THE TRANSCRIPTIONAL AND TRANSLATIONAL RESPONSE OF *Neurospora crassa* TO SALT AND LIGHT

A Dissertation

by

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ABSTRACT

Many different response-pathways enable microorganisms to respond to changes in the environment. Two environmental conditions that elicit responses in the model filamentous fungus *Neurospora crassa* are light and osmotic stress. The light response is controlled by transcription factors (TFs) —including the blue-light photo receptor WC-1. WC-1 forms a heterodimeric complex with WC-2; this complex, referred to as the white collar complex (WCC) binds to the promoters of multiple genes and regulates their transcription. Among the WCC targets are other TFs. To determine the downstream impact of removing WC-1, WC-2, and light-responsive TFs, we grew deletion-strains of 11 known light-responsive TFs (including Δ*wc-1* and Δ*wc-2*) whose promoters are bound by WCC. Strains were grown for 24hr then either exposed to 1hr of light or kept in the dark. RNA-seq analyses were then performed. We discovered that these 11 TFs play important roles in the transcript-level light response and established that two previously uncharacterized genes, NCU00275 and NCU09615, have major roles in multiple light-regulated pathways.

In *N. crassa*, osmotic stress activates an osmosensing mitogen activated kinase (OS MAPK) pathway. This pathway activates numerous kinases, including RCK-2, which phosphorylates translation elongation factor 2 (EF2) in response to salt stress. Phosphorylation of EF2 reduces translation. We examined the effects of both salt stress and deletion of *rck-2* by RNA-seq and ribosome profiling (Ribo-seq). We found that salt stress has large transcript-level and translation-level impacts on wild type *N. crassa* that

differ from what is observed in *S. cerevisiae*. In the *N. crassa* Δ*rck-2* mutant, multiple pathways that are normally repressed following salt stress lose repression at both a transcriptional and translational levels. However, deletion of *rck-2* did not impact transcript levels, but did impact translation in the absence of salt. Genes involved in respiration and the electron transport chain have increased translation efficiency in Δ*rck-2* as compared to wild type.

DEDICATION

I dedicate this work to Bethany Lynn Shockey. She was the love of my life and my inspiration—and still is. Though she is no longer here with me, I will hold her in my heart forever. I love you my sweet baby bear.

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CHAPTER I

INTRODUCTION

The history of *Neurospora crassa*

Neurospora crassa is a model filamentous fungus with traceable genetics that has been paramount in research involving genetics, transcriptional control, translational control, circadian biology, and more. It was first described as a "red bread mold" in France in 1842. It wasn't until the 1920s that *N. crassa* would see much more use in microbiology. A scientist by the name Bernard Dodge first published detailed work on *N. crassa* in 1927 with C.L. Shear. Later, *N. crassa* would be used to describe the "one gene, one enzyme" theory by Beadle and Tatum in a 1941 paper (PERKINS 1992). In 2003, the *N. crassa* genome was completely sequenced and subsequent annotation of its genome revealed 10,082 potential protein coding genes across seven linkage groups (GALAGAN *et al.* 2003). Its genome was found to be 38.6 megabases long (GALAGAN *et al.* 2003). *N. crassa* still remains a model organism for use in genetics and cell-free systems, as well as many other topics, due to its ability to be grown on minimal growth materials, traceable genetics, and relatively small genome (PERKINS 1992; GALAGAN *et al.* 2003).

The *Neurospora crassa* light response

The circadian clock

The circadian clock is responsible for daily biological rhythms in many different organisms. Many metabolic disorders can arise, such as diabetes, heart disease, and cancer, in higher eukaryotes that do not have a functioning clock. Evidence of the

circadian clock was first posited by Jean Jacques d'Ortous deMairan when, in 1729, he noted that plant leaves continue to open and close in the absence or presence of light in a rhythmic manner. Despite this discovery, research on circadian biology did not continue until the 1950s and 1960s when model organisms, such as *Neurospora crassa*, were used in this research.

Circadian clocks across all organisms share a number of defining characteristics (BELL-PEDERSEN *et al.* 2005). First, the expression of genes related to the clock must have a period of approximately 24hr. Second, this period should be able to be entrained by environmental changes, such as light, temperature, or food availability. Third, the clock should be able to run in the absence of any environmental cues (also referred to as "free-running") after entrainment. Finally, the clock should be made-up of a positive element(s) that positively regulates a downstream negative element(s). The negative element(s) should negatively regulate the positive element(s), causing a feedback loop that controls the rhythm of clock-regulated genes (BELL-PEDERSEN *et al.* 2005).

The circadian clock in Neurospora crassa

The filamentous fungus, *Neurospora crassa*, has been the subject of numerous circadian biology studies (CROSTHWAITE *et al.* 1995; DUNLAP 1999; GALAGAN *et al.* 2003; LEE *et al.* 2003; BELL-PEDERSEN *et al.* 2005; CHEN *et al.* 2009; LAMB *et al.* 2011; HURLEY *et al.* 2014; WU *et al.* 2014; CASTER *et al.* 2016; DEKHANG *et al.* 2017). The core clock of *N. crassa* contains a positive and negative arm (CROSTHWAITE *et al.* 1995; DUNLAP 1999; LEE *et al.* 2003). The positive arm of the clock is comprised of white collar 1 (WC-1) and white collar 2 (WC-2) (LEE *et al.* 2003; DUNLAP *et al.* 2007). WC-1

and WC-2 form a heterodimeric complex that is referred to as the White Collar Complex (WCC). WC-1 in WCC acts as the primary blue-light photoreceptor in *N. crassa*. The WCC then acts in a positive manner on the transcription of frequency (FRQ), one component of the negative arm of the clock (CROSTHWAITE *et al.* 1995; DUNLAP 1999). FRQ forms a heterodimeric complex with frequency-interacting RNA helicase (FRH). This complex, referred to as FCC, negatively impacts the production of *wc-1* and *wc-2* mRNA. This interaction of WCC and FCC forms the regulatory feedback loop that is the basis of the *N. crassa* core clock (CROSTHWAITE *et al.* 1995; DUNLAP 1999; LEE *et al.* 2003).

Down-stream impacts of the circadian clock in Neurospora crassa on transcription

Multiple studies have been conducted on the effects of the core clock on transcription in *Neurospora crassa* (CHEN *et al.* 2009; SMITH *et al.* 2010; WU *et al.* 2014; DEKHANG *et al.* 2017). Microarray and RNA-seq studies have found that there is both an early and late light-response that controls the transcription of various genes that are light regulated in wild type *N. crassa* (SMITH *et al.* 2010; WU *et al.* 2014; HURLEY *et al.* 2015b; SANCAR *et al.* 2015b). The early light response can be seen as little as 5min, while the late light-response can continue for hours. Identification of early light responsive genes (ELRGs) and late light-responsive genes (LLRGs) has been accomplished through comparisons of microarray data of wild-type to Δ*wc-1* and Δ*wc-2* strains of *N. crassa* after light induction (SMITH *et al.* 2010). Genes that showed up or down-regulation in response to light in wild type, but not Δ*wc-1* or Δ*wc-2* were deemed

to be blue light-regulated. ELRGs had the strongest response between 15 and 45min, while LLRGs had the strongest response between 45 and 90min (SMITH *et al.* 2010).

RNA-seq analysis of the *N. crassa* light-response revealed numerous pathways to be light-regulated after 60min of light exposure in wild type (WU *et al.* 2014). Genes involved in the oxidative stress response and previously reported light-induced mRNAs were identified in this study. Genes involved in rRNA processing were also strongly down-regulated in response to light in wild type (WU *et al.* 2014). The microarray and RNA-seq studies revealed numerous transcription factors whose promoters are known to be directly bound by WC-1 to be light-responsive (SMITH *et al.* 2010; WU *et al.* 2014). The functions of some of these transcription factors, including SUB-1, CSP-1, and ADV-1, have been explored (CHEN *et al.* 2009; SMITH *et al.* 2010; WU *et al.* 2014; DEKHANG *et al.* 2017). The deletion of *sub-1* has been shown to disrupt the light-response of both LLRGs and ELRGs, with LLRGs being the most strongly impacted (CHEN *et al.* 2009). *ADV-1* has been shown to be involved in the expression of light-responsive genes as well, although the impacted genes have not yet been described as LLRGs or ELRGs (DEKHANG *et al.* 2017).

The salt response in *Neurospora crassa*

The OS-pathway and phosphorylation of EF2

The ability of an organism to adapt to various environmental conditions is important for survival and fitness. One condition that can impact an organism's ability to survive is salinity. In *Neurospora crassa*, adaptation to changes in salinity in the environment is largely regulated by the osmotic sensing (OS) pathway (MISHRA 1977;

SCHUMACHER *et al.* 1997; JONES *et al.* 2007; WATANABE *et al.* 2007; YAMASHITA *et al.* 2007; LAMB *et al.* 2011). This pathway was originally noticed in 1969 when strains of *N. crassa* were noted as being salt sensitive in that specific strains died when exposed to salt stress that would not kill wild type cells (MAYS 1969). Since then it has been demonstrated that the OS pathway is activated through a sensor histidine kinase (OS-1) detecting a change in the environment and sending a signal through a histidine phosphotransferase to response regulator 1 (RRG-1), which begins a cascade of mitogen activated kinases and their kinases; OS-4 (MAP kinase kinase kinase), OS-5 (MAP kinase kinase), and OS-2 (MAP kinase) (MISHRA 1977; SCHUMACHER *et al.* 1997; WATANABE *et al.* 2007; YAMASHITA *et al.* 2007; LAMB *et al.* 2011). The activated MAP kinase OS-2 has been shown to activate multiple transcription factors, translation factors, and other kinases. One such kinase that is activated by OS-2 is radiation sensitivity complementing kinase-2 (RCK-2). RCK-2 is phosphorylated by OS-2 and its phosphorylation and subsequent activation are necessary for normal levels of elongation factor 2 (EF2) phosphorylation (CASTER *et al.* 2016; WU *et al.* 2019). RCK-2 activity has also been demonstrated to reduce global translation-level through the phosphorylation of EF2 (CASTER *et al.* 2016; WU *et al.* 2019).

Transcript and translation-level responses to osmotic stress

Transcript-level changes in *Neurospora crassa* in response to osmotic stress are poorly understood. *Saccharomyces cerevisiae*, however, has been extensively studied regarding its response to salt. The salt response pathway of *S. cerevisiae* shares a number of strong similarities to *N. crassa* in that the sensor histidine kinase (OS-1) of *N. crassa*

is very similar to the sensor histidine kinase (Hog1) of *S. cerevisiae* (SCHUMACHER *et al.* 1997; KRANTZ *et al.* 2006). Both the OS and HOG pathways act as the primary response system to environmental changes—most notably, osmotic stress (POSAS *et al.* 2000; REP *et al.* 2000; HOHMANN 2002; BILSLAND *et al.* 2004; KRANTZ *et al.* 2006).

In *S. cerevisiae*, transcript-level changes in response to salt stress have been explored (POSAS *et al.* 2000; REP *et al.* 2000). Microarray analysis has shown that genes in the transporter facilitation superfamily and involved in glycerol production have increased mRNA levels after exposure to salt. Glycerol production is a known response to osmotic stress, so we hypothesized that genes involved in glycerol production might be impacted after salt exposure in *N. crassa* as well due to the need for glycerol to maintain cell wall stability under osmotic stress (POSAS *et al.* 2000). Genes involved in general translation, amino acid metabolism, and sugar metabolism all show mRNA level decreases in response to 30min exposure to salt in *S. cerevisiae* (POSAS *et al.* 2000; REP *et al.* 2000).

Translation-level responses to salt stress have also been explored in *S. cerevisiae* (MELAMED *et al.* 2008; WARRINGER *et al.* 2010). These studies looked at the amount of polysomes (mRNA with 2 or more ribosome bound to them) for certain genes following salt stress. They found that some genes involved in translation, the general stress response, ribosome biogenesis, and protein catabolism are all translationally induced following salt stress, while genes involved in translation (different from those that were translationally induced), energy production, amino acid catabolism, the cell wall, and ion

homeostasis are translationally repressed following salt stress (MELAMED *et al.* 2008; WARRINGER *et al.* 2010).

RCK-2 and salt stress

Deletion of *rck-2* in *Neurospora crassa* causes the organism to become salt sensitive. Further, deletion of *rck-2* also causes changes in translation in *N. crassa,* with and without salt stress (WU *et al.* 2019). The role of *rck2* on the salt response in S. cerevisiae has been explored (WARRINGER *et al.* 2010). Analysis of ribosome associated mRNAs in rck2Δ and wild type *S. cerevisiae* following 6min of salt stress has revealed that rck2 is important for translation of mRNAs in *S. cerevisiae* following salt stress (WARRINGER *et al.* 2010). Our analysis will be performed on *N. crassa* that has been exposed to salt for 30min as opposed to 6min, which means our results will not be directly comparable. However, despite this it is known that *rck2Δ S. cerevisiae* has more free ribosomes (not associated to mRNA) than wild type (WARRINGER *et al.* 2010). This may indicate a role that *rck2* plays in ribosome binding and may be reflected in our study.

CHAPTER II

THE EFFECTS OF LIGHT-RESPONSIVE TRANSCRIPTION FACTORS ON THE TRANSCRIPTIONAL LIGHT-RESPONSE IN *Neurospora crassa*

Introduction

Circadian clocks are responsible for the regulation of daily biological rhythms and the filamentous fungus *Neurospora crassa* serves as prominent model organism for understanding circadian rhythms (DUNLAP 1999; DUNLAP *et al.* 2007; HURLEY *et al.* 2015b). The *N. crassa* core circadian clock impacts the expression of numerous genes, including transcription factors (TFs) that themselves modulate the expression of downstream clock-controlled genes. The *N. crassa* core clock, like other eukaryotic clocks, is composed of a positive arm and a negative arm that interact through positive and negative feedback loops (LINDEN *et al.* 1997b; LEE *et al.* 2003). The positive arm of the core clock is the white collar complex (WCC), which is a heterodimeric TF composed of white collar 1 (WC-1) and white collar 2 (WC-2) (HURLEY *et al.* 2015a). WCC also functions as the blue light photoreceptor needed for all blue-light photoresponses (LEE *et al.* 2003). WCC activates the transcription of many mRNAs, including the mRNA encoding FRQ (frequency), which functions in the negative arm of the core clock. FRQ interacts with frequency-interacting RNA helicase (FRH) to downregulate the expression of both components of WCC, WC-1 and WC-2 (DUNLAP 1999; HUNT *et al.* 2010; HURLEY *et al.* 2015a).

An environmental dark-to-light transition (dawn) is important for synchronizing *N. crassa*'s clock to events in the real world, connecting the organism's response to light

and to its circadian rhythm. A global analysis of microarray data of wild-type *N. crassa* shows both early and late responses to light (CHEN *et al.* 2009). The transcriptional response to exposure to light can be seen in as little as 5min (early response) and continues for hours (late response) (SOMMER *et al.* 1989; CROSTHWAITE *et al.* 1995; LINDEN *et al.* 1997a). Identification of early light responsive genes (ELRGs) and late-light responsive genes (LLRGs) has been accomplished through microarray analyses of wild-type, Δ*wc-1*, and Δ*wc-2* strains of *N. crassa* after light induction (CHEN *et al.* 2009). These findings revealed numerous ELRGs and LLRGs that were seen to be either up- or down-regulated after exposure to light in the wild-type strain that were not regulated in the Δ*wc-1* or Δ*wc-2* strains, indicating a transcriptional blue light-response. ELRGs were most strongly affected between 15 and 45 minutes of light exposure, while LLRGs were most strongly affected between 45 and 90 minutes of light exposure (CHEN *et al.* 2009). Most light regulated genes returned to basal levels of gene expression after 4 hours of light exposure, indicating photoadaptation to prolonged light exposure (CHEN *et al.* 2009). Interestingly, not all light responsive genes were functionally non-responsive to light in the Δ*wc-1* and Δ*wc-2* strains, with some remaining light-induced in those strains (CHEN *et al.* 2009).

A subsequent global analysis of the wild-type *N. crassa* response to light using RNA-seq (WU *et al.* 2014) enabled a more sensitive analysis of transcriptional changes occurring during light exposure. These analyses confirmed that exposure of *N. crassa* to light causes a strong increase in levels of mRNA for genes involved in the oxidative stress response and in previously described light-regulated and clock-controlled genes

(CHEN *et al.* 2009; WU *et al.* 2014). In addition, down-regulation of mRNAs for genes involved in rRNA processing (WU *et al.* 2014) was observed. In both microarray and RNA-seq studies, numerous TFs that are directly regulated by WC-1, are also up-regulated after exposure to 60min of light (CHEN *et al.* 2009; SMITH *et al.* 2010; WU *et al.* 2014). The functions of some of these light responsive TFs, including SUB-1, ADV-1, and CSP-1, have been explored (CHEN *et al.* 2009; SANCAR *et al.* 2011; SANCAR *et al.* 2012; SANCAR *et al.* 2015b; DEKHANG *et al.* 2017).

SUB-1 is a late light responsive TF (CHEN *et al.* 2009). The first phenotype discovered for a *N. crassa* mutant lacking functional *sub-1* (Δ*sub-1*) was that the mutant made protoperithecia (the *N. crassa* female reproductive structure) within the agar (submerged protoperithecia) during the sexual reproductive cycle, indicating it has roles beyond the light response (COLOT *et al.* 2006). The *sub-1* promoter is a direct target of WCC and the affinity of WCC for the *sub-1* promoter is increased when *N. crassa* cells are exposed to light (CHEN *et al.* 2009). While SUB-1 is not required for the activation of *wc-1* transcription, it is necessary for the regulation of many LLRGs and some ELRGs (CHEN *et al.* 2009). SUB-1 is not necessary for core clock function, as Δ*sub-1* maintains a circadian rhythm (CHEN *et al.* 2009). Taken together, these data indicate an interplay between WCC and SUB-1 regulatory pathways.

