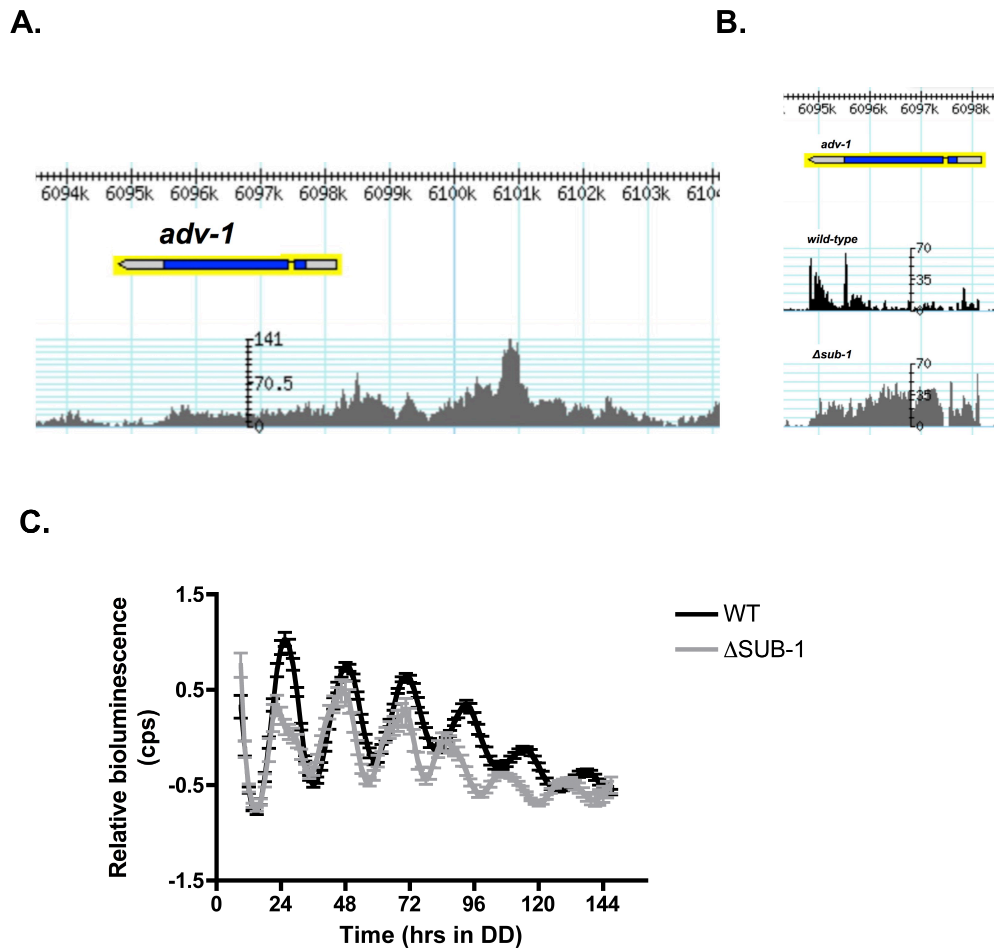

Figure 8. The effect of ΔSUB-1 on ADV-1::LUC. A.) RNA-seq data showing *adv-1* transcript levels in wild-type and *Δsub-1* cells. B.) ChIP-seq showing the binding of SUB-1 to the promoter of *adv-1*. C.) ADV-1::LUC protein has a significantly shorter period in *Δsub-1* cells (21.1±0.1 hr) than in wild-type cells (22.6±0.1 hr) . (n= 21 p<0.0001).

Deletion of *csp-2* (*conidial separation-2; ncu06095*), like csp-1 also exhibits a phenotype in which the conidia fail to separate(Springer & Yanofsky, 1989). CSP-2 is critical for the proper localization of BGT-2, a β-13-endoglucanase that is important for cell wall remodeling in *N. crassa* during the spore development(Martinez-Nunez & Riquelme, 2015). CSP-2 binds to the promoter

of adv-1 (Figure 9B), and deleting *csp-2* leads to a misregulation of *adv-1* transcript levels by RNA-seq obtained from a light induction experiment (Figure 9A). Knocking out CSP-2 in *N. crassa* leads to a significant phase delay of 5.1±0.2 hrs in ADV-1::LUC protein rhythms (Figure 9C and Table 2). Deleting *csp-2* does not affect the period of ADV-1::LUC.