ADV-1 (arrested development 1) is another TF that is also directly regulated by WCC [6]. The Δ*adv-1* strain has a phenotype of poor protoperithecial differentiation and shortened aerial hyphae (COLOT *et al.* 2006; FISCHER *et al.* 2018). Further, ADV-1 is required for normal vegetative growth, with Δ*adv-1* having a vegetative hyphal fusion

phenotype (COLOT *et al.* 2006). RNA-seq analysis of Δ*adv-1* reveals that ADV-1 is important for the expression of genes critical for metabolism, development, and cell fusion (DEKHANG *et al.* 2017; FISCHER *et al.* 2018). The Δ*adv-1* mutant has a hyphal fusion defect (DEKHANG *et al.* 2017; FISCHER *et al.* 2018). Importantly, a number of direct targets of ADV-1 are also direct targets of SUB-1 and CSP-1 (another light-induced TF), which strongly suggest that TFs act combinatorially and downstream of WCC in the *N. crassa* light-response (SMITH *et al.* 2010; SANCAR *et al.* 2011; SANCAR *et al.* 2015b; DEKHANG *et al.* 2017). Of the functional categories of direct targets of ADV-1, the transcription of genes related to cellular metabolism was the most strongly impacted (DEKHANG *et al.* 2017). Interestingly, ADV-1 binds distal to the *adv-1* 3'UTR, indicating the possibility of self-regulation from a downstream site (DEKHANG *et al.* 2017).

CSP-1 (conidial separation 1) is a light-responsive TF (LAMBREGHTS *et al.* 2009; SMITH *et al.* 2010). The phenotype of Δ*csp-1* is poor conidial separation (LAMBREGHTS *et al.* 2009). Transcription of *csp-1* is both directly regulated by WC-1 and directly regulates the transcription of *wc-1* (SMITH *et al.* 2010; SANCAR *et al.* 2011). Overexpression of CSP-1 causes the core clock to lose rhythmicity due to an impact on the expression of *wc-1* (SANCAR *et al.* 2012). CSP-1 was identified as a TF that acts as a transcriptional repressor (SANCAR *et al.* 2011). This TF has a short half-life that helps regulate the circadian response to glucose, similarly to yeast NRG1 and NRG2 (SANCAR *et al.* 2011; SANCAR *et al.* 2012). CSP-1 acts by repressing transcription during

subjective morning and, as CSP-1 is degraded and no longer produced, regulated genes are able to peak in the subjective evening (SANCAR *et al.* 2012).

Other light-regulated TFs, including CSP-2, CLR-1, SAH-1, and VOS-1, have not been investigated with respect to their function regarding clock regulation or in controlling the light response. The TFs NCU00275 and NCU09615, while identified as light-responsive, have not been well characterized regarding impact to the clock. Nevertheless, other functions of these TFs are in some cases established. CSP-2 (conidial separation 2) is a TF that controls aerial hyphae growth and conidial separation whose promoter is directly bound by WCC and is light responsive (CHEN AND LOROS 2009; SMITH *et al.* 2010; CARRILLO *et al.* 2017). CLR-1 is a TF that is known to be involved in cellulose degradation (SMITH *et al.* 2010; CORADETTI *et al.* 2012; CRAIG *et al.* 2015). CLR-1 also directly binds to the *frq* promoter, indicating a possible link between the core clock and cellulose degradation (CRAIG *et al.* 2015). *SAH-1* is a TF that is also a direct target of WCC with a phenotype of shortened aerial hyphae (COLOT *et al.* 2006; CHEN AND LOROS 2009; SMITH *et al.* 2010; WU *et al.* 2014). VOS-1 is a light-induced TF that is a direct target of WCC (SMITH *et al.* 2010; WU *et al.* 2014). It is regulated by FLB-3 during asexual development with a phenotype of delayed conidial germination (BONI *et al.* 2018). VOS-1 negatively regulates other developmentally important genes including *aba-1* and *wet-1* (BONI *et al.* 2018). NCU00275 and NCU09615 are both putative TFs that are light-responsive (SMITH *et al.* 2010; WU *et al.* 2014) . Both are proposed to be TFs due to the presence of zinc finger DNA binding domains (COLOT *et al.* 2006; CARRILLO *et al.* 2017).

The mechanisms of activation of many downstream light-responsive TFs—and how this activation is connected to the action of WCC—remains unresolved. To understand these relationships, we examined the effects of disrupting 11 light-responsive TFs that are known to be light-responsive and that are directly regulated by WCC: *adv-1, clr-1, csp-1, csp-2, sah-1, sub-1, vos-1, ncu00275, ncu09615, wc-1,* and *wc-2*). We accomplished this by comparing their mRNA levels to a wild-type strain (OR74A). These mutant strains were grown in the dark for 23 hours and then either exposed to 60 minutes of light, or kept in the dark for an additional 60 minutes, totaling 24 hours of growth. By exposing these strains to 60 minutes of light, we are activating WCC (except in the *wc-1* and *wc-2* mutants), which, in turn, activates light-responsive TFs. By disrupting various TFs, we can observe the changes in transcription that occur as a result, and then determine how these TFs are involved in transcriptional regulation past the activation of WCC. Disrupting WCC through the removal of *wc-1*/*wc-2* will allow us to see which TFs work in concert with WCC by comparing mRNA expression changes between the various TF mutants, WCC mutants, and wild-type *N. crassa*.

Materials and Methods

Strains and culture conditions

The Fungal Genetics Stock Center (FGSC, Kansas State University, Manhattan, KS; http://www.fgsc.net) is the source for all *Neurospora crassa* strains used in this study, including wild type (OR74A, FGSC #987) and deletion *N. crassa* strains (Δ*adv-1* (FGSC #11041), Δclr-1 (FGSC #11028), Δ*csp-1* (FGSC #11348), Δ*csp-2* (FGSC #13563), Δ*ncu00275* (FGSC #12217), Δ*ncu09615* (FGSC #19000), Δ*sah-1* (FGSC

#11132), Δ*sub-1* (FGSC #11127), Δ*vos-1* (FGSC #13536), Δ*wc-1* (FGSC #11711), and Δ*wc-2* (FGSC #11124)). Deletion strains were created by the *Neurospora* genome project (COLLOPY *et al.* 2010). Conidia for inoculation of flasks for RNA-seq studies were generated as described (WU *et al.* 2014). Conidia for RNA-seq experiment were harvested with 200ml sterile water and counted for concentration under a hemocytometer. For light induction, 200ml of Bird's media (METZENBERG 2004) in a 500ml flask was inoculated to a final concentration of 107 conidia per ml. Cells were then grown for 23hr in the dark, at 30oC, shaking at 170rpm, and subsequently kept in the dark for 60min or exposed to light for 60min for a total of 24hr of growth while still shaking at 170rpm at 30^{o} C. Cells were collected by centrifugation in an IEC clinical centrifuge at 1,000g for 1min. The resulting mycelial mass was poured onto sterile filter paper layered on top of paper towels and pressed between another sheet of sterile filter paper. The mycelial pads were washed with sterile ice cold water and pressed with sterile filter paper a second time. The resulting washed mycelial pads were then cut into ~100mg pieces using sterile razor blades and were placed in 50ml falcon tubes and flash-frozen in liquid nitrogen. The flash-frozen pads were then stored at -80o C until used in RNA-seq library preparation.

RNA-seq procedure

Total RNA extraction, cDNA library preparation, sequencing library preparation, and sequencing for were conducted as described for the wild-type, Δ*adv-1*, Δclr-1, Δ*csp-1*, Δ*csp-2*, Δ*ncu00275*, Δ*ncu09615*, Δ*sah-1*, Δ*sub-1*, and Δ*vos-1* strains (WU *et al.* 2014). The Δ*wc-1* and Δ*wc-2* cells' RNA extraction and cDNA library preparation were

performed as described (WU *et al.* 2014). RNA-seq library preparation was performed using the Lexogen CORALL Total RNA-seq Library Prep Kit.

NGS alignment and analysis

Illumina reads were aligned to *Neurospora crassa* assembly 12 annotation using HISAT2 version 2.0.5 (TRAPNELL *et al.* 2009) with the –no-softclip and –S options. Output SAM files were then converted into BAM files, sorted, and indexed using samtools version 1.7 (LI *et al.* 2009). The BAM files were then initially analyzed through the Cuffdiff program from the Cufflinks Suite version 2.2.1 (TRAPNELL *et al.* 2012) to obtain FPKM and differential expression (DE) values for each gene in each strain. This was done using the –b and –L options. The BAM files were also analyzed with DESeq2 version 1.22.2 (LOVE *et al.* 2014) in RStudio for a differential expression analysis that is considered to be more robust. Obtaining read counts for DESeq2 was done through the use of HTSeq-count (ANDERS *et al.* 2015). The resulting DE tables from DESeq2 and the FPKM tables from the Cufflinks suite were then combined to generate a master table with each FPKM and DE value associated with its NCU identifier. This table was uploaded to a SQL database for data analyses. Cross comparisons of wild-type and mutant strains at 0min and 60min of light exposure were done in MySQL Server through a local installation of MySQL Workbench. These lists were then compared through inner-joining to examine differential expression patterns at 0min, 60min, or both. Each mutant strain was compared to the wild-type strain at 0min and at 60min separately. Once completed, the genes were sorted based on if they were up- or down-regulated in the mutant strain as compared to wild-type at 0min, 60min, or

at both time points. The resulting lists would show genes that are up- or down-regulated in each mutant strain as compared to wild-type at either 0min or 60min, or at both time points, while also taking into account difference between the mutant strains and the wild-type with regards to FPKM. Once comparisons were done, differentially expressed genes at time 0, 60, or at both time-points were analyzed through Functional Analysis using FungiFun2 (PRIEBE *et al.* 2015). Gene Ontology analysis was completed using Panther GO (MI *et al.* 2019).

Heat map construction

Differential gene expression patterns were visualized using heat maps in R through the package heatmap.2 and colorRampPalette. Strains and genes were clustered using Ward's minimum variance method before being displayed on heat maps. Strains were mapped with dendrograms to assess similarities between mutant strains. Dendrograms were generated in heatmap.2 using the dend() and hclust() functions in R through the Pearson's clustering method.

Results

RNA-seq of light-responsive TFs reveals transcript levels regulation of light-regulated genes by light-responsive TFs.

To see the effects of light-responsive TFs on global transcript levels in *N. crassa*, we used RNA-seq data collected from biological duplicates of deletion strains of 8 known, *wc-1* regulated TFs, Δ*wc-1*, Δ*wc-2*, and wild type that were grown in the dark for 24hr then either left in the dark or exposed to light for an additional 60min. All sequencing data were aligned to the FungiDB *N. crassa* genome annotation (release 38)

using HISAT2. Normalized reads (FPKMs) were calculated from the alignment files through Cuffdiff from the Cufflinks package. From this we obtained highly correlated biological duplicate data sets for 8 TFs knock-out strains (Δclr-1, Δ*csp-1*, Δ*csp-2*, Δ*ncu00275*, Δ*ncu09615*, Δ*sub-1*, Δ*sah-1*, and Δ*vos-1*) and highly correlated biological triplicate data sets for 2 TF knock-out strains (Δ*wc-1*, and Δ*wc-2* (Table S1). We also reanalyzed the primary data for wild type and Δ*adv-1* that were previously published (WU *et al.* 2014; DEKHANG *et al.* 2017). The alignments of the wild type and Δ*adv-1* raw reads again produced highly correlated duplicate data sets as expected. Data from DESeq2 and Cuffdiff analyses are summarized in Table S2. To assess the effects of deletion of the genes for light-responsive TFs, we analyzed the 8221 annotated genes that had FPKM expression values of greater than 0 in all strains (Table S3).

We initially looked for genes that were statistically significantly ($q \leq 0.05$) regulated ≥2-fold up or down at the transcript level after light exposure in wild type by reanalysis of the primary Illumina sequence data provided by Wu et al. (WU *et al.* 2014). DESeq2 analysis of these data revealed 806 up-regulated genes and 659 down-regulated genes using these criteria (Figure 1, Table S4). For comparison, the original Cuffdiff analysis of these data using different criteria completed by Wu et al found 249 genes to be up-regulated and 166 genes to be down-regulated in response to 60min of light ($q \leq 0.2$) (Table S4). Of the 806 up-regulated genes found in this study, 235 are shared with the Wu et al analysis, 571 are unique to our analysis, and 14 are unique to Wu et al. Of the 659 down-regulated genes reported in this analysis, 164 are shared with Wu et al, 495 are unique to our analysis, and 2 are unique to Wu et al.

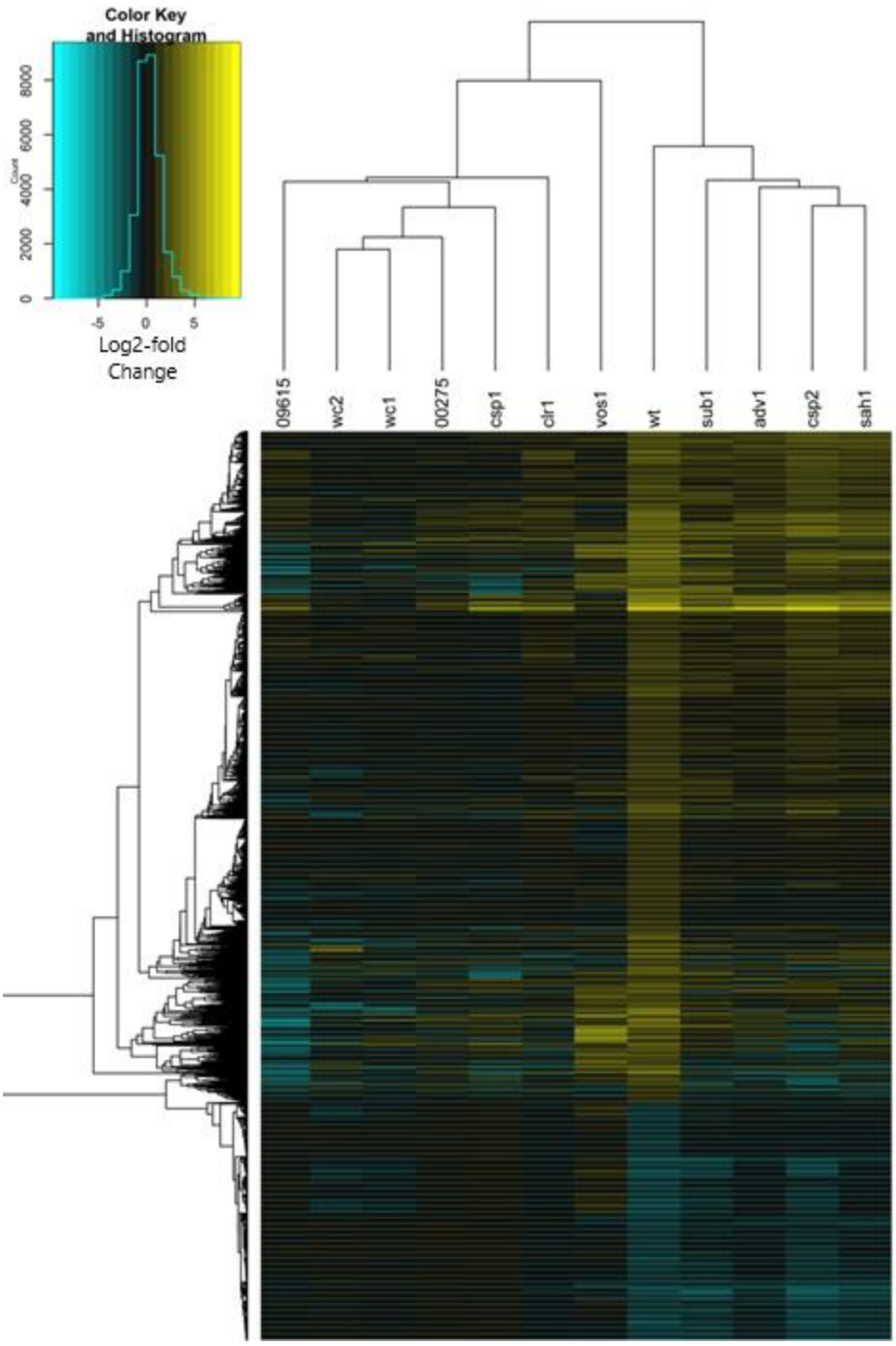

Figure 1: Heat map of all light-regulated genes in wild type. Genes that were found to be light-regulated if they had a fold-change of log2-fold ≥ 1 or log2-fold ≤ -1 ($q \leq 0.05$) through DESeq2 analysis comparing RNA-seq of wild type cells either exposed to 60min of light or kept in the dark. Heat map was generated in R using heatmap.2. Clustering and dendrogram creation was done using Pearson method within R.

Functional analysis (FunCat) using FungiFun2 was then performed on all of the light-regulated genes that we found and the results compared to the FunCat analyses performed by Wu et al. While both up- and down-regulated categories were shared between the two analyses, there were differences. These differences are likely due to differences in statistical significance cut-off values used for DE analysis and to differences in the annotations used for analysis. For example, Wu et al study used a cut-off of $q \leq 0.2$, while we used a cut-off of $q \leq 0.05$. Two of the up-regulated categories that we found that were not reported in the Wu et al analysis include the synthesis of vitamins, cofactors, and prosthetic groups and the metabolism of vitamins, cofactors, and prosthetic groups. Importantly, genes were found to be up-regulated within these categories in the Wu et al study, but the category itself did not meet the statistical cut-off required for significance ($p \leq 0.05$).

There were also multiple differences between down-regulated categories reported in Wu et al and our analysis. As with up-regulated category differences, more down-regulated categories were called in the Wu et al FunCat analysis. Some of the down-regulated categories found in our analysis ($q \leq 0.05$) that were not reported in Wu et al ($q \leq 0.2$) include rRNA synthesis, ribosome biogenesis, DNA topology, and DNA synthesis/replication. These differences are likely due to changes made in alignment algorithms, differential expression algorithms, and the *N. crassa* genome annotation that have been made to improve their accuracy.