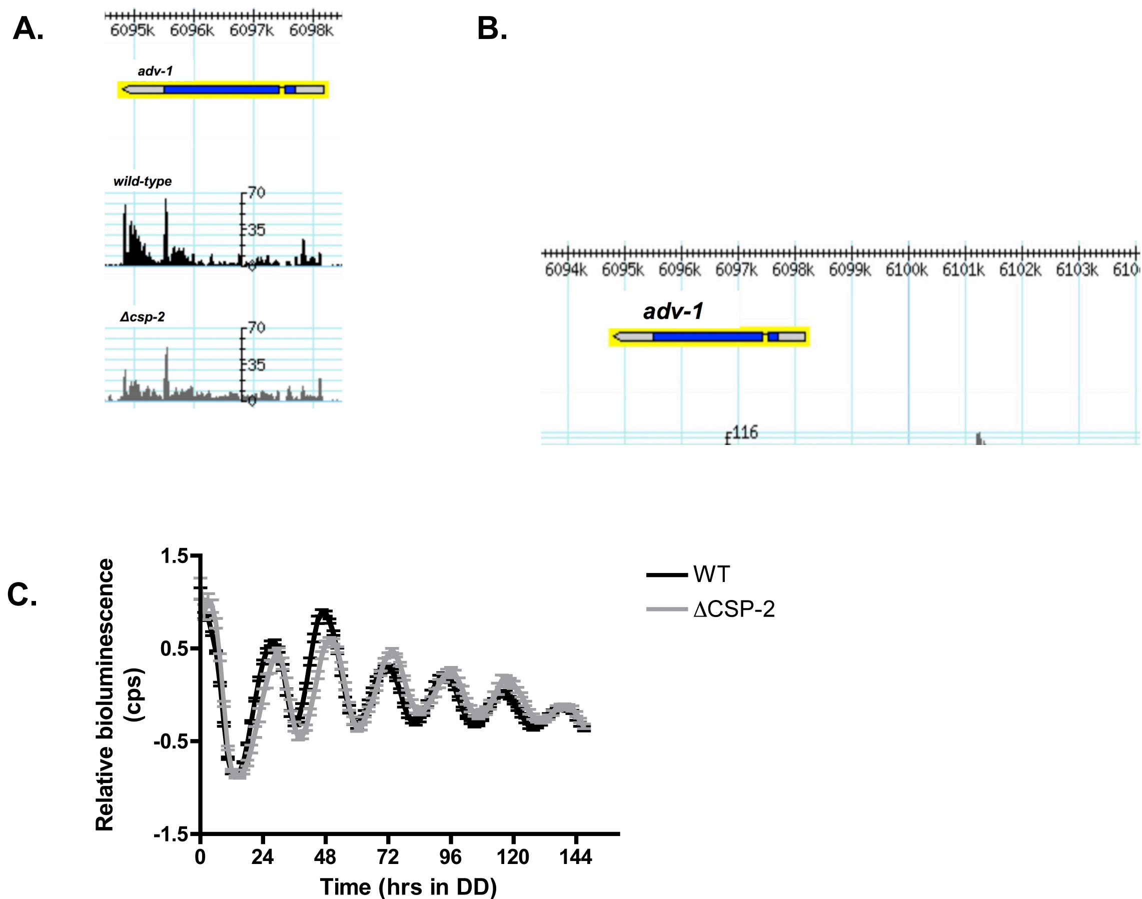

Figure 9. The effect of ΔCSP-2 on ADV-1::LUC. A.) RNA-seq data showing *adv-1* transcript levels in wild-type and *Δcsp-2* cells. B.) ChIP-seq showing the binding of CSP-2 to the *adv-1* promoter. C.) Knocking out the TF CSP-2 (NCU06095) causes a significant phase delay of 5.1±0.2 hrs in ADV-1::LUC rhythms. (n=36 p<0.001).

The role of *znf-21* (*zinc finger transcription factor-21; ncu03184*) in *N. crassa* is currently unknown but ZNF-21 has been studied in other fungi. In *Aspergillus nidulans*, ZNF-21 TF is involved in secondary metabolism and morphogenesis(Ramamoorthy et al., 2013). ZNF-21 binds to the *adv-1* promoter (Figure 10A), but we do not know how deleting *znf-21* affects adv-1 transcript levels due to the lack of RNA-seq. ZNF-21 Deleting *ncu03184* leads to a ~0.5 hr increase in ADV-1::LUC period compared to wild-type cells, but not effect on phase (Figure 10B and Table 2).

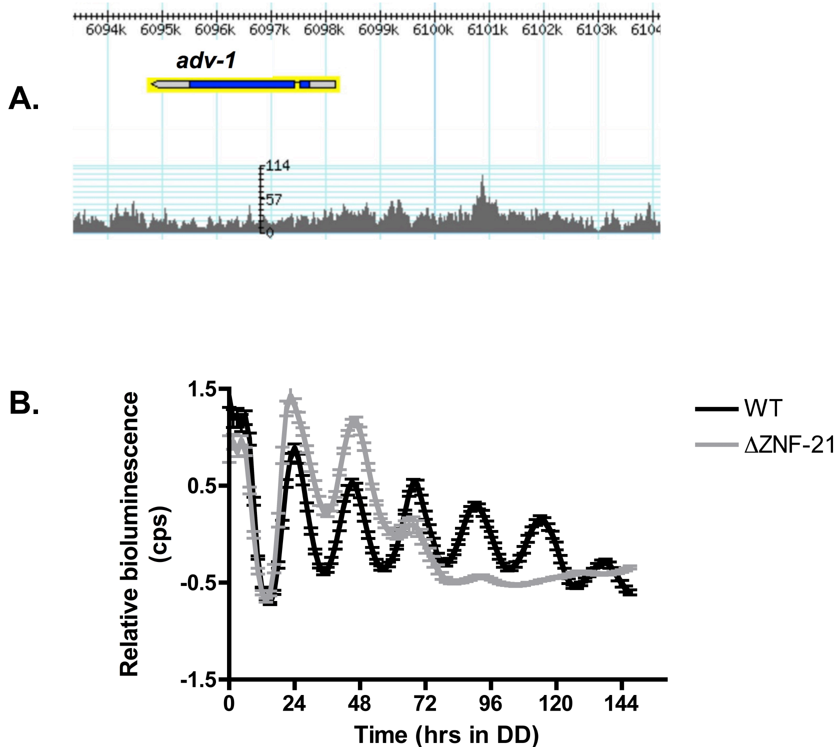

Figure 10. The effect of ΔZNF-21 on ADV-1::LUC. A.) ChIP-seq showing the binding of ZNF-21 to the promoter of *adv-1*. B.) ADV-1::LUC rhythms have a significant longer period in *Δadv-1* cells than in wild-type cells. P=0.0106 according to student's t-test.

clr-1 (*cellulose degradation regulator-1*) encodes for TF CLR-1 and regulates, along with two other TFs (CLR-1 and XLR-1), genes that are involved in cell wall degradation in *N. crassa* in the presence of plant cell wall material(Craig, Coradetti, Starr, & Glass, 2015). Even though CLR-1 binds to the promoter of *adv-1* (Figure 11B), deleting *clr-1* does not affect the transcript levels of *adv-1* in comparison to wild-type cells by RNA-seq (Figure 11A). In addition, deleting *clr-1* does not affect the rhythmicity of ADV-1::LUC protein rhythms in comparison to wild-type cells (Figure 11C and Table 2). Similar to the effect of in *Δclr-1* deletion, deleting 5 additional TFs FKH-1 (NCU00019), SAH-1 (NCU04179), VOS-1 (NCU05964), TAH3 (NCU03686), NCU09615 did not affect the levels of ADV-1::LUC rhythmicity (Table 2). As expected for the core clock component, deleting *wc-1* and *wc-2* abolished ADV-1::LUC protein rhythmicity (Table 2). Currently strains containing single deletions of *Δncu01871* and *Δncu08159* are being crossed to a strain containing the ADV-1 translational fusion construct. Additionally, a colleague in the lab is working on overexpressing *cre-1* (*ncu08807*) using a copper responsive promoter(Lamb et al., 2013) because a deletion of *cre-1* is lethal.

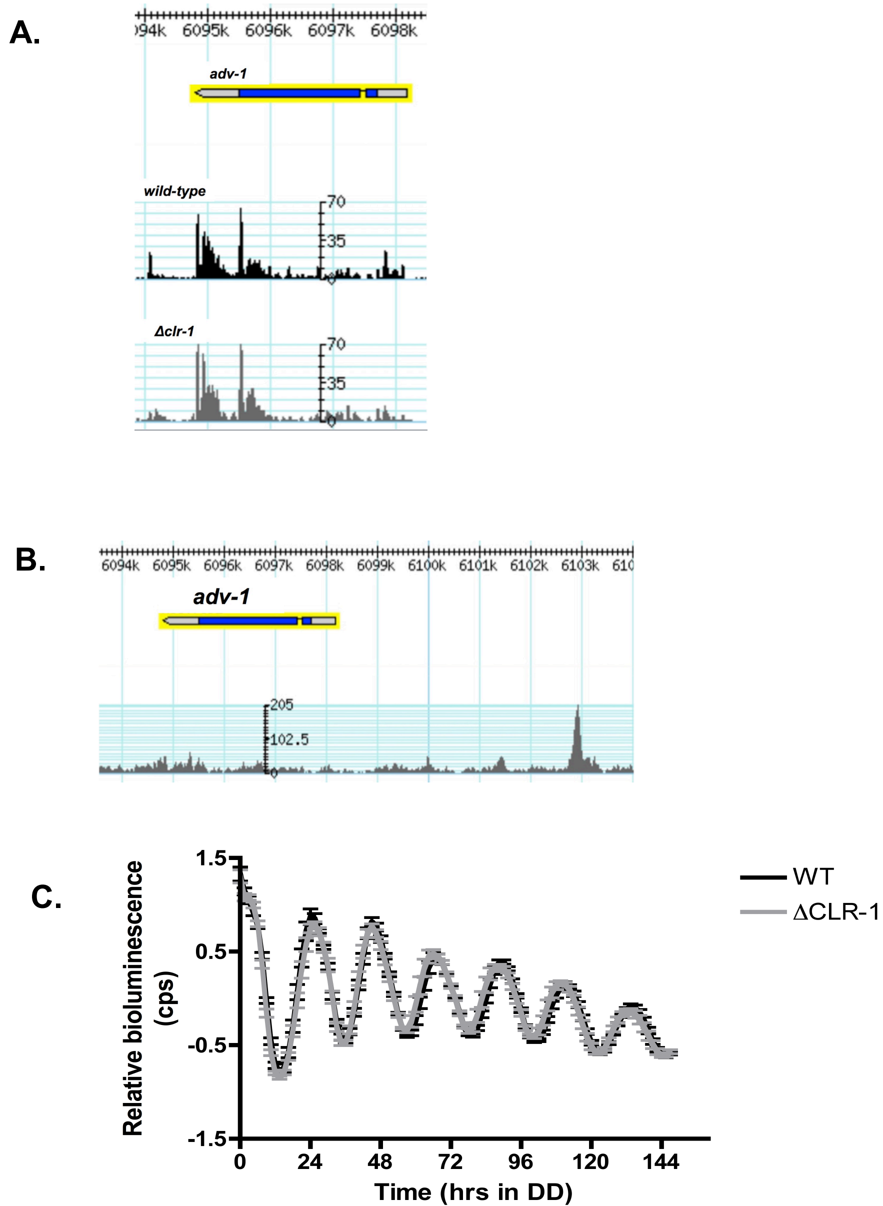

Figure 11. The effect of ΔCLR-1 on ADV-1::LUC. A.) RNA-seq data showing *adv-1* transcript levels in wild-type and *Δsub-1* cells. B.) ChIP-seq showing the binding of CLR-1 at the *adv-1* promoter. C.) Knocking out CLR-1 (NCU07705) did not alter the period or phase of ADV-1::LUC protein rhythms.

Table 2. Effect of ADV-1::LUC protein rhythms in TF knockouts.