We next analyzed the genes that either lost or gained light regulation in ΔTF strains as compared to wild type. Very few genes that were not light-regulated in wild-

type gained light-regulation in mutants. Therefore, we focused on genes that were light regulated in wild type and lost this regulation in at least one ΔTF strain as there were substantially more genes impacted in this way. After discerning which genes are responsive to light in wild type, we looked to see which of these lost the wild type light-response in the ΔTF strains. This analysis revealed numerous genes that were up-regulated in wild-type after exposure to light, but lost regulation in at least one deletion strain. The two strains with the greatest number of genes that lost up-regulation were Δ*wc-1* (626) and Δ*wc-2* (575), while Δ*csp-2* had the least number of genes lose up-regulation (112) (Table S5). Conversely, numerous genes that were down-regulated in wild type also lost this regulation in at least one deletion strain. The strain with the greatest number of genes that lost down-regulation was Δ*ncu00275* (638), while the strain with the least number of genes that lost down-regulation was Δ*csp-2* (83) (Table S5).

The effects of wc-1 and wc-2 on transcript levels.

Of the 626 and 575 genes that lost up-regulation in Δ*wc-1* and Δ*wc-2*, respectively, 478 are shared, with 148 unique to Δ*wc-1* and 97 unique to Δ*wc-2*. The genes that lost up-regulation in both of these TFs yielded two categories: homeostasis of metal ions (16 genes) and metabolism of vitamins, cofactors, and prosthetic groups (17 genes). Importantly to note is that FunCat does not have categories for light-regulated genes, but many known light-regulated genes, such as the blue light inducible genes and albino genes, were found to be strongly up-regulated in the wild-type strain and were not regulated in the white collar deletion strains. Genes that lost light-regulation that were

not shared between the two TFs did not yield any statistically significant FunCat categories.

Next we looked at the effects of Δ*wc-1* and Δ*wc-2* by looking for genes that are down-regulated in wild type. There were 475 genes that were down-regulated in wild type and lose regulation in Δ*wc-1*; 411 genes lost down-regulated in Δ*wc-2* (Table S6). Of these, 370 were shared between Δ*wc-1* and Δ*wc-1*; 105 were unique to Δ*wc-1* and 41 were unique to Δ*wc-2*. There were 20 FunCat categories that were shared between Δ*wc-1* and Δ*wc-2*. These include DNA topology (15 genes) and DNA recombination/repair (37). There is a great deal of overlap between the 20 categories, but most are involved with DNA binding and the DNA damage response. Interestingly, translation initiation was the only FunCat category that lost down-regulation exclusively in Δ*wc-1*. The genes that comprise this category include eukaryotic initiation factor 4 subunits B and E, and eukaryotic elongation factor 3. The annotation for eukaryotic elongation factor 3 may be incorrect, as eukaryotic elongation factor 3 is not involved in translation initiation. The genes involved in DNA topology and DNA recombination/repair include RNA helicase mak-5 and dbp-2, and a RecQ family helicase. One category was statistically significant exclusively for Δ*wc-2*: tRNA modification. Only 4 genes significantly matched this category, three of which are annotated as methyl transferases.

Functional analysis of genes regulated by light-responsive TFs.

A Cuffdiff analysis was previously performed by Dekhang et al to analyze the effects of Δ*adv-1* on transcript levels after exposure to light (DEKHANG *et al.* 2017). They reported that 93 genes were up-regulated by light in Δ*adv-1* but not wild type

(log2-fold ≥ 1, q-value ≤ 0.05). Reanalysis of the Dekhang et al primary data using DESeq2 yielded 82 of the 93 genes, as well as an additional 220 genes that were up-regulated by light. Dekhang et al reported 117 genes that were down-regulated in Δ*adv-1* but not wild type. DESeq2 reanalysis yielded 98 of the 117 down-regulated genes, as well as an additional 394 down-regulated genes.

We next analyzed transcript levels of Δclr-1, Δ*csp-1*, Δ*csp-2*, Δ*ncu00275*, Δ*ncu09615*, Δ*sah-1*, Δ*sub-1*, and Δ*vos-1* using DESeq2, and determined the functional categories whose light-response was impacted by Δ*wc-1*, Δ*wc-2*, Δ*adv-1*, or any of these eight mutants. Here we focused on functional categories that lost statistically significant up-regulation or down-regulation across multiple ΔTF strains (Table S7, S8). FunCat categories that lost up-regulation in multiple strains included (i) metabolism of vitamins, cofactors, and prosthetic groups, (ii) synthesis of vitamins, cofactors, and prosthetic groups, and (iii) homeostasis of metal ions. Altered regulation of all 22 genes in the metabolism of vitamins, cofactors, and prosthetic groups category was observed in four ΔTF strains (Δ*ncu00275*, Δ*vos-1*, Δ*wc-1*, and Δ*wc-2*) (Figure 2, Table S7). This category was also affected in Δclr-1 (13 of 22), Δ*csp-1* (11 of 22), Δ*adv-1* (8 of 22), Δ*ncu09615* (8 of 22), Δ*sub-1* (8 of 22), Δ*csp-2* (6 of 22), and Δ*sah-1* (5 of 22). Altered regulation of all 30 genes in the synthesis of vitamins, cofactors, and prosthetic groups category was seen in four ΔTF strains (Δ*ncu00275*, Δ*vos-1*, Δ*wc-1*, and Δ*wc-2*). Approximately half of the 30 genes were found to lose regulation in 7 of the 11 ΔTF strains (Δ*adv-1*, Δclr-1, Δ*csp-1*, Δ*csp-2*, Δ*ncu09615*, Δ*sah-1*, and Δ*sub-1*) (Figure 2). The 20 genes found to lose up-regulation in the homeostasis of metal ions category were

varied across the ΔTF strains, with only Δ*ncu00275*, Δ*wc-1*, and Δ*wc-2* showing a loss of light-regulation in most of these genes. Δ*vos-1*, which had similar effects on genes involved in the synthesis and metabolism of vitamins, cofactors, and prosthetic groups as Δ*ncu00275*, Δ*wc-1*, and Δ*wc-2*, had no impact on genes involved in homeostasis of metal ions.

FunCat categories that were found to lose down-regulation in multiple ΔTF strains included (i) chromatin remodeling, (ii) rRNA synthesis, (iii) rRNA processing, and (iv) ribosome biogenesis. Chromatin remodeling in response to light has been explored both in *N. crassa* and higher eukaryotes (CROSIO *et al.* 2000; SANCAR *et al.* 2015a). Our DESeq2 analyses of the light response of chromatin remodeling genes in *N. crassa* revealed 45 genes that are down-regulated in wild type that are either not down-regulated, or are up-regulated, in ΔTF strains (Figure 3, Table S7, S8).

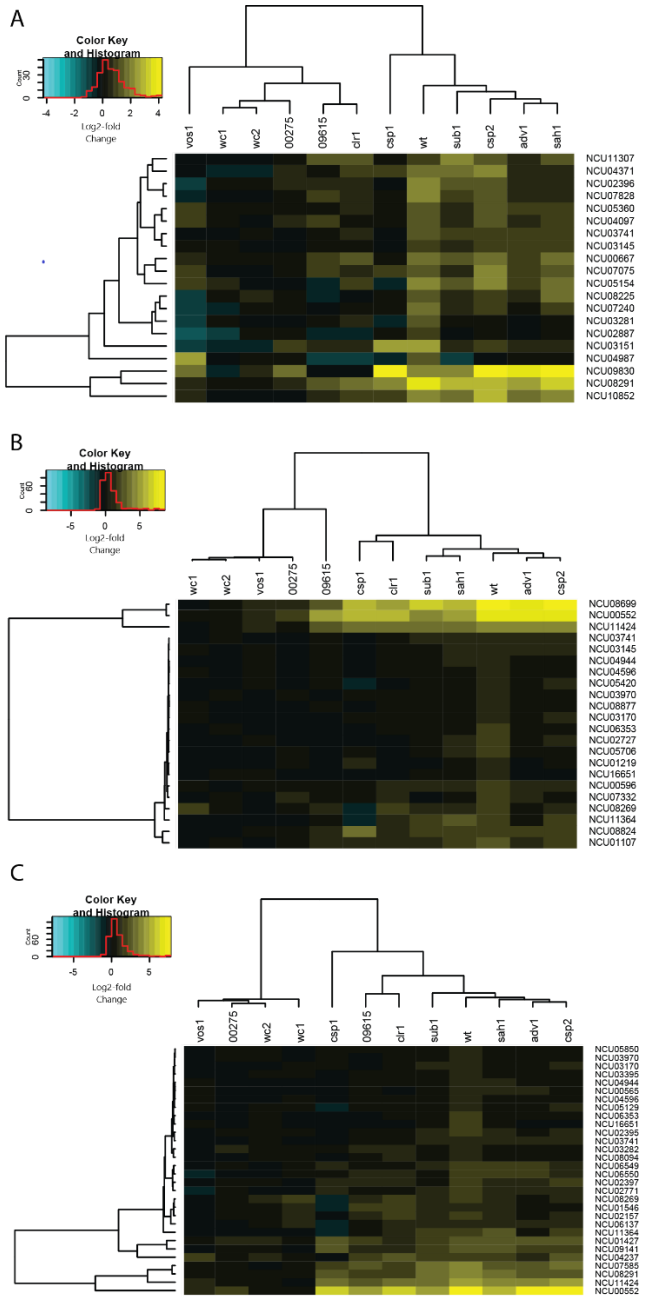

Figure 2 Heat map of gene categories which have transcript-level increases in response to light in wild type. Log2-fold changes of genes of three FunCat categories were found to have transcript-level increases (log2-fold ≥ 1, $q \leq 0.05$) in response to light in wild type: (A) homeostasis of metal cations, (B) metabolism of vitamins, cofactors, and prosthetic groups, and (C) synthesis of vitamins, cofactors, and prosthetic groups. All genes that were up-regulated in wild type lost regulation in at least one ΔTF strain. Heat map was generated in R using heatmap.2. Clustering and dendrogram creation was done using Pearson method within R.

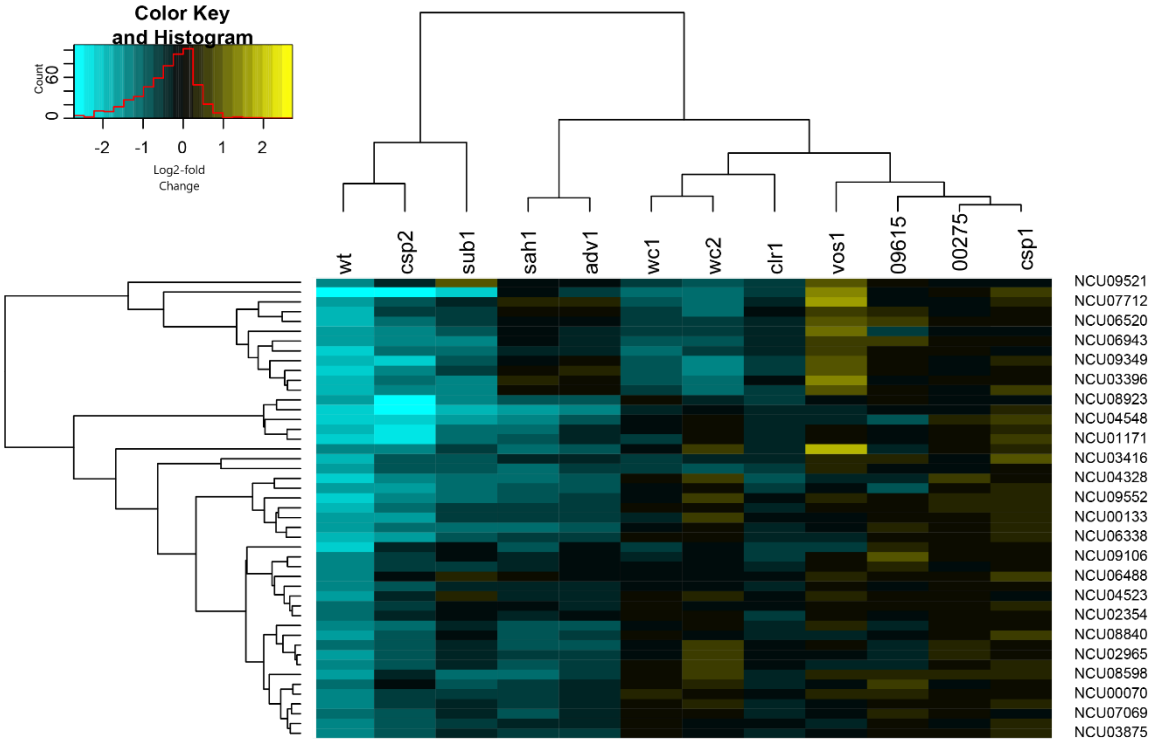

Figure 3: Heat map of chromatin remodeling genes which have transcript-level increases in response to light in wild type. Genes of the chromatin remodeling FunCat category were found to have transcript-level increases (log2-fold ≥ 1, $q \leq 0.05$) in response to light in wild type. All genes that were up-regulated in wild type lost regulation in at least one ΔTF strain. Heat map was generated in R using heatmap.2. Clustering and dendrogram creation was done using Pearson method within R.

The three FunCat categories, (i) rRNA processing, (ii) rRNA synthesis, and (iii) ribosome biogenesis, contain a combined total of 423 genes, 13 of which are shared by all categories (Figure 4A). rRNA processing and rRNA synthesis share 11 genes; rRNA processing and ribosome biogenesis share 54 genes; and rRNA synthesis and ribosome biogenesis share two genes. The rRNA processing category has 104 unique genes; rRNA

synthesis has 41 unique genes; and ribosome biogenesis has 41 unique genes. Due to this complexity, we first considered each of the three categories separately.

There are 186 genes in the rRNA processing FunCat category; 76 of these are down-regulated in wild type in response to light (Figure 4). All 76 lose this down-regulation in one or more ΔTF strain (TABLE S7, S8). Interestingly, nearly all of these lose regulation in Δ*csp-1* (60 of 76), Δ*ncu00275* (75 of 76), and Δ*ncu09615* (70 of 76), with 55 losing regulation in all three ΔTF strains. The Δ*wc-1* and Δwc-2 strains have fewer genes in this category that lose their light response (16 and 11 of the 76 genes, respectively). For Δ*vos-1*, all 76 of these genes showed altered regulation; for 47, transcript levels remain unchanged in response to light. For 29 genes, transcript levels paradoxically increased in response to light. Δ*sub-1* and Δ*csp-2* have similar expression levels to wild type (Figure 2).

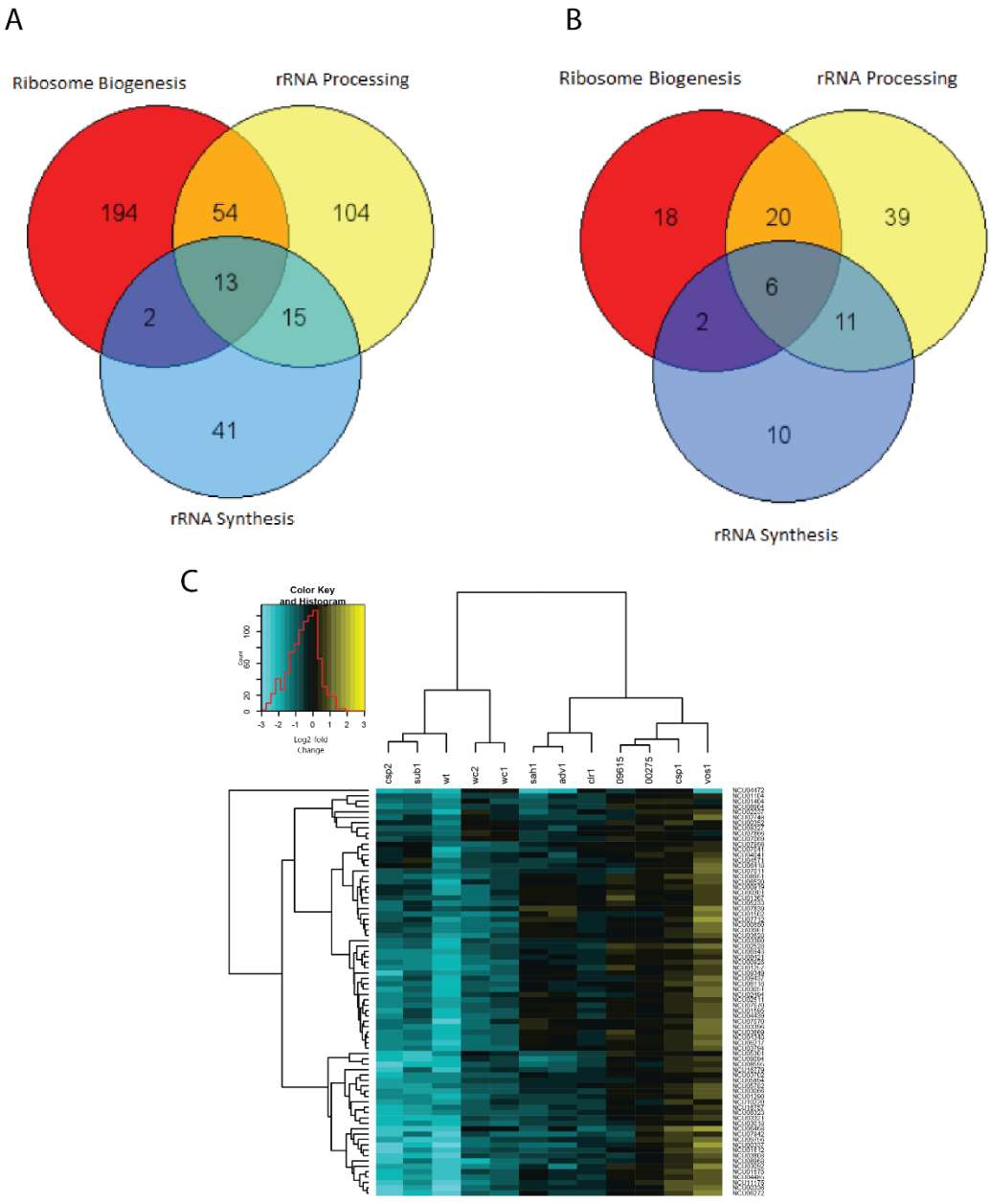

Figure 4: Analysis of rRNA-related pathways. (A) A comparison of all genes found within the (i) ribosome biogenesis, (ii) rRNA processing, and (iii) rRNA synthesis FunCat categories was done. We then compared the genes we found to have transcript-level decreases in wild type in each of those categories to each other (B). This revealed overlap of genes between the three categories. A heat map was constructed to look at the changes occurring in each ΔTF strain following light for genes in the rRNA processing FunCat category (C). All genes that were down-regulated in wild type lost regulation in at least one ΔTF strain. Heat map was generated in R using heatmap.2. Clustering and dendrogram creation was done using Pearson method within R.