TF knockout	NCU #	Effect on ADV-1::LUC
ΔCSP-1	ΔNCU02713	Effect on phase (>6 hrs)
ΔSUB-1	ΔNCU01154	Effect on period (>1 hr)
ΔCSP-2	ΔNCU06095	Effect on phase (>2 hrs)
ΔZNF-21	ΔNCU03184	Effect on period (~1 hr)
ΔCLR-1	ΔNCU07705	No effect
ΔFKH-1	ΔNCU00019	No effect
ΔSAH-1	ΔNCU04179	No effect
ΔVOS-1	ΔNCU05964	No effect
ΔNCU09615	ΔNCU09615	No effect
ΔTAH3	ΔNCU03686	No effect
ΔWC-1	ΔNCU02356	Arrhythmic
ΔWC-2	ΔNCU00902	Arrhythmic
ΔNCU08159	ΔNCU08159	Generating strain
ΔNCU01871	ΔNCU01871	Generating strain
ΔCRE-1	ΔNCU08807	No knockout

3.2 The effect of the TF network surrounding ADV-1 on the clock

ChIP-seq data of the first tier TFs suggested that several TFs that bind to the promoter regions of *wc-1*, *wc-2*, or *frq*. VOS-1, CSP-1, and FKH-1 bind to the promoter of *frq*. VOS-1, NCU01871, CLR-1, CSP-1, CSP-2, SAH-1, FKH-1 bind to the promoter of *wc-1*. VOS-1 and FKH-1 bind to the *wc-2* promoter (Figure 12). These data support a network model in which some of the TFs that are regulated by the WCC feedback to control the clock. To determine if the first tier TFs in the -1 network surrounding affect the clock, I examined FRQ::LUC rhythmicity in single TFs knockouts (Figure 13). FRQ is the negative component of the molecular oscillator; thus, an alteration on FRQ rhythmicity is expected to affect all downstream rhythms(Cheng et al., 2001). Therefore, it was important to determine if the TFs that alter ADV-1 rhythms similarly affect the clock. If FRQ

rhythms are not altered, these data would suggest that the changes in ADV-1 rhythms are due to the effects downstream of the clock.

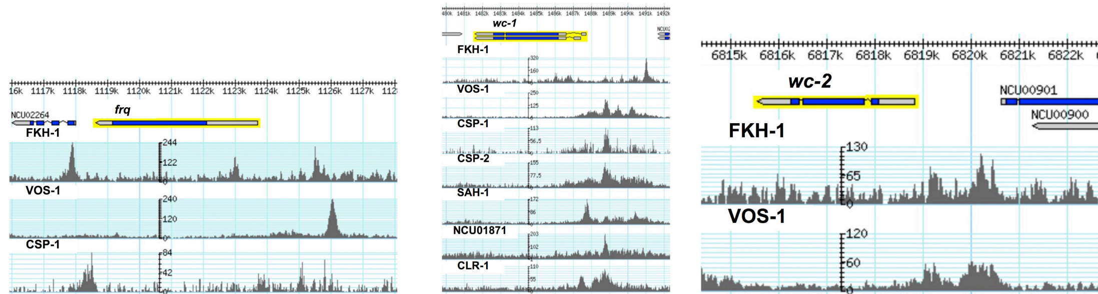

Figure 12. ChIP-seq data showing the binding of first tier TFs on clock components, *frq*, *wc-1* and *wc-2*.

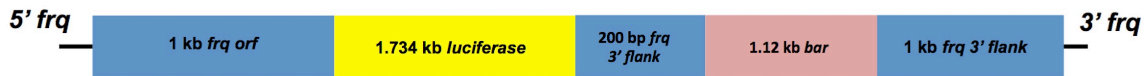

Figure 13. FRQ::LUC translational fusion construct.

Deleting *znf-21 (ncu03184)* lengthened the period of FRQ::LUC protein rhythms by ~1.5 hr (Figure 14). This change in period was longer that what was observed for ADV-1::LUC in *znf-21* cells (Figure 10). Similarly, the period of FRQ::LUC protein rhythms was shortened by 0.9 hr in *Δsub-1* (*ncu01154*) cells in comparison to wild-type cells (Figure 15), whereas the period of ADV-1::LUC in *Δsub-1* was 1.5 hr shorter (Figure 8). Taken together, these data suggest that

the downstream TF network somehow either buffers (ZNF-21) or exacerbates (SUB-1) period changes in the oscillator (ZNF-21)

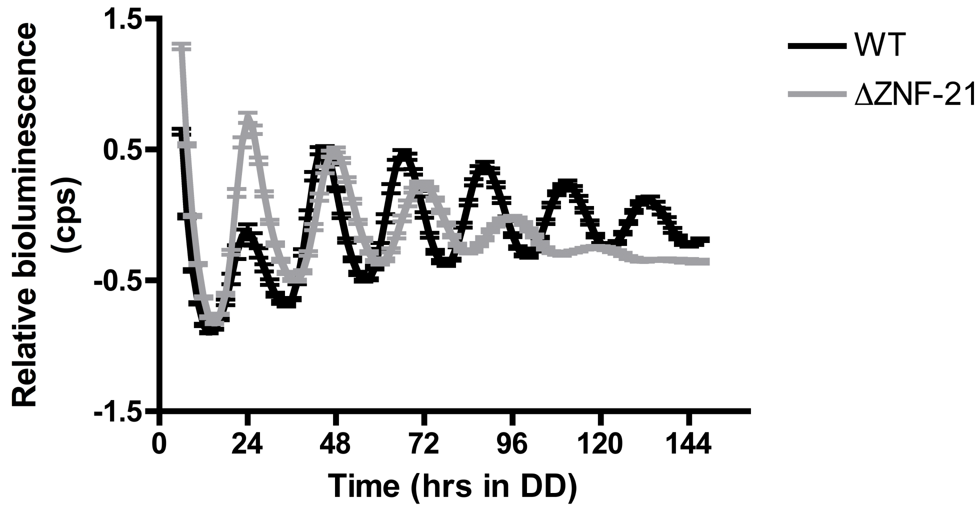

Figure 14. The effect of ΔZNF-21 on FRQ::LUC. Deleting *znf-21* leads to a longer period in FRQ::LUC rhythms (23.5±0.1) than in wild-type cells (21.8±0.04). (n=48, p<0.0001).