There are 263 genes in the ribosome biogenesis FunCat category; 46 of these are down-regulated in wild type in response to light. All 46 lose this down-regulation in one or more ΔTF strain (Figure 5, Table S7, S8). Again, nearly all of these lose regulation in Δ*csp-1* (38 of 46), Δ*ncu00275* (45 of 46), and Δ*ncu09615* (34 of 46), with 29 losing regulation in all three ΔTF strains. The Δ*wc-1* and Δ*wc-2* strains have fewer genes that lose their light response with seven and six of the 46 genes losing regulation, respectively (5 are shared). For Δ*vos-1*, all 46 of these genes showed altered regulation; for 12, transcript levels remain unchanged in response to light and for 34, transcript levels increase in response to light. Just as for the rRNA processing category, Δ*sub-1* and Δ*csp-2* have similar expression levels to wild type.

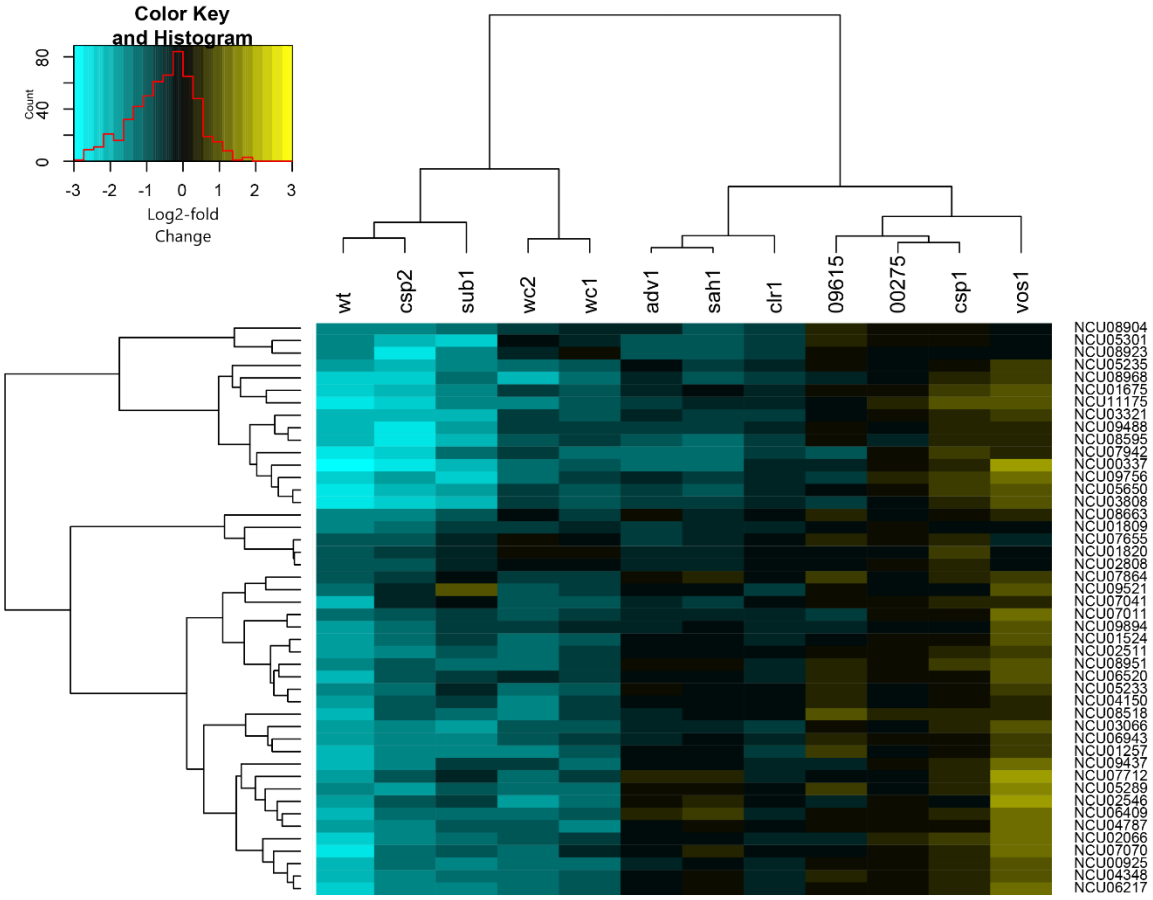

Figure 5: Heat map of rRNA synthesis genes which have transcript-level decreases in response to light in wild type. Genes of the rRNA synthesis FunCat category that were found to have transcript-level decreases (log2-fold ≤ -1, q ≤ 0.05) in response to light in wild type. All genes that were down-regulated in wild type lost regulation in at least one ΔTF strain. Heat map was generated in R using heatmap.2. Clustering and dendrogram creation was done using Pearson method within R.

There are 73 genes in the rRNA synthesis FunCat category; 29 of these are down-regulated in wild type in response to light (Figure 6, Table S7, S8). All 29 lose this down-regulation in one or more ΔTF strain. All or nearly all of these lose regulation in

Δ*csp-1* (23 of 29), Δ*ncu00275* (29 of 29), and Δ*ncu09615* (23 of 29), with 17 losing regulation in all three ΔTF strains. Interestingly, all genes that lose regulation in these three ΔTF strains are shared with at least one other ΔTF strain. The Δ*wc-1* and Δ*wc-2* strains have fewer genes that lose their light response with four and three of the 46 genes losing regulation, respectively, with all three found to lose regulation in Δ*wc-2* losing regulation in Δ*wc-1*. For Δ*vos-1,* all 29 of these genes showed altered regulation; for six, transcript levels remain unchanged in response to light. For 23 genes, transcript levels increase in response to light. Just as for the rRNA processing and ribosome biogenesis categories, Δ*sub-1* and Δ*csp-2* have similar expression levels to wild type.

In summary, the ΔTF strains had similar effects on light regulation for each of these three categories. Δ*sub-1* and Δ*csp-2* clustered with wild type did not appear to have an impact on light response. Δ*csp-1*, Δ*ncu00275*, and Δ*ncu09615* clustered together in each heat map and affected the regulation of the majority of genes in each category. This indicates that these three TFs could impact the production of ribosomes through effects on rRNA synthesis and processing, and ribosome assembly. Δ*wc-1* and Δ*wc-2* also clustered, but their effects on genes are less pronounced. Δ*vos-1* was the most distinct from wild type in each category and the mutant showed a paradoxical increase in transcript levels for numerous genes in each category in response to light. This indicates that *vos-1* could play an important role in the light response of genes involved in rRNA synthesis and processing, and ribosome assembly.

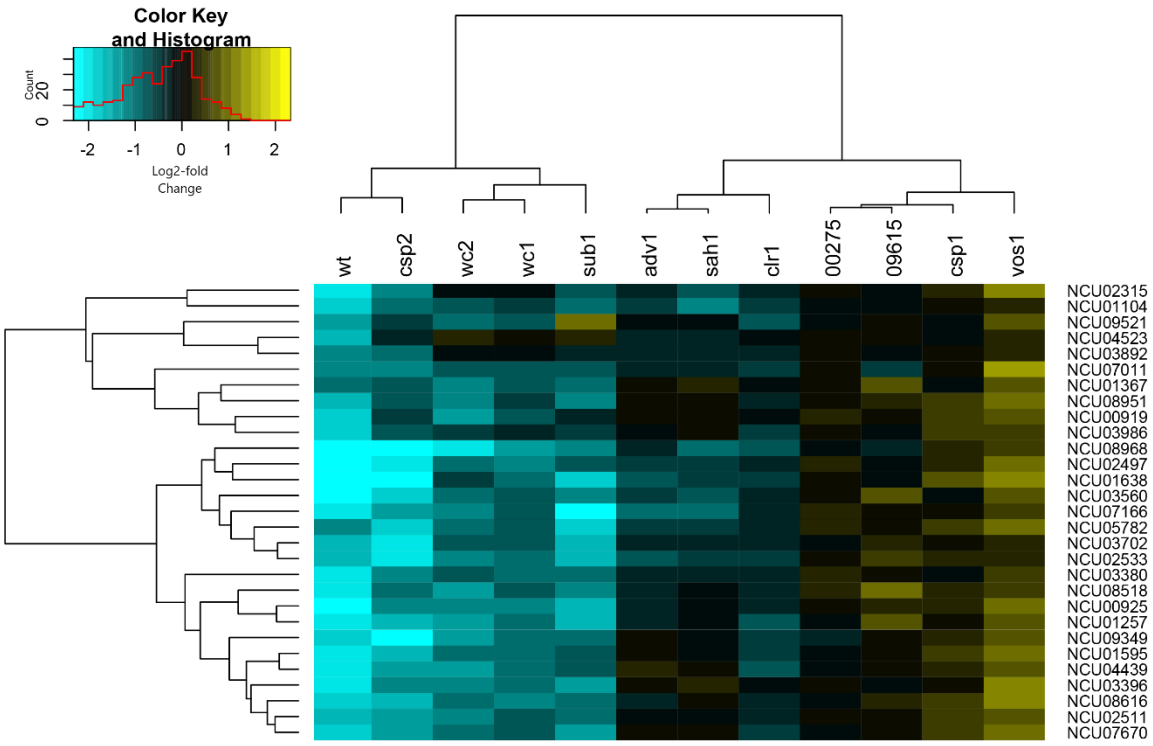

Figure 6: Heat map of ribosome biogenesis genes which have transcript-level decreases in response to light in wild type. Genes of the ribosome biogenesis FunCat category that were found to have transcript-level decreases (log2-fold ≤ -1, q ≤ 0.05) in response to light in wild type. All genes that were down-regulated in wild type lost regulation in at least one ΔTF strain. Heat map was generated in R using heatmap.2. Clustering and dendrogram creation was done using Pearson method within R.

Expression of transcript levels of core clock components depends on light-responsive transcription factors

Due to the known WC-1 binding sites near each of the TFs analyzed as determined by ChIP-seq (SMITH *et al.* 2010), we looked at the impact of each TF deletion on the expression levels of *wc-1*, *wc-2*, *frq*, and *frh* to assess the possibility of feedback control (Figure 7, Table S9). First we looked at one portion of the negative arm of the clock, *frq*. Consistent with previous work, the wild-type had a strong induction of *frq* transcript levels (log-2 fold = 2.67) after exposure to light. The Δ*adv-1*, Δ*sub-1*, and Δ*vos-1* strains *frq* transcript levels and induction resembled what was seen in wild type (Figure 5). Interestingly, the Δ*clr-1*, Δ*csp-1*, Δ *csp-2*, and Δ*sub-1* strains all showed induction for *frq* transcript levels in response to light, but had significantly reduced FPKM values at both time points (0min and 60min) as compared to wild-type. This suggests that these TFs are important for establishing basal expression of *frq* transcripts, but are not involved in its differential expression. The Δ*ncu00275* and Δ*ncu09615* strains showed a significant reduction in light response as compared to wild-type expression, while having similar transcript levels in the dark. This indicates that these two TFs involved in proper induction of *frq* transcript levels, but not for basal expression.

Next we looked at the one portion of the positive arm of the clock, *wc-1*. Consistent with previous work, we saw an induction in *wc-1* transcript levels in wild type after exposure to light. Δ*adv-1*, Δ*sah-1*, Δ*sub-1*, and Δ*vos-1* all have transcript levels and induction that resembled what was seen in wild type. The Δ*csp-1* and Δ*csp-2* induction for *wc-1* transcript levels in response to light, but had significantly reduced

FPKM values at both time points (0min and 60min) as compared to wild-type. Interestingly, the Δ*clr-1*, Δ*ncu00275*, and Δ*ncu09615* strains showed a significant reduction in light response as compared to wild-type expression, while having similar transcript levels in the dark (Figure 5). This indicates that these two TFs involved in proper induction of *wc-1* transcript levels, but not for basal expression.

The components of the core clock that are not light-responsive (*frh* and *wc-2*) were then analyzed. Consistent with previous work, *frh* was not observed to have a statistically significant increase or decrease in transcript levels in wild-type cells in response to light. Additionally, none of the mutant strains showed any regulation of *frh* expression in response to light. Similarly to *frh*, *wc-2* did not have any expression changes in the wild-type strain and the mutant strains were all similar to the wild-type strain with regards to both normalized expression and induction (no induction).

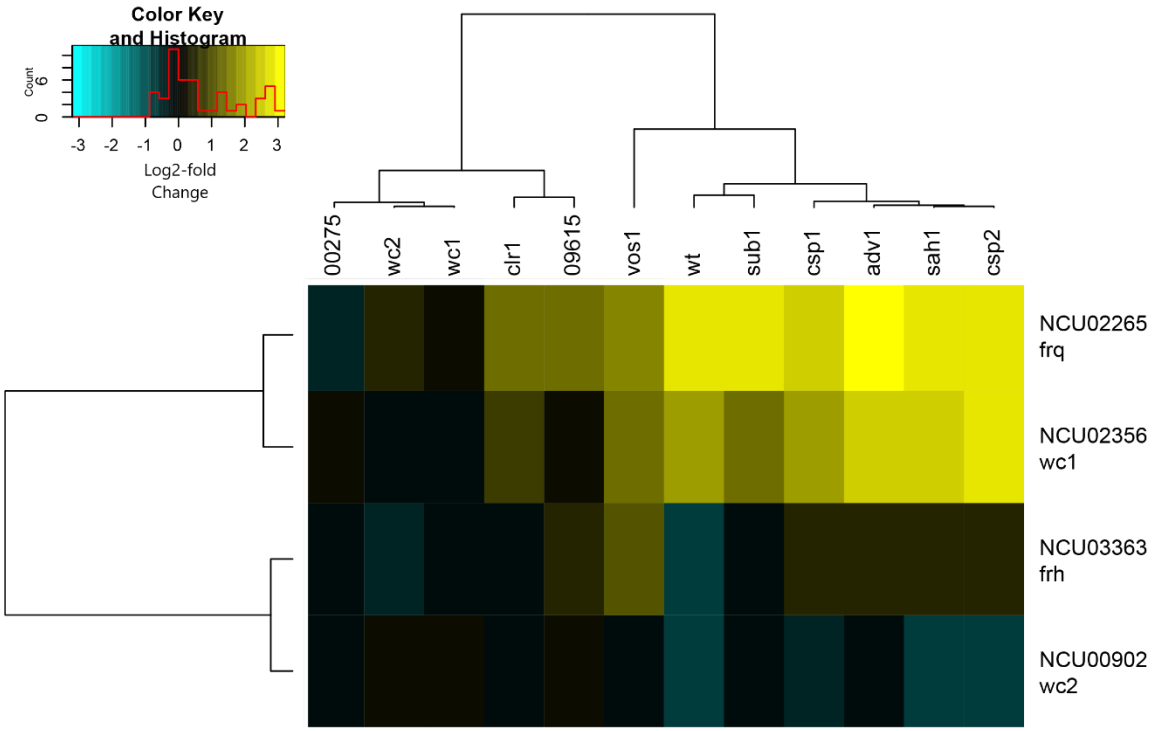

Figure 7: Heat map of the transcript-level changes for the core clock genes. Heat map of log2-fold changes in transcript levels for each of the four major core clock genes (*wc-1*, *wc-2*, *frh*, and *frh*). Heat map was generated in R using heatmap.2. Clustering and dendrogram creation was done using Pearson method within R.

Taken together we can see a complex relationship these TFs play with the core clock. The TFs *adv-1*, *sub-1*, and *vos-1* impact neither of the differential expression of *frq* or *wc-1* transcript levels. CSP-1 and CSP-2 impact basal transcript levels of *wc-1*, but have no impact on the *wc-1* light response or *frq*. NCU00275 and NCU09615 impact the light response of both *wc-1* and *frq*, but do not impact the basal transcript levels of either. Clr-1 impacts the basal expression levels of *frq* and the light-response of *wc-1*.

Δ*sah-1* affects the basal levels of *frq*, but has no impact and *wc-1*. WC-2 impacts both the basal expression levels and light-response of *frq* and *wc-1*.

Cross-regulation of light-responsive transcription factors

Next we wanted to see how the loss of each of these light-responsive TFs impacted the transcript levels of the other TFs. First we looked at differential expression values of these TFs in wild type after exposure to light. Five of the TFs (*ncu00275, ncu09615, sah-1, vos-1,* and *wc-1*) had greater than 2-fold up-regulation in the wild-type strain in response to light. The upregulation of four of these five TFs (*ncu00275, ncu09615, vos-1,* and *wc-1*) was absent in both the Δ*wc-1* and Δ*wc-2* strains, indicating a requirement of the core clock's positive for proper induction of transcript levels of these TFs in response to light (Figure 7). Interestingly, *sah-1* was still upregulated in the Δ*wc-2* strain, but not the Δ*wc-1* strain. This may indicate control of transcript levels of *sah-1* by WC-1 that is independent of WC-2. In addition to the Δ*wc-1* strain, the Δ*ncu00275* and Δ*ncu09615* strains showed a lack of regulation of the previously mentioned five TFs (*ncu00275, ncu09615, sah-1, vos-1,* and *wc-1*). Further, the Δclr-1 strain showed no regulation of four of the 5 previously mentioned TFs (*ncu00275, ncu09615 vos-1,* and *wc-1*). This may indicate a role that these three TFs play with regards to proper up-regulation of transcript levels of light-regulated TFs in concert with *wc-1* and *wc-2* and that these three TFs are necessary for proper *wc-1* expression. Δ*csp-1* and Δclr-1 showed a loss of up-regulation of *ncu00275* and *ncu09615*, while the Δ*vos-1* showed a loss of up-regulation of only *ncu00275* (Figure 8). 5 of the 11 TFs were not light-responsive in wild type (clr-1, *csp-1, csp-2, sub-1,* and *wc-2*). Only one of the TFs (*adv-*

1) was observed to have a 2-fold down-regulation in response to light in wild-type (Figure 8, Table S10). This down-regulation was not present in any TF mutant strain, indicating that each of these TFs are necessary for proper regulation of *adv-1* following exposure to light. Taken together, these findings reveal a complex web of connections between the expression of light-regulated TFs and their effects on the transcript level expression of other light-regulated TFs that appear to be rooted in the positive arm of the core clock, *wc-1* and *wc-2* (Figure 8).