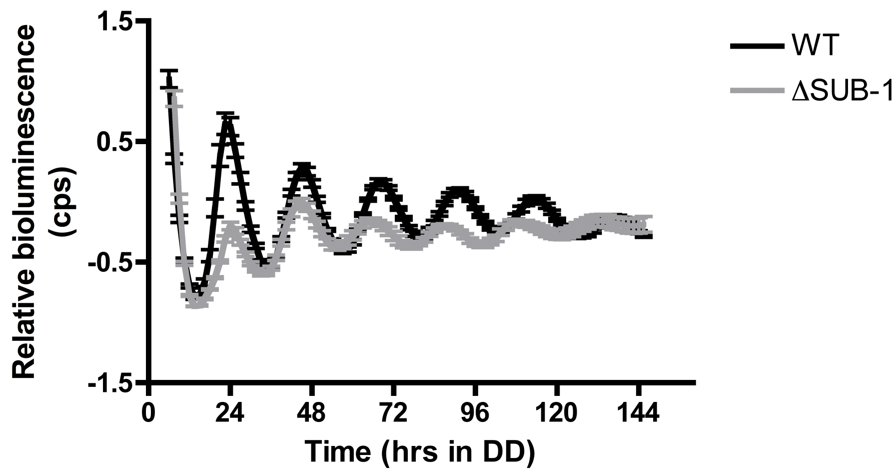

Figure 15. The effect of ΔSUB-1 on FRQ::LUC. Knocking out SUB-1 shortens the period of FRQ::LUC (21.5±0.2 hr) compared to wild-type cells (22.4±0.1 hr) (n=23, p≤0.0002)

Alternatively, knocking out CSP-1 does not affect FRQ::LUC rhythmicity (Figure 16), which indicates that although there are binding sites for CSP-1 on *frq* and *wc-1* (Figure 12), CSP-1 deletion does not alter the clock. FRQ proteins rhythms were not affected in other TFs knockouts, such as ΔFKH-1 (ΔNCU00019), ΔNCU09615, ΔVOS-1 (ΔNCU05964), ΔTAH3 (ΔNCU03686), ΔSAH-1 (ΔNCU04179), and ΔCLR-1 (ΔNCU07705) (data not shown). Experiments are currently in progress to examine the effects of CSP-2 on the clock.

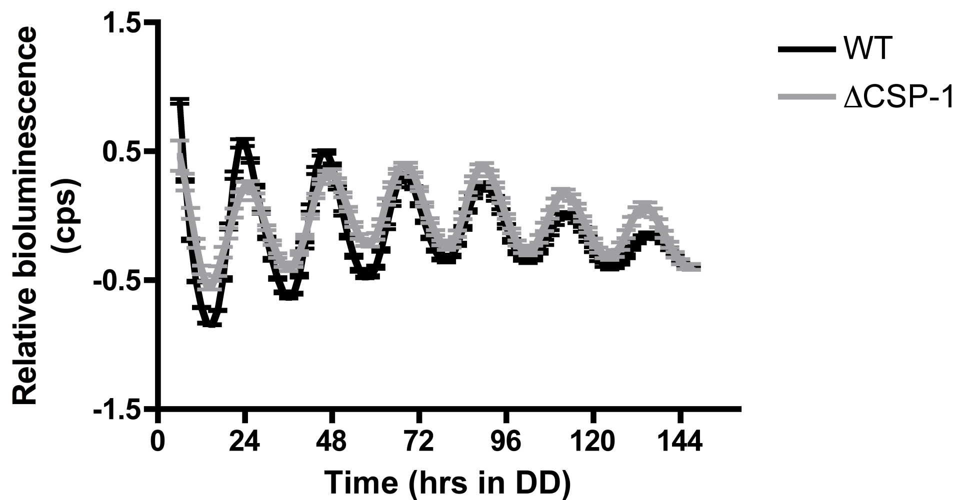

Figure 16. The effect of ΔCSP-1 on FRQ::LUC. A knockout of CSP-1 (NCU02713) does not alter FRQ::LUC rhythms.

3.3 The effect of *Δadv-1* on downstream target clock-controlled genes

ADV-1 regulates genes involved in metabolic and developmental processes(Dekhang et al., 2017). Computational modeling by our collaborators, Dr. James Galagan at Boston University, predicted that deleting *adv-1* either directly, or indirectly through the TF network, affects the phase of several downstream direct target genes. One of these genes predicted to have an altered when ADV-1 is deleted is *mitogen-activated protein kinase* (*mak-1*). MAK-1 is a key protein in the mitogen-activated protein kinase (MAPK) pathway(Bennett et al., 2013), and it important for developmental processes, such as cell fusion(Fu, Ao, Dettmann, Seiler, & Free, 2014; Fu et al., 2011). The predictions, which were based on rhythmic microarray data(Dong et al., 2008) and our ChIP-seq data, indicated that *mak-1* mRNA has a 5.49 phase advance

in *Δadv-1* cells in comparison to wild-type cells. To attempt to validate this prediction, I examined MAK-1::LUC protein rhythmicity in *Δadv-1* versus wild-type cells. In *Δadv-1* cells, MAK-1::LUC protein rhythms have about a 1 hr phase advance (Figure 17A), whereas the model predicts a 5.5 hr phase advance (Figure 17B). Although differences in the magnitude of the phase advance differ between the model and the *in vivo* data, these data, and additional validations carried out in the lab by graduate student Jennifer Jung suggest that the modeling is making progress.

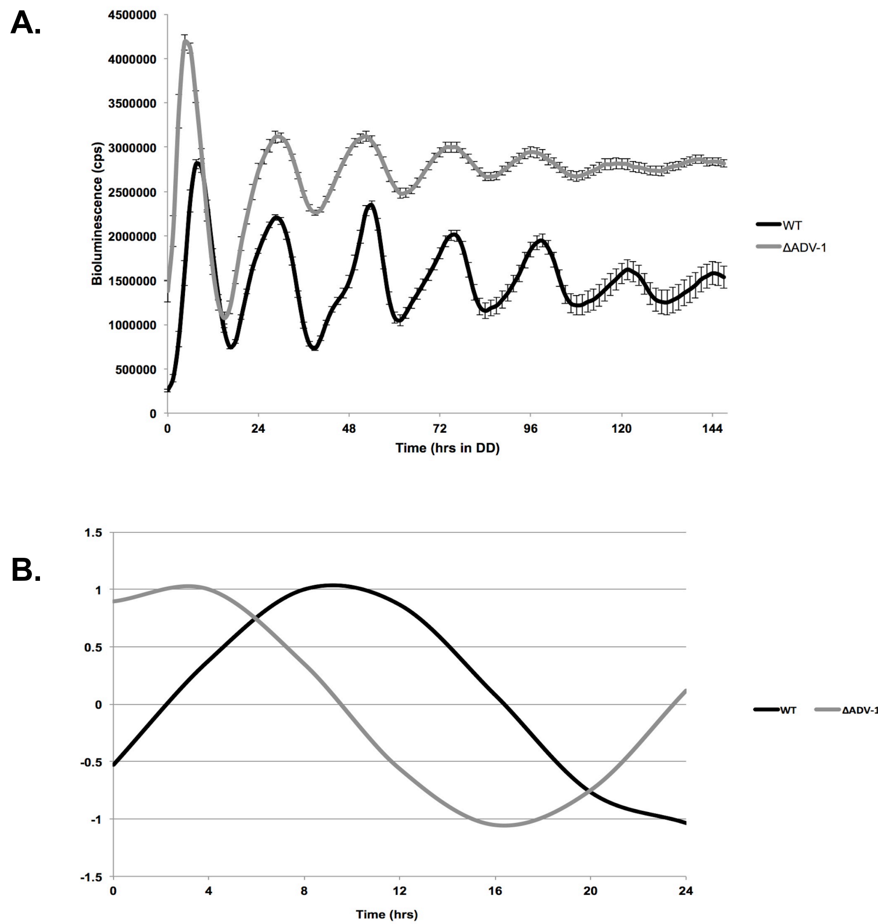

Figure 17. The effect of ΔADV-1 on MAK-1::LUC. A.) Deleting *adv-1* causes a significant 1 hr phase advance in MAK-1::LUC rhythms in comparison to wild-type cells. (n= 34, P≤0.0004). B.) Model prediction of 5.5 hr phase advance for *mak-1*.

The difference in phase between the model and the experimental data may be due to binding by other first TFs (Figure 18), MAD-1 (NCU07430) and ZNF-24 (NCU06503), to the *mak-1* promoter, differences in rhythmic transcript levels uses to generate the model versus translation used to validate the model, or errors in the model due to weakness in the rhythmic microarray data.

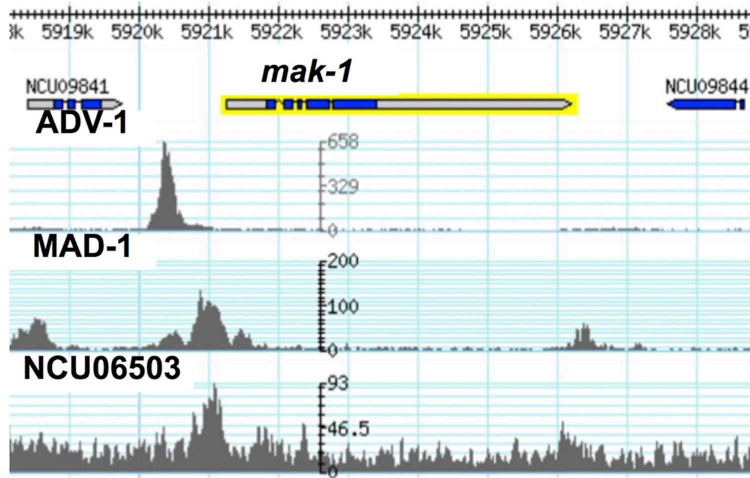

Figure 18. ChIP-seq data showing binding of TFs ADV-1, MAD-1, and NCU06503 at the *mak-1* promoter.

The model would be better improved by a better estimate of phase of ccgs in wild-type cells, and identification of ccgs that require ADV-1 for normal period and phase. To accomplish this goal, I harvested wild-type and *Δadv-1* cells every 2 hr over a 48 hr period in DD and isolated total RNA in duplicate. Rhythmicity of the cells was verified by examining FRQ protein rhythms in western blots (Figure 19). Once rhythmicity was validated, strand-specific RNA-seq libraries were prepared by Dr. Cheng Wu. These libraries are currently being sequenced and analyzed. Dense sampling, along with strand-specific sequencing, will provide key information for modeling the TF network surrounding ADV-1, and on the effects of deleting ADV-1 on rhythmic gene expression. In addition, strand-specific RNA-seq from wild-type *N. crassa* cells will provide information on alternative splicing and antisense transcription that will be valuable to the research community.