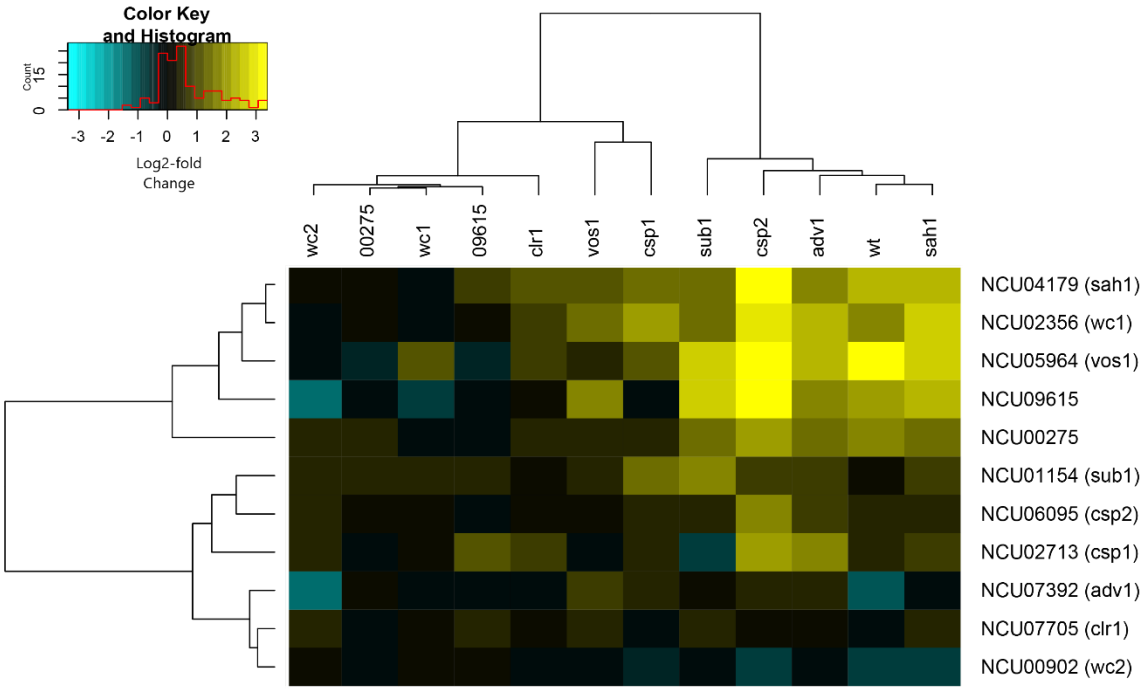

Figure 8: Heat map of transcript level changes for each transcription factor reviewed. Heat map of log2-fold changes in transcript levels for each of the 11 TFs reviewed here. Heat map was generated in R using heatmap.2. Clustering and dendrogram creation was done using Pearson method within R.

Light-responsive transcription factor effects on transcript levels in dark grown cultures

With the knowledge that these light-responsive TFs are responsible for the proper light-response of numerous genes, we wanted to see whether they are also responsible for basal expression (i.e. without light) in *N. crassa*. To do this we compared the expression of genes in the deletion strains to wild-type and removed any genes that appeared in either other light-regulation analysis. We found that a number of these TFs play a role in the production of ribosomal proteins and ribosome biogenesis in the dark. The Δclr-1, Δ*csp-2*, and Δ*ncu00275* strains all have ribosomal protein genes that are downregulated in the dark as compared to wild-type (Table S11). The Δclr-1 strain has 41 down-regulated genes, the Δ*csp-1* strain has 30 down-regulated genes, and the Δclr-1 strain has 22 down-regulated genes, all of which are ribosomal proteins (Figure 7). These down-regulated ribosomal protein genes total 60 ribosomal proteins, both mitochondrial and cytosolic, of the 91 annotation ribosomal proteins in *N. crassa*. Of these down-regulated ribosomal protein genes, there is some overlap, indicating that more than one of these TFs may act on single ribosomal protein genes. Importantly, there was a total of 48 ribosome biogenesis genes shown as down regulated in these three deletion strains. Of these 48 down-regulated genes, 44 of them were ribosomal proteins, indicating a different role these TFs play in ribosome biogenesis in the dark than was shown in our earlier light induction analysis.

Constitutive effects of light-responsive transcription factors on gene expression

The effects of these TFs appear to vary greatly between TFs with very little overlap between them. Further, changes in gene expression at both 0min and 60min

involves fewer genes than either 0min or 60min individually. Of note, most genes that are constitutively up-regulated in a deletion strain as compared to wild-type are involved in secondary metabolism processes, such as nitrogen metabolism, metabolism of amines, cellular respiration, or carbohydrate metabolism (Table S10, S11). The only notable up-regulated functional category that is altered in any of these TFs both in the dark and after exposure to 60min of light is ribosomal proteins in the Δclr-1 strain. There are 15 ribosomal protein genes that are up-regulated in the Δclr-1 strain, indicating a further role that clr-1 may play in the production of ribosomes. Similarly to the up-regulated genes, there are very few down-regulated functional categories. These down-regulated categories also vary greatly between transcript levels factors with most being involved in various metabolic pathways, such as carbon metabolism.

Discussion

We examined the consequences of deleting known light-responsive TFs on the *N. crassa* transcriptional light response. To do this we analyzed RNA-seq from wild type, Δ*adv-1*, Δclr-1, Δ*csp-1*, Δ*csp-2*, Δncu002785, Δ*NCU09615*, Δ*sah-1*, Δ*sub-1*, Δ*vos-1*, Δ*wc-1*, and Δ*wc-2* that were grown for 23hr then either exposed to 1hr of light or kept in the dark. DESeq2 and CuffDiff analyses were completed on all datasets to examine the effects of deletions of these TFs on basal levels of gene expression and the light response. We found that these light-responsive TFs have a small effect on expression of genes in the dark these, but all have roles in proper expression of multiple light-regulated pathways, components of the core clock, and each other.

Overall, our observed transcript-level changes in wild type after exposure to light were similar to those described by Wu et al (WU *et al.* 2014). Our analyses of the wild type light-response are based on the raw data obtained previously in our lab (WU *et al.* 2014). We found 1465 genes whose transcript levels were increased or reduced in response to light (FPKM ≥ 1, q ≤ 0.05, and log2-fold change ≥ 1). Wu et al reported 233 genes whose transcript levels were increased or reduced using q ≤ 0.05 as the cutoff and 415 genes using q ≤ 0.2 as the cutoff. The latter cutoff was used for Wu et al's detailed analyses of the wild type light response (WU *et al.* 2014). Wu et al reported that 2353 genes were light regulated (FPKM ≥ 1 and log2-fold change ≥ 1) when observed without using statistical cutoffs; our analysis revealed 2526 to be light regulated when similarly analyzed.

Two of the most interesting findings in our analysis were that NCU00275 and NCU09615 have critical roles in controlling the expression of genes whose transcript levels decrease in response to light. NCU00275 and NCU09615 cluster together in every functional analysis, both for positively and negatively regulated light-responsive genes. Also, importantly, both genes are needed for wild type light-regulated changes in transcript levels for the known light-responsive TFs examined here. Relatively little is known about NCU00275 or NCU09615, but some phenotypes of Δ*ncu09615* have been reported (CARRILLO *et al.* 2017). The Δ*ncu09615* strain has shorter hyphae and a slower growth rate as compared to wild type (CARRILLO *et al.* 2017). Further analysis of NCU09615 reveals that there is another gene in *N. crassa*, NCU02386, that shares 38.91% amino acid identity and 93% coverage with it (data not shown). This related

gene, which does not appear to be essential, is not light-responsive in wild type at the transcript level, nor is its expression affected by any of the TFs examined here.

The genes *wc-1* and *wc-2*, which specify the blue-light photoreceptors and compose the positive arm of the *N. crassa* core clock, have been extensively studied (SOMMER *et al.* 1989; LINDEN *et al.* 1997b; LEE *et al.* 2003; CHEN *et al.* 2009; HUNT *et al.* 2010; SMITH *et al.* 2010; HURLEY *et al.* 2015a). Previous studies have shown that *wc-1* is responsible for regulation of a majority of blue-light responses in *N. crassa* (LINDEN *et al.* 1997b; LEE *et al.* 2003; CHEN *et al.* 2009; SMITH *et al.* 2010) Our analysis revealed that 1101 of the 1465 light-regulated genes lose light-regulation in Δ*wc-1* and 986 lose light-regulation in Δ*wc-2*. A slight majority of genes that lose regulation are those that are normally up-regulated in light. WCC is known to bind the promoters of many genes, including the 11 light-regulated TFs examined in this study (SMITH *et al.* 2010; SANCAR *et al.* 2015a). Six of the 11 light-responsive TFs (*adv-1*, *ncu00275*, *ncu09615*, *sah-1*, *vos-1*, and *wc-1*) were observed to show transcript level up or down-regulation in response to light and all six lose light-regulation in Δ*wc-1* and Δ*wc-2*. Three of the remaining five TFs (*csp-1*, *csp-2*, and *sub-1*) show up-regulation at 15min of light exposure, but lose up-regulation after 60min of light exposure (WU *et al.* 2014). The remaining two TFs, clr-1 and *wc-2*, do not show a light response. Sancar et al reported 80 genes to be associated with *wc-2* using stringent cutoffs. Of these 80 genes, 56 that are reported to be associated *wc-2* are also light-regulated in our study (log2-fold ≥ 1). All 56 of these genes lost this regulation in either Δ*wc-1* or Δ*wc-2*.

Another finding of this study is that multiple TFs impact down-regulation of many different genes. We looked more closely at the impact these TFs have on down-regulation of genes involved in the rRNA processing, rRNA synthesis and ribosome biogenesis pathways (henceforth referred to as rRNA-related pathways) by performing a clustering analysis on genes which lost down-regulation in at least one ΔTF strain. This revealed that the TFs can be placed in four different groups based on their effects on down-regulation.

TF group 1 strains (Δ*wc-1*, and Δ*wc-2*, Δ*csp-2*, and Δ*sub-1*) were observed to be the most similar to wild type with respect to down-regulation of transcript level in rRNA-related pathways (Figure 3, 4, and 5). That Δ*wc-1* and Δ*wc-2* did not impact down-regulation of these genes in response to light, despite the dramatic loss of many up-regulated genes, is surprising (Table S6). This indicates that this light response may be regulated by a WCC-independent pathway.

TF group 2 strains (Δ*ncu00275*, Δ*ncu09615*, and Δ*csp-1*) were observed to lose down-regulation of all genes involved in rRNA-related pathways that were down-regulated in wild type. *csp-1* is known to function as a negative regulator (SANCAR *et al.* 2011), and our data indicate this is the case for this group of genes. Further, *csp-1* has been demonstrated to be needed for normal conidiation and to be involved in glucose-dependent feedback of the circadian clock (LAMBREGHTS *et al.* 2009; SANCAR *et al.* 2011; SANCAR *et al.* 2012; SANCAR *et al.* 2015b). Our data indicate that, like *csp-1*, NCU00275 and NCU09615 also have major roles in regulating the transcriptional light response, but their functions outside of the light-response remain to be elucidated.

TF group 3 strains (Δclr-1, Δ*sah-1*, and Δ*adv-1*,) showed a loss of down-regulation for approximately half of the genes in rRNA-related pathways and retained down-regulation for the other half. Outside of our data indicating their role in rRNA-related pathways, little is known concerning the roles of clr-1 and *sah-1* in the light response. Much more is known about *adv-1*. Dekhang et al performed RNA-seq on Δ*adv-1* and ChiP-seq for ADV-1 and demonstrated that *adv-1* is responsible for the proper expression of multiple functional categories of genes, including those necessary for cell fusion, in response to light (DEKHANG *et al.* 2017). While Dekhang et al reported changes in transcript levels, they did not discuss genes that lost light-regulation in Δ*adv-1*. Our data show that *adv-1* is important for down-regulation of genes involved in rRNA-related pathways.

TF group 4 contained a single gene: *vos-1*. The Δ*vos-1* mutant did not display simply loss of down-regulation. Paradoxically, nearly all down-regulated genes in the rRNA-related pathways became up-regulated in Δ*vos-1*. This suggests that *vos-1* either directly or indirectly plays a role in repressing the expression of these genes in response to light. Additionally, *vos-1* is established to have an important role in the expression of developmental and metabolic genes and its promoter is directly bound by WCC (CHEN *et al.* 2009; BONI *et al.* 2018). Studies have explored the role of *vos-1* and its activity in the Velvet complex, which contains VOS-1, VE-1, and VE-2 (SARIKAYA BAYRAM *et al.* 2019). Our data show that neither *ve-1* nor *ve-2* are transcriptionally light-responsive (Table S2) and that Δ*vos-1* does not affect expression of either gene at a basal level (Table S2). The *Aspergillus nidulans* homolog, vosA, is critical for proper control of

developmental genes and for conidiation in response to light (BAYRAM *et al.* 2016; PARK *et al.* 2017). We observed a loss of up-regulation of conidiation genes, including *con-8* (NCU09235) and *con-10* (NCU07325), and developmental genes, such as *eas* (NCU08457, also called *bli-7* and *ccg-2*), in Δ*vos-1* as compared to wild type (Table S3). The large changes in the transcriptome that occur in response to light would be expected to be accompanied by up-regulation of chromatin remodeling genes. Such changes are well established and WCC and SUB-1 have roles in the up-regulation of some chromatin remodeling genes in response to light (SANCAR *et al.* 2015a). Our data generally confirm the up-regulation of these previously described genes and, importantly, our analyses revealed a group of different chromatin remodeling genes that are down-regulated in response to light. This group of down-regulated chromatin remodeling genes loses regulation in Δ*wc-1* and Δ*wc-2*, but not Δ*sub-1*. This indicates that WCC is important for both up and down-regulation of chromatin remodeling genes, while *sub-1* is necessary for only the genes that are up-regulated.

Clustering analysis to examine the roles of TFs in up-regulation did not reveal consistent groups such as those observed for the rRNA-related pathways, except for *wc-1* and *wc-2* which impacted expression in every statistically significantly enriched functional category of genes that were up-regulated in wild type in response to light. Functional analysis of genes that lost up-regulation in at least one ΔTF strain revealed very few categories, the largest of which are (i) homeostasis of metal cations, (ii) metabolism of vitamins, cofactors, and prosthetic groups, and (iii) synthesis of vitamins, cofactors, and prosthetic groups (Table S7, S8). These groups have been reported to be

up-regulated in response to light in wild type (WU *et al.* 2014) and our analyses indicate that this is the case. Notably, Δ*ncu00275* and Δ*ncu09615* did not cluster together in the metabolism of vitamins, cofactors, and prosthetic groups analysis, which is the first time we have seen these TFs act very differently from each other.

We found that transcript levels for two of the core clock components, *frq* and *wc-1*, were impacted by the loss of one or more TFs. *frq* loses up-regulation in response to light in Δ*wc-1*, Δ*wc-2*, and Δ*ncu00275*. The loss of up-regulation in Δ*ncu00275* indicates that this TF is important for normal light-regulation of *frq*. The requirement of *wc-1* and *wc-2* for proper *frq* expression in response to light has been previously described, but the role of NCU00275 has not (CROSTHWAITE *et al.* 1995; LEE *et al.* 2003). Additionally, there was a log2-fold increase in FPKM of *frq* in Δ*ncu00275* in the dark, while Δ*ncu09615*, Δ*csp-1*, Δ*csp-2*, and Δclr-1 showed a decrease in expression of *frq* in the dark. Furthermore, we initially hypothesized that *frq* light-regulation might be impacted by clr-1, due to known binding sites for CLR-1 in *frq* (CRAIG *et al.* 2015). Although we did not see a complete loss of up-regulation of *frq* in response to light in Δclr-1 , we did see that the normalized expression of *frq* is approximately 3-fold lower in Δclr-1 than in wild type (both after 60min of light and in the dark) and its light induction is slightly reduced. CLR-1 has also been shown to directly bind and regulate the expression of many genes involved in cell wall and cellulose degradation (CORADETTI *et al.* 2012; CRAIG *et al.* 2015), which is also reflected in our RNA-seq analyses (Table S3).

The other known light-regulated component of the core clock, *wc-1*, also had altered transcript-level expression in multiple ΔTF strains. The light response of *wc-1* is lost in Δ*wc-2*, Δ*ncu00275*, and Δ*ncu09615*. The impact of Δ*wc-2* on *wc-1* expression in response to light has previously been described, but the roles of *NCU00275* and *NCU09615* for proper *wc-1* expression have not.

In summary, through the use of RNA-seq we have demonstrated the role 11 light-responsive TFs play on transcription in response to light in *N. crassa*. We have shown that a number of these TFs play a significant role in the proper expression of rRNA processing, rRNA synthesis, ribosome biogenesis, and chromatin remodeling genes in response to light. These 11 TFs were also shown to be involved in the proper expression of each other in a complex manner. Further, we have shown that *NCU00275* and *NCU09615* may play a very impactful role in the expression of core clock components, as well as other light-regulated TFs. Additional research into the role of *NCU00275*, such as searching for direct binding sites, may reveal a more detailed role that this TF plays in both the transcriptional light-response and proper function of the core clock.