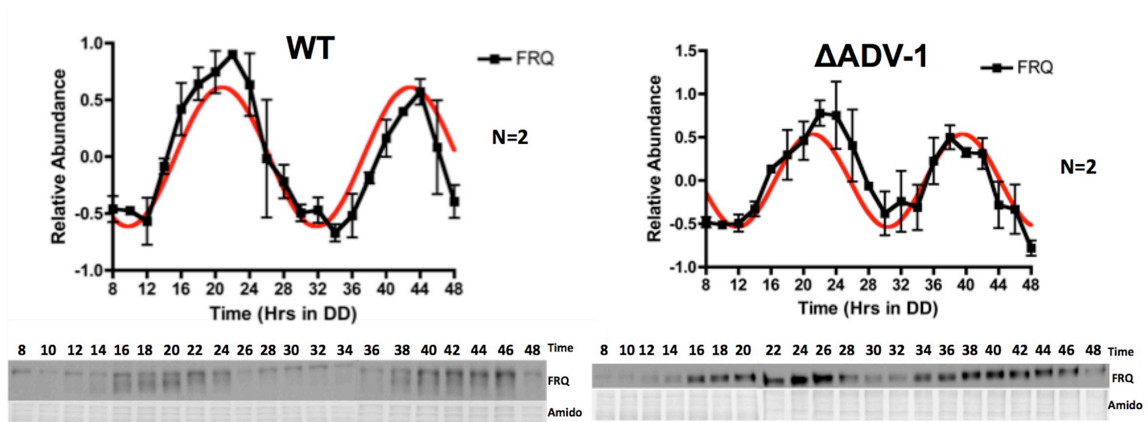

Figure 19. FRQ is rhythmic in 48 hr timecourses in wild-type and *Δadv-1* cells, grown in DD, indicating that the clock is functional.

4. SUMMARY

4.1 The first-tier TF network

The circadian clock controls approximately 40% of genes at the level of transcript abundance, and individual genes peak at all phases of the day(Hurley et al., 2014), but the mechanism by which the clock regulates phase is not known. The clock controls important physiological processes, and one key process regulated by the clock is metabolism. For example, genes that peak at dawn are mostly involved in catabolic processes, while genes that peak at dusk are involved in anabolic processes(C. Sancar, Sancar, et al., 2015). Using experimental and computational biology in *N. crassa*, this study focuses on determining the mechanism by which the clock might be regulating phase. In *N. crassa*, the core clock component WCC binds to the promoter 24 genes that encode for TFs and controls their rhythmic expression. These TFs are predicted to regulate downstream ccgs that are expressed at different times throughout the day; however, it was not clear if this was through flat hierarchical pathways as originally proposed(Smith et al., 2010) or through a network containing feed forward and feed back loops. One of the TFs regulated by the WCC is ADV-1. Loss of ADV-1 alters the expression of genes that are involved in development and metabolic processes(Dekhang et al., 2017).

Using ChIP-seq and microarray data(Dong et al., 2008), a complex, but preliminary, network was modeled for ADV-1. The network, which contains the WCC and 13 clock-controlled TFs (first tier TFs) that either bind near the *adv-1*

gene and potentially directly regulate *adv-1* expression, or bind near one of the genes encoding a TF that binds near adv-1 to potentially indirectly control *adv-1* expression. The TFs in the network potentially regulate other TFs within the network and/or regulate their own transcription. By examining an ADV-1 translational fusion construct in strains containing individual TF knockouts, I determined how each individual TF in the network affects the rhythms of ADV-1::LUC protein. Knocking out four of the TFs, CSP-1, CSP-2, SUB-1, and ZNF-21, alter the phase or period of ADV-1::LUC protein rhythmicity. CSP-1 binds in two places on the *adv-1* promoter and possibly at the 3' flank of *adv-1*. Knocking out CSP-1 leads to a phase delay of ADV-1::LUC protein rhythmicity (Figure 6C), but deleting one CSP-1 binding site on the ADV-1::LUC translational construct does not affect the rhythms of ADV-1::LUC protein (Figure 7). The expectation was that deleting a CSP-1 binding site would affect phase; however, this was not the case. Other potential CSP-1 binding sites exist near *adv-1*, suggesting the possibility that CSP-1 binding at these sites may be responsible for the phase regulation of *adv-1.* Alternatively, the change in *adv-1* phase in the CSP-1 knockout strain may be due to indirect effects through the network. Mutating each of the three CSP-1 binding sites located near *adv-1* individually and altogether would help distinguish these possibilities. It is also important to note that the first tier TF model was built on ChIP-seq and transcriptome data, whereas all of our experiments have examined protein rhythms. Our current

plans are to extend these studies using an *adv-1* promoter::*luc* fusion in the TF knockout strains.

Currently, we cannot distinguish between a linear versus network model for phase regulation. However, our data strongly support a network. For example, I found that the period defect of FRQ::LUC (3 hrs longer that wild-type) was more severe in cells deleted for ZNF-21, than ADV-1::LUC rhythm (1.5 hrs longer). Furthermore, deletion of ZNF-21 had no effect on the period of the conidiation rhythm (Jennifer Jung, unpublished data). These results support the idea that the TF network partially compensates for long period of FRQ::LUC. To further test the network Jennifer Jung has identified TF network motifs. She found that VOS-1, CSP-1, and ADV-1 form a feed forward loop (FFL) within the TF network (Figure 20). Both CSP-1 and VOS-1 bind to *adv-1* regulatory regions. CSP-1 represses *adv-1* transcript levels (Figure 6A), while CSP-1 activates *vos-1* mRNA (unpublished data). As predicted for this motif, deletion of CSP-1, which is at the head of the FFL has a greater effect on ADV-1::LUC rhythms that deletion of VOS-1 (which does not alter ADV-1::LUC rhythms). To further test this FFL module, ADV-1::LUC protein rhythms are currently being examined in a double CSP-1 and VOS-1 deletion strain. In addition, CSP-1 and VOS-1 binding sites near *adv-1* can be deleted to determine if they directly affect ADV-1::LUC protein rhythms. Current work in the lab is focusing on how this motif affects ADV-1 protein.

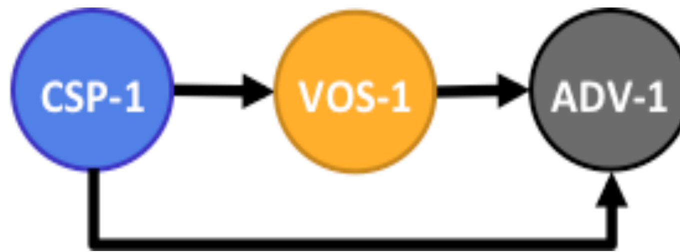

Figure 20. Feed forward loop (FFL) with CSP-1, VOS-1, and ADV-1.