CHAPTER III

THE EFFECTS OF SALT STRESS AND RCK-2 ON GLOBAL TRANSCRIPTION AND TRANSLATION LEVELS AS ASSESSED BY RNA-SEQ AND RIBO-SEQ

Introduction

The ability for organisms to adapt to their environment is important for survival and fitness. One such environmental factor that can impact an organism's ability to survive is salinity. In the filamentous fungus *Neurospora crassa* adaptation to osmotic stress is accomplished largely through the activation of the osmotic sensing (OS) pathway (ZHANG *et al.* 2002; JONES *et al.* 2007). This pathway is activated through a sensor histidine kinase (OS-1) detecting a change in the environment and sending a signal through a histidine phosphotransferase to RRG-1, which begins a cascade of mitogen activated kinases and their kinases; OS-4 (MAP kinase kinase kinase), OS-5 (MAP kinase kinase), and OS-2 (MAP kinase) (JONES *et al.* 2007). The activated MAP kinase OS-2 regulates transcription factors, translation factors, and other kinases (BARDWELL 2006; CHEN AND THORNER 2007; CASTER *et al.* 2016). One such example of a kinase regulated by OS-2 is radiation sensitivity complementing kinase-2 (RCK-2) (CASTER *et al.* 2016). The kinase RCK-2 is phosphorylated by OS-2 and is necessary for the phosphorylation of eukaryotic elongation factor 2 (eEF-2) following salt stress (TEIGE *et al.* 2001; BILSLAND *et al.* 2004; SWAMINATHAN *et al.* 2006; CASTER *et al.* 2016). Once eEF-2 is phosphorylated by RCK-2, translation is repressed (TEIGE *et al.* 2001; WARRINGER *et al.* 2010). Despite this knowledge, there are no published studies on either the effects of salt or RCK-2 on transcription or translation in *N. crassa*.

The yeast *Saccharomyces cerevisiae*, however, has been studied extensively regarding both transcriptional and translational salt response (POSAS *et al.* 2000; TEIGE *et al.* 2001; HOHMANN 2002; MELAMED *et al.* 2008; WARRINGER *et al.* 2010; WORLEY *et al.* 2015). Further, the salt response pathway in *S. cerevisiae* shares a number of strong similarities to that of *N. crassa* (POSAS *et al.* 2000; ZHANG *et al.* 2002; KRANTZ *et al.* 2006). The sensor histidine kinase in *N. crassa* (OS-1) is very similar to the sensor in *S. cerevisiae* (HOG1) (SCHUMACHER *et al.* 1997; ZHANG *et al.* 2002; KRANTZ *et al.* 2006) and both the OS pathway and HOG pathway act as the primary response system to outside environmental changes—most notably salt (POSAS *et al.* 2000; HOHMANN 2002; JONES *et al.* 2007). Unlike *N. crassa*, the translational and transcriptional osmotic stress response in *S. cerevisiae* has been shown to be tied to numerous different pathways. Msn2p and Msn4p are examples of two transcription factors whose regulation is tied to Hog1 (HOHMANN 2002). In Δ*msn2p* and Δ*msn4p* strains, there are a number of genes that show a reduced transcription in response to salt as compared to the wild-type (REP *et al.* 2000; HOHMANN 2002). These genes have overlap with Δ*hog1p* strain, but not complete overlap (REP *et al.* 2000; HOHMANN 2002). This indicates that multiple pathways are acting on transcriptional regulation of salt stress in *S. cerevisiae.*

Salt stress also has a significant impact on translation in *S. cerevisiae.* Polysome analysis showed that salt stress causes large changes in the translation of genes reliant on Rck2 and Hog1(BILSLAND *et al.* 2004; SWAMINATHAN *et al.* 2006; WARRINGER *et al.* 2010). Translationally active ribosomes are reduced and there is repression of a large class of osmoresponsive stress genes in a Δ*hog1* strain of *S. cerevisiae* as compared to

the wild-type (WARRINGER *et al.* 2010). Paradoxically, however, there are a number of genes that are translationally upregulated as well (MELAMED *et al.* 2008). This may indicate that the salt response is involved in both up- and down-regulation of numerous genes, some of which are directly regulated by the HOG pathway. In addition to Hog1, the MAP kinase *rck2* is required for normal protein synthesis under osmotic stress (BILSLAND *et al.* 2004). Just as in *N. crassa*, *rck2* is required for phosphorylation of eEF2, which, when phosphorylated by *rck2*, reduces translation (TEIGE *et al.* 2001; SWAMINATHAN *et al.* 2006).

The mammalian stress response also functions similarly to *N. crassa* and *S. cerevisiae*. The mammalian MAP kinase, p38, is structurally similar to *S. cerevisiae* and both genes fall under the MAP kinase protein family. Similarly to both *N. crassa* and *S. cerevisiae*, the mammalian osmotic response pathway is activated by external environmental stimuli, such as salt, heat shock, or UV (HAN *et al.* 1994; ROUSE *et al.* 1994). Mammalian p38 functions as a MAP kinase that is responsive to various stimuli (ROUSE *et al.* 1994; COHEN 1997). Further, p38 is involved in the activation of many transcription factors through phosphorylation (ZARUBIN AND HAN 2005). Microarray analysis has also shown that p38 is involved in the transcriptional regulation of many genes, including proinflammatory cytokines (ZER *et al.* 2007). Additionally, the classes of genes that are regulated by p38 depend on the stimulus being applied to the cells (FERREIRO *et al.* 2010). Asinomycin treatment of mouse embryonic fibroblasts resulted in a large number of up and down-regulated genes as compared to salt treatment (FERREIRO *et al.* 2010).

Despite the abundance of research that has been done on osmotic stress and *rck-2*'s role in translation elongation, very little is understood regarding global transcriptional and translational response of the deletion of *rck-2* or following salt stress. By exposing wild type cells to 4% NaCl for 30min following a 24hr growth period and performing RNA-seq and Ribo-seq on the cells, we can begin to elucidate both the transcriptional and translational response to salt in wild type. We can begin to determine the role of *rck-2* in translation with and without salt stress by exposing Δ*rck-2* to 4% NaCl for 30min, then performing RNA-seq and RIbo-seq on the cells. Then, through comparing RNA-seq and Ribo-seq data from salt stressed Δ*rck-2* to Δ*rck-2* grown in minimal media and comparing Δ*rck-2* to wild type grown in minimal media, we can begin to discern the role of *rck-2* in translation under, and independent from, salt stress.

Materials and Methods

Strains and culture conditions

The Fungal Genetics Stock Center (FGSC, Kansas State University, Manhattan, KS; http://www.fgsc.net) is the source for all *Neurospora crassa* strains used in this study, including Wild type (OR74A, FGSC #987) and Δ*rck-2* (FGSC #11545). The *rck-2* deletion strain was created by the *Neurospora* genome project. Conidia for inoculation of flasks for RNA-seq studies were generated as described (WU *et al.* 2014). Conidia for RNA-seq and ribosome profiling (Ribo-seq) experiments were harvested with 200ml sterile water and counted for concentration under a hemocytometer. For salt treatment, 200ml of Vogel's media (METZENBERG 2004) in a 500ml flask was inoculated to a final concentration of 10^7 conidia per ml. Cells were then grown for 24hr in constant light, at

30°C, shaking at 170rpm, and subsequently exposed to NaCl to a final concentration of 4% or not exposed to NaCl while still shaking at 170rpm at 30°C. Cells were collected by vacuum filtration. The mycelial pads were washed with sterile ice cold water, cut into ~100mg pieces using sterile razor blades, and were placed in 50ml falcon tubes and flash-frozen in liquid nitrogen. The flash-frozen pads were then stored at -80° C until used in RNA-seq and ribosome profiling library preparation.

RNA-seq and Ribo-seq protocols

Total RNA extraction, cDNA library preparation, sequencing library preparation, and sequencing for were conducted for wild-type and Δ*rck-2* as described (WU *et al.* 2014). Ribo-seq was completed as described (WEI *et al.* 2013).

Alignment and analysis of RNA-seq data

Illumina reads were aligned to FungiDB *Neurospora crassa* assembly 38 annotation using STAR version 2.7 (DOBIN *et al.* 2013). Output BAM files were then initially analyzed through the Cuffdiff program from the Cufflinks Suite version 2.2.1 (TRAPNELL *et al.* 2012) to obtain FPKM and differential expression (DE) values for each gene in each strain. This was done using the –b and –L options. The BAM files were also analyzed with edgeR version 3.26.5 in RStudio for a differential expression analysis that is considered to be more robust (ROBINSON *et al.* 2010). The resulting DE tables from edgeR and the FPKM tables from the Cufflinks suite were then combined to generate a master table with each FPKM and DE value shown by ncu number. This table was uploaded to a SQL database to be mined. Cross comparisons of wild-type and Δ*rck-2* with and without salt treatment were done in MySQL Server through a local

installation of MySQL Workbench. These lists were then compared through inner joining to obtain differences in differential expression between both strains and conditions. Once comparisons were done, differentially expressed genes between each strain and condition were analyzed through Functional Analysis using FungiFun (PRIEBE *et al.* 2015). Gene Ontology analysis was completed using Panther GO (MI *et al.* 2019).

Alignment and analysis of Ribo-seq data

Ribo-seq data were handled in a similar manner to RNA-seq data with one exception. Due to the enrichment of reads that are ribosome protected between 28 and 32 nucleotides in length, we selected for that length using Trimmomatic prior to any alignment and subsequent analysis (BOLGER *et al.* 2014).

Translation efficiency calculation methods

Translation efficiencies were calculated using the FPKM values from Cuffdiff. The calculation for determining the translation efficiency of all samples was as follows:

$$TE = (Ribo - seq) \div (RNA - seq)$$

The calculation for determining the fold-change in translation efficiencies between strains was as follows:

$$Fold\ change = (TE1) \div (TE2)$$

Where TE1 is the translation efficiency of the delta condition and TE2 is the translation efficiency of the wild type condition.

Results

Transcript level response to salt in wild type and Δrck-2 N. crassa

To see the effects of salt on *N. crassa*, we took wild type cells and grew them for 24 hours, then either exposed them to 4% NaCl for 1hr or not. The cells were then collected and RNA-seq libraries were prepared. All sequencing data were aligned to the FungiDB *N. crassa* genome annotation (release 38) using STAR. Normalized read counts (FPKMs) were calculated from the alignment files through the use of Cuffdiff from the Cufflinks package. From this we obtained highly correlated biological duplicate data sets for wild type and Δ*rck-2*, both exposed and not exposed to salt (Table S12). Differential expression values looking at salt for each strain and comparing basal expression levels for each strain were then obtained through the use of EdgeR. The FPKM and differential expression data were then compiled and analyzed through the use of SQL queries that pulled genes with specific statistical and differential expression value cutoffs.

To see the effects that just deleting *rck-2* has on transcription, we first looked for genes that were statistically significantly ($q \leq 0.05$) regulated at least 2-fold at the transcript level without exposure to salt in Δ*rck-2* as compared to wild type *N. crassa*. This gave us an idea as to which changes in transcript levels are the result of the deletion of *rck*-2 alone and which changes are the result of both salt stress and the deletion of *rck*-2. EdgeR analysis yielded no genes that were statistically significantly up or down-regulated between wild type and Δ*rck-2*. Notably, *rck-2* expression does not significantly change (Figure 9). This is likely due to the presence of reads in the 3' and 5' UTRs that

remain after knocking out *rck-2*. Importantly, this lack of change between *rck-2* and wild type indicates that any salt induced differences between Δ*rck-2* and wild type are not the result of the deletion of Δ*rck-2* itself, but rather due to differences occurring in response to salt.

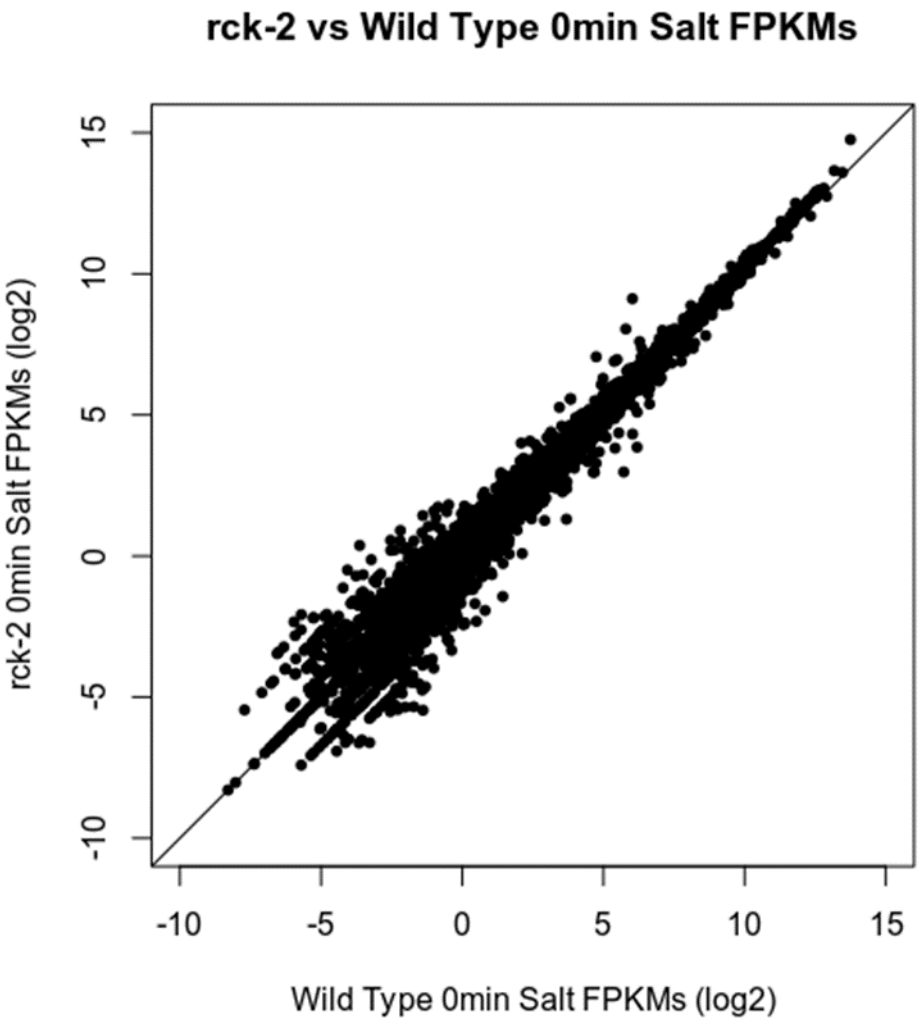

Figure 9: Scatterplot comparing FPKM values of Δ*rck-2* to wild type cells that were not treated with salt. Scatterplot was generated in R using ggplots. FPKM values were generated by normalizing read counts and distributions across each gene and the genome through the use of Cuffdiff and cummeRbund. FPKM values were log2-transformed prior to plotting. Ab line was used to place a "corner to corner" line.

We next wanted to test if salt stress induced a response in wild type *N. crassa*. To do this, we quantified for genes that were statistically significantly ($q \leq 0.05$) regulated at least 2-fold at the transcript level after exposure to salt. These cutoff values revealed 781 genes that were up-regulated and 162 genes that were down-regulated in response to salt (Figure 10, Table S13). To identify which biological functions were being impacted by salt at a transcript level, we did a functional category (FunCat) enrichment analysis on the genes that were up or down-regulated.

9 statistically significant FunCat categories were found that contained 235 of the 781 genes that were up-regulated in response to salt in wild type. The major categories found included (i) C-compound and carbohydrate metabolism (76/711 genes in this category), (ii) C-compound and carbohydrate transport (23/159), (iii) transport facilities (34/323), (iv) homeostasis of cations (10/54), and (v) pH stress response (5/9) (Table S14). Of the 162 genes that were down-regulated in response to salt at the transcript level, 82 were found in 21 statistically significant FunCat categories. Statistically significant categories that contained the largest number of down-regulated genes relative to other statistically significant categories included (i) rRNA processing (33/198), (ii) rRNA synthesis (16/76), (iii) rRNA modification (6/19), (iv) ribosome biogenesis (25/202), and (v) DNA damage repair (16/264) (Table S15).

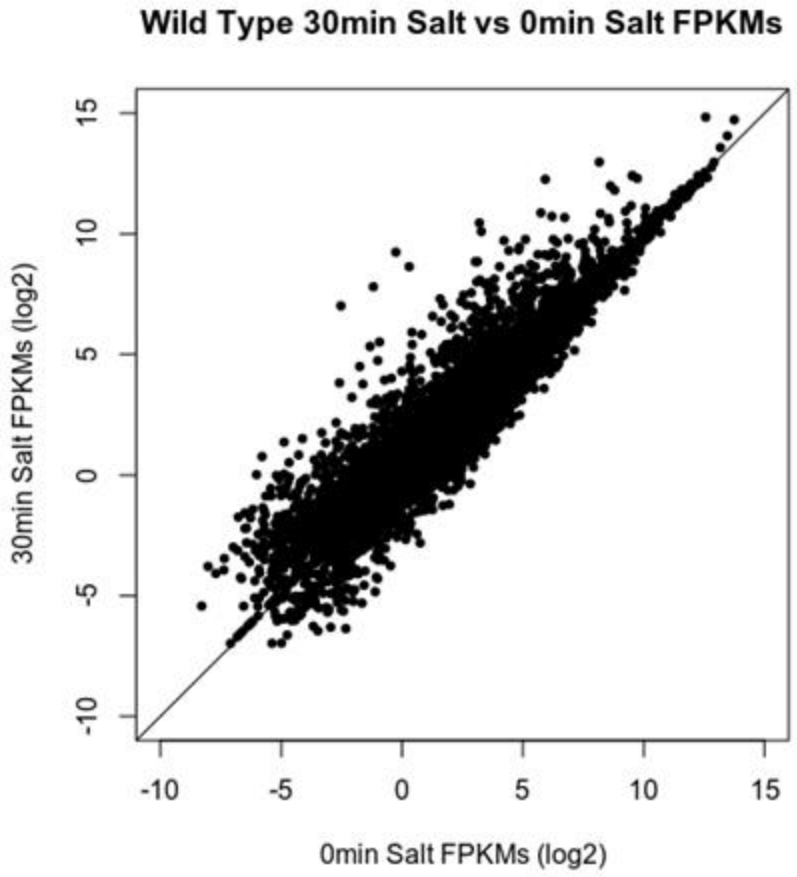

Figure 10: Scatterplot comparing FPKM values of salt treated and untreated wild type cells. Scatterplot was generated in R using ggplots. FPKM values were generated by normalizing read counts and distributions across each gene and the genome through the use of Cuffdiff and cummeRbund. FPKM values were log2-transformed prior to plotting. Ab line was used to place a "corner to corner" line.

We next wanted to assess how salt impacted transcription in Δ*rck-2*. To do this we looked for genes that were statistically significantly ($q \leq 0.05$) regulated as we did for wild type. This revealed 470 up-regulated genes and 37 down-regulated genes after exposure to salt (Figure 11, Table S16). FunCat analysis revealed two up-regulated

functional categories: (i) C-compound and carbohydrate metabolism (49/711) and (ii) protein antiporters (6/25) (Table S17). Both of these categories were also up-regulated in wild type after exposure to salt (Table S14). FunCat revealed 13 transcriptionally down-regulated categories. Important among these groups are (i) rRNA processing (11/198), (ii) rRNA synthesis (4/76), (iii) rRNA modification (3/19), (iv) ribosome biogenesis (10/202), and (v) DNA damage repair (6/264) (Table S18). All 5 of these categories were also down-regulated in wild type after exposure to salt (Table S15).

Next we looked to see transcript level differences between wild type and Δ*rck-2* after salt. To do this we compared the genes that were up or down-regulated in wild type to those that were up or down-regulated in Δ*rck-2*. 419 of the 781 genes found to be up-regulated in wild type were also found to also be up-regulated in Δ*rck-2* and 51 were unique to Δ*rck-2*. Of the 9 categories observed to be up-regulated in wild type, one was up-regulated in Δ*rck-2*. In Δ*rck-2*, some genes that were up-regulated in wild type were also up-regulated, but not as strongly (Table S13, S16). Again, others lost their regulation after exposure to salt entirely. Importantly, some genes that were observed in all statistically significant up-regulated categories in wild type were observed to be up-regulated in Δ*rck-2*, even though those categories were not considered to be statistically significant in the Δ*rck-2* analysis. This difference in up-regulated functional categories indicates that *rck-2* plays a role in the normal expression of these genes in response to salt.

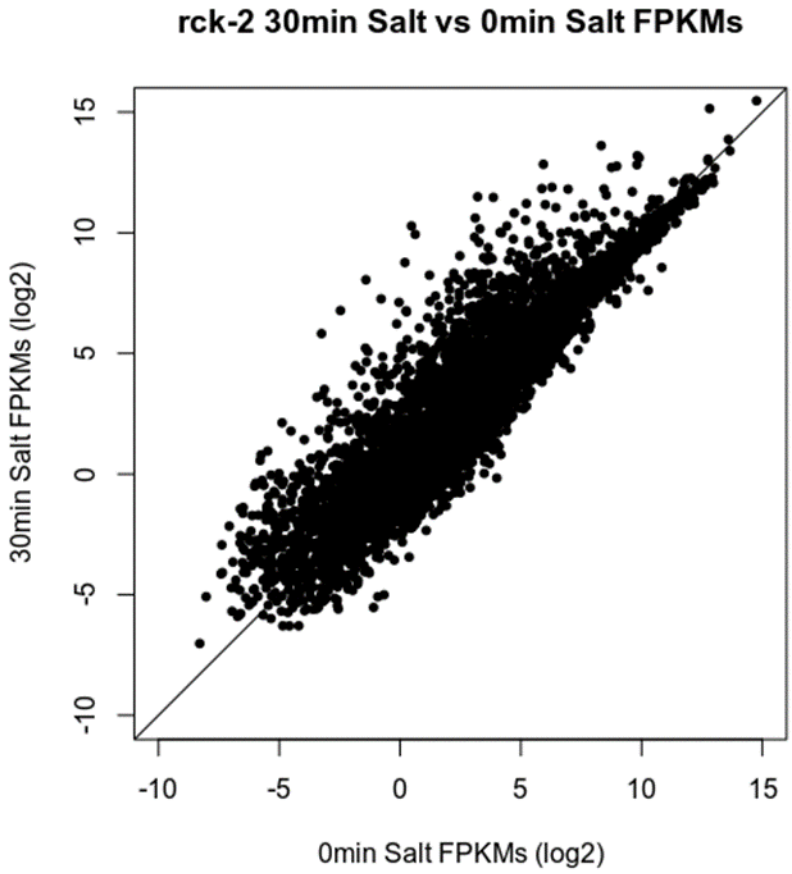

Figure 11: Scatterplot comparing FPKM values of salt treated and untreated Δ*rck-2* cells. Scatterplot was generated in R using ggplots. FPKM values were generated by normalizing read counts and distributions across each gene and the genome through the use of Cuffdiff and cummeRbund. FPKM values were log2-transformed prior to plotting. Ab line was used to place a "corner to corner" line.

Of the 162 down-regulated genes in wild type, 23 are also down-regulated in Δ*rck-2*. The remaining 14 of the 37 total down-regulated genes in Δ*rck*-2 were unique to Δ*rck-2*. In Δ*rck-2*, some genes that were down-regulated in wild type were observed to

be somewhat down-regulated. Others lost regulation entirely. Importantly, some genes that were observed in all statistically significant up-regulated categories in wild type were observed to be up-regulated in Δ*rck-2*, even though those categories were not considered to be statistically significant in the Δ*rck-2* analysis. The differences between the down-regulation of various categories in Δ*rck-2* indicate that *rck-2* plays a role in the production of rRNAs and ribosomes, as well as DNA repair in response to salt.

Translation level response to salt in N. crassa

Due to the role that both salt and *rck-2* have been shown to play on translation in *S. cerevisiae* and *N. crassa*, we next wanted to test the effects of both salt stress and the deletion of *rck-2* on translation in *N. crassa*. To do this, we took the cells analyzed by RNA-seq and performed standard Ribo-seq on them. All Ribo-seq sequencing data were then aligned and analyzed in the same manner as the RNA-seq data. The alignments and analysis yielded highly correlated duplicate datasets (Table S23). Phasing analysis (a process that allows us to see the frame of each ribosome in a Ribo-seq data set) of Ribo-seq data revealed that each data set has good phasing with reads peaking between 28 and 32 nucleotides in length. Additionally, independent gene prediction through the use of RiboCode was able to establish the presence of 6342 of the 9730 known protein coding genes in *N. crassa*. As with previous data, queries were handled in SQL and analysis was done independently of RNA-seq.

We first looked for genes in wild type that were statistically significantly ($q \leq 0.05$) regulated at least 2-fold at the translation level after exposure to salt. Changes observed with these cutoffs could be due to an increase in the amount of translated

mRNA, an increase in ribosome load on the mRNA, or both. These cutoff values revealed 460 genes that were up-regulated by Ribo-seq and 116 genes that were down-regulated in response to salt (Table S19). To identify which biological functions were being impacted in wild type by salt at a translational level, we did a FunCat analysis on the genes that were up or down-regulated.

FunCat analysis of up-regulated genes revealed three statistically significant categories: (i) C-compound and carbohydrate metabolism, (ii) pH stress response, and (iii) sugar, glucoside, polyol and carboxylate catabolism (Table S20). All of these categories were also transcriptionally up-regulated in response to salt. Importantly, a number of categories that were up-regulated as assessed by RNA-seq were not seen to be up-regulated as assessed by Ribo-seq. Further inspection of the data revealed that many genes had low read coverage in Ribo-seq but had high mRNA levels, contributing to differences in RNA-seq and Ribo-seq analyses. This lack of reads results in a low translation efficiency (TE), and consequently a loss of statistical significance in evaluating changes in gene expression.

FunCat analysis of down-regulated genes as assessed by Ribo-seq also revealed three statistically significant categories: (i) ori recognition and priming complex formation, (ii) rRNA processing, and (iii) rRNA modification, all of which were also transcriptionally down-regulated (Table S21). Similarly to the categories observed to be up-regulated by Ribo-seq, a number of categories were not down-regulated, but showed reduced mRNA levels. Again, this is due to low read coverage for multiple genes whose mRNA is down-regulated, resulting in low TE.

Next we repeated these analyses for Δ*rck-2*. This revealed 704 genes that were up-regulated and 92 genes that were down-regulated in response to salt (Table S22). FunCat analysis of up-regulated genes did not reveal any statistically significantly enriched FunCat categories. FunCat analysis of down-regulated genes revealed 14 categories (Table S23). Of these 14 categories, (i) DNA and (ii) rRNA processing were notable in that they were the only categories that were also transcriptionally down-regulated. The other 12 categories did not show down-regulation at the transcript level. Again, as with the wild type data, the Δ*rck-2* Ribo-seq data had very low read counts on many genes, resulting in low TE.

Effects of salt on translation efficiency in N. crassa

We next wanted to test how salt and *rck-2* impact TE in *N. crassa*. To do this we used the FPKMs from Cuffdiff to calculate translation efficiency ratios to test which genes showed changes in TE after exposure to salt or in the absence of *rck-2*. We first looked at genes whose mRNA levels have minimal change (less than 0.2-fold in either direction), but showed a 1.5-fold change in reads by Ribo-seq.

We found 159 genes that had an increase in TE, but did not have a change in mRNA levels, in wild type after salt (Figure 12, Table S24). FunCat analysis did not yield any statistically significantly enriched categories. As a result, we decided to run a gene ontology (GO) analysis to see if we could get at the biological functions being impacted by salt at a translational level. This yielded no statistically significant categories for genes that were impacted translationally by salt.

Next we looked for genes in wild type that had a reduction in TE, but did not have changes in mRNA levels in response to salt. We observed 313 genes that met these criteria (Table S24). Unlike the FunCat analysis of up-regulated genes, the FunCat analysis of down-regulated genes yielded three categories: (i) cell wall (21/262 total genes), (ii) budding, cell polarity, and filament formation (22/319), and (iii) polysaccharide metabolism (17/229) (Table S25).

We next wanted to test how Δ*rck-2* impacted translation in response to salt. Using the same cutoffs as previously mentioned, 225 genes were revealed to have increased TE in Δ*rck-2* (Figure 13, Table S15). FunCat and GO analysis did not result in any statistically significantly enriched categories. Additionally, we looked for genes in Δ*rck-2* that were translationally down-regulated genes but whose mRNA levels did not change in response to salt. We observed 286 genes that met these criteria (Table S26). Similarly to the up-regulated genes, FunCat analysis yielded no statistically significantly enriched categories.

Lastly, we looked at the impact that deleting Δ*rck-2* had on TE as compared to wild type. We accomplished this by comparing the TE values of wild type and Δ*rck-2* that were not exposed to salt. There were 210 genes that showed an increase in TE in the Δ*rck-2* strain as compared to wild type (Figure 14, Table S27). FunCat analysis revealed six categories that were up-regulated with no change in mRNA levels in response to salt. These categories were (i) polysaccharide metabolism (17/229 total genes in this category), (ii) respiration (10/105), (iii) proteins necessary for transposon movement (5/17), (iv) electron transport and membrane-associated energy conservation (10/121),

(v) extracellular polysaccharide degradation (5/34), and (iv) sugar, glucoside, polyol and carboxylate catabolism (10/191) (Table S28).

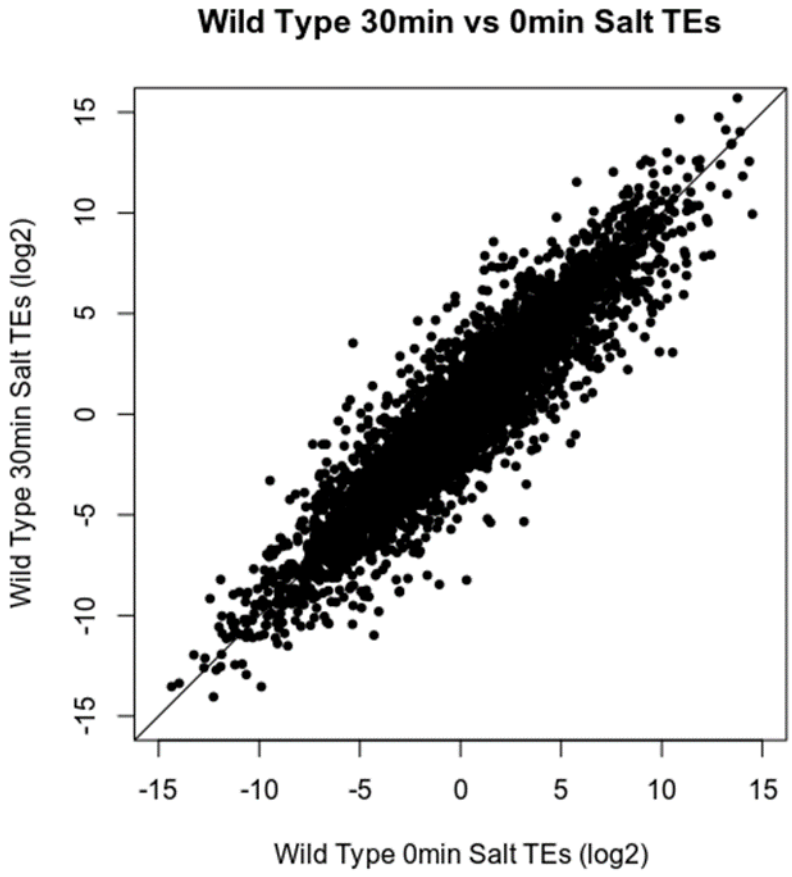

Figure 12: Scatterplot comparing TE values of salt treated and untreated wild type cells. Scatterplot was generated in R using ggplots. TE values were calculated as described previously using FPKM values from Cuffdiff. TE values were log2-transformed prior to plotting. Ab line was used to place a "corner to corner" line.

We next looked at translationally down-regulated genes in Δ*rck-2* as compared to wild type and found 185 genes (Table S27). FunCat analysis of these genes revealed one category that was translationally down-regulated in Δ*rck-2*, but not wild type: degradation / modification of foreign (exogenous) polysaccharides (4/26 total genes within this category) (Table S29).

Next we wanted to look at genes whose mRNA levels may have increased, but whose Ribo-seq levels increased further, or genes whose mRNA levels may have decreased, but whose Ribo-seq levels decreased further. To do this we looked only at genes whose FPKMs were greater than 1 in all datasets and had either a TE increase or decrease of 1.5-fold. This yielded 445 genes that had an increase in TE in wild type after exposure to salt (Figure 12, Table S30). Functional analysis of these genes revealed 7 categories: (i) ribosomal proteins (23/187 total genes within this category), (ii) electron transport and membrane-associated energy conservation (16/121), (iii) rRNA processing (20/198), (iv) ribosome biogenesis (20/202), (v) respiration (13/105), (vi) rRNA synthesis (10/76), and (vii) aerobic respiration (12/112) (Table S31).

This same analysis revealed 387 genes that had a decrease in TE (Table S20). FunCat analysis of these genes revealed 9 statistically significant categories: (i) cell wall (39/262), (ii) homeostasis of metal ions (Na, K, Ca etc.) (21/144), (iii) homeostasis of cations (11/54), (iv) polysaccharide metabolism (24/229), (v) transport ATPases (12/80), (vi) cation transport (H+, Na+, K+, Ca2+ , NH4+, etc.) (16/131), (vii) ion transport (8/48), (viii) chitin anabolism (5/19), and (ix) budding, cell polarity and filament formation (26/319) (Table S32). Interestingly, (i) cell wall, (iv) polysaccharide

metabolism, and (ix) budding, cell polarity and filament formation were all down-regulated with regards to both RNA-seq and Ribo-seq. This indicates that under salt stress, these categories are more strongly down-regulated with regards to Ribo-seq than RNA-seq, which may indicate a level of translational control in response to salt.

This analysis was then repeated for the Δ*rck-2* data. 909 genes, twice as much as in wild type, showed an increase in TE (Figure 13, Table S33). Funcat analysis revealed 10 statistically significant categories: (i) ribosome biogenesis (69/202), (ii) ribosomal proteins (58/187), (iii) rRNA processing (44/198), (iv) translation (43/292), (v) rRNA synthesis (18/76), (vi) RNA binding (51/343), (vii) rRNA modification (8/19), (viii) protein binding (180/1663), (ix) metabolism of primary metabolic sugar derivatives (4/5), (x) electron transport and membrane-associated energy conservation (22/121) (Table S34). All categories directly related to ribosome production are shared with wild type, but all other categories do not show statistically significant enrichment for an increase in TE. This indicates that these remaining 5 categories have an increase in TE exclusively in Δ*rck-2*.