Similar experiment will be carried out for other TFs that affect ADV-1::LUC phase and period. For example, CSP-2 also binds at the *adv-1* promoter (Figure 9A), and deleting *csp-2* alters the phase of ADV-1::LUC protein rhythms (Figure 9C). To determine if this effect is direct or indirect, the CSP-2 binding site near *adv-1* will also be mutated. If mutating the CSP-2 binding site affects phase, this would indicate that CSP-2 is critical for the proper phase regulation for ADV-1::LUC protein rhythms. If no change in phase is observed after the mutation of the CSP-2 binding site, these data would suggest that other TFs in the network compensate for the loss of CSP-1. Alternatively, CSP-2 may interact with another TF, within or outside the network, to regulate phase of ADV-1::LUC protein rhythms. For example, SUB-1 interacts with a TF that is not in the network (FF7) to activate late light-induced genes(C. Sancar, Ha, et al., 2015).

Both SUB-1 and ZNF-21 affect the period length of ADV-1::LUC protein rhythms (Figure 8C and Figure 10B). Deleting *sub-1* shortens ADV-1::LUC protein rhythms, while deleting *znf-21* lengthens ADV-1::LUC protein rhythms. Single deletions of *sub-1* and *znf-21* also shorten and elongate the period of FRQ::LUC protein rhythms, respectively. However, neither SUB-1 nor ZNF-21

bind to the promoter regions of frq, wc-1, wc-2, *frq,* suggesting that the regulation of *frq* is indirect. Additionally, ADV-1 binds to the promoter region of *wc-2*. Therefore, one possible mechanism to explain our results is that SUB-1 and ZNF-21 feed forward in the network to regulate the period of ADV-1::LUC protein rhythms, and then ADV-1 in turn feeds back to the clock to control FRQ::LUC protein rhythms. This hypothesis can be tested by mutating the SUB-1 and/or ZNF-21 binding site near the *adv-1* gene. If the model is correct, then I predict that mutation of the binding site would lead to FRQ::LUC period lengthening.

Deletions of *csp-1* or *csp-2* do not affect the rhythms of FRQ::LUC protein, indicating that CSP-1 and CSP-2 do not feed back to the clock. Several TFs, such as CSP-1, VOS-1, FKH-1, CSP-2, SAH-1, NCU01871, and CLR-1, in the TF network bind to clock genes (Figure 12); however, only a deletion of SUB-1 and ZNF-21 alters FRQ::LUC protein rhythms. These data further support the idea that the network provides robustness to the clock mechanism.

4.2 Future prospects

Our long-term goal is to determine what regulates phase. The ChIP-seq and RNA-seq data obtained in this study will be used to model the TF network, using ADV-1 to train the model. Experiments will then be done to test predictions of the model. The model will be revised as needed. Eventually, we hope that the model will allow us to predict how the phase of any of the TFs in the network and

its downstream targets are controlled. In addition, we hope that the model will be applicable to other TF networks.

In addition to modeling, our plan is to engineer genes to be expressed at any phase that we desire. This will be accomplished by identifying consensus binding sequences for each of the TFs in the network. Using this information, we will add any combination of these consensus binding sequences to engineer a reporter gene to get peak reporter activity at all different phases of the day.

4.3 Phase regulation of ADV-1 target genes

ChIP-seq was used to identify genes that are bound by ADV-1, and RNA-seq was used to identify genes whose regulation is altered when ADV-1 is missing from cells(Dekhang et al., 2017). Additionally, early computational modeling of the TF network, which was based on ChIP-seq and microarray data(Dong et al., 2008), suggested that several target ccgs of ADV-1 would have altered phase in *Δadv-1* cells compared to wild-type cells. Early predictions from the model indicated that *mak-1* mRNA would have a 5.5 hr phase advance in *Δadv-1* cells. Consistent with the model, I found that the of MAK-1::LUC protein rhythms in *Δadv-1* cells was advanced when compared to wild-type cells; however, the magnitude of the change was less than predicted. Additional tests of the model by Jennifer Jung and myself revealed that the model was correct in 2/4 tests, although similar to MAK-1, the magnitude of the change differed between the model and the experimental data. This may be because the model was

constructed based on transcript levels, and we are testing protein levels. Future experiments will need t be done to assay the phase of the transcripts. Alternatively, other TFs in the downstream network may compensate for the deletion of a single TF on a particular ccg. For example, MAD-1 and ZNF-24, both accumulate rhythmically(Hurley et al., 2014) also bind to the promoter region of *mak-1* and may participate with ADV-1 to control the phase of MAK-1 rhythms. Future work will continue to validate predictions for other ADV-1 direct targets; however, we anticipate the model will be greatly advanced with our high density rhythmic strand-specific RNA-seq data from wild-type and *Δadv-1* cells. In addition, the current model will be expanded to include ChIP-seq data from 9 additional TFs, whose promoter regions are bound by ADV-1 (Jennifer Jung, unpublished data).

4.4 Future prospects

Through characterization of the role of ADV-1 and it associated upstream and downstream regulatory networks in the circadian regulation of phase, we are positioned to use the regulatory network models to guide genetic changes in order to selectively alter the phase of expression of specific genes that are rhythmically controlled by ADV-1 or any other TF in the network. For example, by combining computational modeling and experimental validation of the models, we expect to be able to determine the impact of the upstream and downstream TF network on ADV-1 rhythms and its target ccgs. By integrating

the upstream and downstream models, we will be, for the first time, in a position to predict, and then experimentally validate, how a change in the network alters the phase of activity of a specific pathway, such as a metabolic pathway. This information may provide exciting avenues to explore for treating human disease, such as metabolic syndrome, associated with a defective clock and living against the clock (e.g. shift work).

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