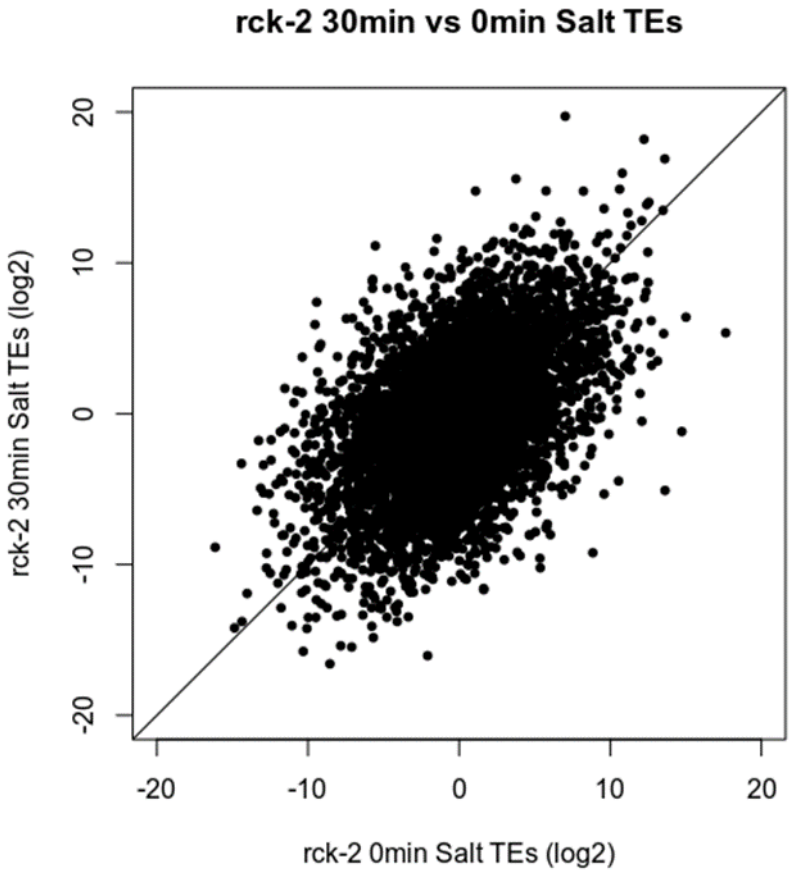

Figure 13: Scatterplot comparing TE values of salt treated and untreated Δ*rck-2* cells. Scatterplot was generated in R using ggplots. TE values were calculated as described previously using FPKM values from Cuffdiff. TE values were log2-transformed prior to plotting. Ab line was used to place a "corner to corner" line.

Next we looked for genes that show a decrease in TE in Δ*rck-2* in response to exposure to salt. 834 genes were observed to have a decrease in TE in response to exposure to salt (Table S33). FunCat analysis of these genes revealed 3 categories: (i) cell wall (65/262), (ii) modification with sugar residues (e.g. glycosylation,

deglycosylation) (18/66), and (iii) homeostasis of cations (15/54) (Table S35). Interestingly, only the cell wall category also showed a reduction of TE in wild type. Importantly, fewer genes within the cell wall category showed a decrease in TE in wild type (39) as compared to Δ*rck-2* (65). This may indicate a role for *rck-2* in cell wall processes under salt stress. Paradoxically, other categories that are associated with cell wall development ((i) budding, cell polarity and filament formation, (ii) polysaccharide metabolism, and (iii) chitin anabolism) do not show a reduction in Δ*rck-2*, but do show a reduction in wild type. This may mean that *rck-2* has a complex role that in translation of cell wall genes under salt stress.

Next, we wanted to see the effects deleting *rck-2* had on TE. To do this we repeated the analysis as done in wild type and Δ*rck-2* exposed to salt, but compared wild type and Δ*rck-2* TEs with no exposure to salt. This will allow us to see basal level changes that deleting *rck-2* would cause. The analysis yielded 71 genes that showed an increase in TE (Figure 14, Table S36). FunCat analysis of these genes revealed 8 statistically significant categories: (i) respiration (10/105), (ii) electron transport and membrane-associated energy conservation (10/121), (iii) proteins necessary for transposon movement (4/17), (iv) accessory proteins of electron transport and membrane-associated energy conservation (4/58), (v) aerobic respiration (5/112), (vi) Fe/S binding (3/43), (vii) FAD/FMN binding (4/93), (viii) electron transport (6/255) (Table S37). All of these categories are involved in cellular respiration and the electron transport chain. This may indicate a role that *rck-2* plays in proper expression of genes involved in the electron transport chain.

Lastly we looked for genes that showed a decrease in TE after deletion of *rck-2*. This yielded 98 genes that have a decrease in TE in the Δ*rck-2* strain as compared to wild type (Table S36). FunCat analysis did not yield any statistically significantly enriched categories.

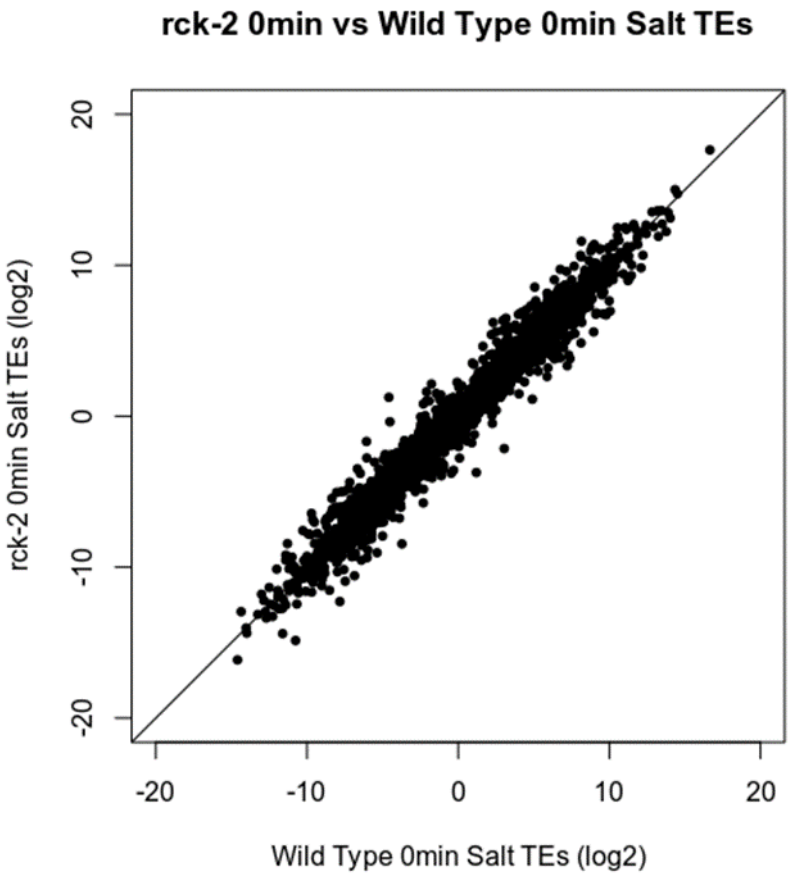

Figure 14: Scatterplot comparing TE values of untreated Δ*rck-2* to untreated wild type cells. Scatterplot was generated in R using ggplots. TE values were calculated as described previously using FPKM values from Cuffdiff. TE values were log2-transformed prior to plotting. Ab line was used to place a "corner to corner" line.

Discussion

The osmotic stress response pathway has been described in both *N. crassa* and *S. cerevisiae* (MAYS 1969; MISHRA 1977; HAN *et al.* 1994; ROUSE *et al.* 1994; DAVIS AND RISTOW 1995; POSAS *et al.* 2000; TEIGE *et al.* 2001; HOHMANN 2002; ZHANG *et al.* 2002; KRANTZ *et al.* 2006; LAMB *et al.* 2011; CASTER *et al.* 2016). In both organisms, a MAPK (mitogen activated protein kinase) pathway responds to salt stress. This pathway has been characterized as the OS pathway in *N. crassa* and the Hog pathway in *S. cerevisiae* (MAYS 1969; MISHRA 1977; ROUSE *et al.* 1994; SCHUMACHER *et al.* 1997; REP *et al.* 2000; ZHANG *et al.* 2002; BILSLAND *et al.* 2004; WARRINGER *et al.* 2010). As a result of the activation of this MAPK pathway in these fungi, RCK-2 is phosphorylated and activated in response to salt (TEIGE *et al.* 2001; BILSLAND *et al.* 2004; SWAMINATHAN *et al.* 2006; WARRINGER *et al.* 2010; LAMB *et al.* 2011; CASTER *et al.* 2016). In *N. crassa* and *S. cevervisiae*, RCK-2 has been demonstrated to be involved in the phosphorylation of the translation elongation factor EF2, as reduced phosphorylation of EF2 is seen in the absence of *rck-2* (TEIGE *et al.* 2001; BILSLAND *et al.* 2004; SWAMINATHAN *et al.* 2006; CASTER *et al.* 2016; WU *et al.* 2019). Further, in both organisms this phosphorylation event has been demonstrated to reduce translation (TEIGE *et al.* 2001; SWAMINATHAN *et al.* 2006; CASTER *et al.* 2016; WU *et al.* 2019). We examined the transcriptional and translational effects of salt stress, and the deletion of *rck-2* on transcriptional and translational responses to salt stress, in *N. crassa*. To do this we grew wild type and Δ*rck*-2 cells for 24hr, exposed them to 4% NaCl for 30min, and then analyzed these cells by RNA-seq and Ribo-seq. Analysis of these data revealed

multiple different pathways of genes impacted by salt and the absence of *rck-2* at either the transcriptional level, the translational level, or both.

Effects of salt on transcript-levels as assessed by RNA-seq

We first examined the effects of salt stress on transcript levels in wild type cells. These analyses revealed 781 genes have increased mRNA levels after salt stress ($q \leq 0.05$, log2-fold ≥ 1). FunCat analysis of these genes showed transcript-level increases for 10 categories, including general transport, cation homeostasis, sugar metabolism, and polysaccharide metabolism (Table S13, S14). In *S. cerevisiae*, genes that have increased mRNA levels after osmotic stress fall into 13 different categories, including the transporter facilitation superfamily and glycerol production. Glycerol production is a known osmotic stress response in *N. crassa* and *S. cerevisiae*, and genes regulating this pathway have increased mRNA levels after salt exposure in our analyses (ALBERTYN *et al.* 1994; YALE AND BOHNERT 2001). Thus, overall, categories of genes that show increases in mRNA levels are similar between *N. crassa* and *S. cerevisiae*. However, *N. crassa* lacks clear homologs of genes specifying transcription factors and pathway regulators involved in the *S. cerevisiae* salt response, including Hot1p, Pde2, and Msn2/4, indicating that, while the transcriptional responses are similar, different mechanisms must exist to enable them.

In contrast, *N. crassa* shows both differences and similarities to *S. cerevisiae* for genes that have decreased mRNA levels after 30min exposure to salt. Decreases in transcript levels of numerous classes of genes in response to salt have been described in *S. cerevisiae* (POSAS *et al.* 2000; REP *et al.* 2000). Genes involved in general translation,

amino acid metabolism, and sugar metabolism all show mRNA level decreases in response to 30min exposure to 3% salt in *S. cerevisiae*. Analyses of wild type *N. crassa* revealed 162 genes that show reduced mRNA levels ($q \leq 0.05$, log2-fold ≤ -1) in response to 30min exposure to 4% salt. FunCat analyses of these genes revealed 10 categories of genes, including those that have roles in rRNA synthesis and processing, ribosome biogenesis, and sugar metabolism (Table S15). We also saw a decrease in mRNA levels for genes involved in DNA synthesis and repair, which were not reported in S. cerevisiae (POSAS *et al.* 2000; REP *et al.* 2000).

Δ*rck-2* had a major impact on the transcriptional response to salt stress. Interestingly, when looking at the transcriptional consequences of deleting *rck-2*, we observed no statistically significant changes in any mRNA in cells that were not exposed to salt. This indicates that RCK-2 does not have a large role in determining transcript levels in cells grown in minimal media under these conditions, but, in striking contrast, has a significant role in the transcript-level salt response. This suggests that, although RCK-2 has been previously shown to be involved in translation, it may also have affects on genes that impact transcription, such as transcription factors or transcription factor regulators.

Our FunCat analyses revealed that far fewer genes and gene pathways show increases in mRNA in response to salt stress in Δ*rck-2* (470 genes ($q \leq 0.05$, log2-fold ≥ 1) and 2 categories) as compared to wild type (781 genes and 10 categories). Notably, genes involved in the categories of the general stress response, cation homeostasis, sugar metabolism, and polysaccharide metabolism are all absent in Δ*rck-2*, indicating the

presence of *rck-2* is necessary for the normal regulation of these genes in response to osmotic stress. Genome-wide transcript-level effects of deleting *rck-2* in *S. cerevisiae* as assessed by microarray has shown that up-regulation of many mRNAs following osmotic stress relies on the presence of RCK-2 (WARRINGER *et al.* 2010). Our findings in *N. crassa* are consistent with these findings in yeast and, thus, RCK-2 appears to have similar functions in these two organisms to control transcript levels in response to salt.

Does Δ*rck-2* also impact genes whose transcript levels decrease in response to salt stress? Analysis of *N. crassa* Δ*rck-2* revealed 37 genes ($q \leq 0.05$, log2-fold ≥ 1) and 13 FunCat categories to have reduced mRNA levels after exposure to salt. This is significantly lower than the 162 genes and 21 categories seen to have reduced mRNA levels in response to salt in wild type. Multiple genes in the sugar metabolism and polysaccharide metabolism categories are not down-regulated in Δ*rck-2*, which causes these categories to lose statistical significance. Importantly, the major categories that remain down-regulated in Δ*rck-2* at the transcript-level are involved in rRNA processing and synthesis, ribosome biogenesis, and DNA synthesis and repair. Interestingly, however, these categories that remain down-regulated have approximately half of the genes that show down-regulation in Δ*rck-2* as they do in wild type. Despite losing down-regulation in approximately half of the genes within those categories, there are enough genes to remain statistically significantly enriched. This indicates that *rck-2* has specific, strong impacts on some, but not all, genes in these categories.

Effects of salt on ribosome occupancy of mRNA as assessed by Ribo-seq

We next examined the effects salt stress in wild type and the deletion of *rck-2* (with and without salt stress) had on translation as assessed by Ribo-seq. We analyzed Ribo-seq data obtained from the same biological replicates of cells that were used for RNA-seq, then compared the normalized expression levels (FPKMs) obtained from RNA-seq data to the FPKMs of the Ribo-seq data to obtain translation efficiencies (TE) as previously described (see Materials and Methods). Then we looked for genes which had TE changes of 1.5-fold (either up or down) in response to salt.

This process revealed 445 genes that showed a TE increase (≥ 1.5-fold) in wild type *N. crassa* in response to salt revealed and FunCat analysis showed ribosome biogenesis, rRNA processing and synthesis, and ribosomal protein categories to have an increase in TE (Table S19, S20). Looking strictly at genes that were up-regulated ($q \leq 0.05$, log2-fold ≥ 1) by Ribo-seq (not by TE) in wild type in response to salt revealed stress response and sugar catabolism genes to be up-regulated. This is likely due to increases in mRNA as these categories are also up-regulated ($q \leq 0.05$, log2-fold ≥ 1) at the transcript level, and without TE calculations, Ribo-seq normalized expression should correspond with RNA-seq normalized expression if there is no translational regulation.

The translation level osmotic stress response has been described in *S. cerevisiae* through polysome profiling and quantifying the amount of polysomal mRNA for mRNAs with 2 or more ribosomes before and after salt stress (WARRINGER *et al.* 2010). Multiple genes are found through this method to be translationally induced in *S. cerevisiae* following osmotic stress including those in the categories of translation, the

general stress response, ribosome biogenesis, and protein catabolism (MELAMED *et al.* 2008). Of these categories, only ribosome biogenesis was observed to be translationally induced in response to salt in wild type *N. crassa*.

We next wanted to see what genes have a reduction in TE in response to salt in wild type *N. crassa*. FunCat analyses of the 313 genes that had a reduced TE in wild type in response to salt stress revealed three categories of genes: (i) cell wall, (ii) budding, cell polarity, and filament formation, and (iii) polysaccharide metabolism. Looking strictly at genes that were down-regulated by Ribo-seq ($q \leq 0.05$, log2-fold ≤ -1) in wild type in response to salt revealed rRNA synthesis and processing genes to be down-regulated. Again, this is likely due to decreases in mRNA as these categories are also down-regulated ($q \leq 0.05$, log2-fold ≤ -1) at the transcript level. The effect of osmotic stress to reduce translation has also been described in *S. cerevisiae* through polysome analysis (WARRINGER *et al.* 2010). Genes involved in the cell wall (also seen in *N. crassa*), translation, energy production, amino acid catabolism, and ion homeostasis are translationally repressed in *S. cerevisiae* following salt stress (MELAMED *et al.* 2008). Interestingly, no overlap exists between the functional categories discovered to be down-regulated in *S. cerevisiae* and *N. crassa.* This may indicate that repression of translation is quite different between these two organisms when they are exposed to osmotic stress.

While it was established that EF2 phosphorylation in response to salt is reduced in Δ*rck-2*, the downstream consequences of the deletion of *rck-2* is not very well understood in *N. crassa*. Polysome analysis of S. cerevisiae *rck2*Δ after 6min of osmotic stress demonstrated that the loss of this gene caused a reduction in polysomal mRNA of

genes involved in respiration, carbohydrate metabolism, ribosome biogenesis, and general transport machinery (WARRINGER *et al.* 2010). Our analyses, performed on *N. crassa* after 30min of osmotic stress, revealed a substantially larger number of genes to be translationally up-regulated in Δ*rck-2* (902 genes) as compared to wild type (445 genes). These changes are very different than what has been reported in *S. cerevisiae* (WARRINGER *et al.* 2010), but the results, unlike those for wild type cells at 30min of salt stress (MELAMED *et al.* 2008), cannot be directly compared because the translational response of *rck2*Δ was analyzed after 6min of salt exposure.

FunCat analysis of the 902 genes with increased TE in Δ*rck-2* revealed that categories of genes involved in rRNA production, ribosome biogenesis, and sugar metabolism all have an increase in TE in response to salt in the Δ*rck-2* mutant. Interestingly, we found that 59 of the 187 genes in the ribosomal protein category have reduced TE in Δ*rck-2* in response to salt. Examination of the 59 genes reveals that 30 of them are annotated as true ribosomal proteins, both mitochondrial and cytoplasmic, and the other 29 have roles in ribosome biogenesis. The dramatic changes we see at the level of translation when comparing wild type and Δ*rck-2* after 30min of osmotic is consistent with *rck-2*'s involvement in the translational changes in the *N. crassa* salt response. Due to the role of RCK-2 on EF2 phosphorylation, the increased TE observed for these genes could mean they are less well translated when EF2 is phosphorylated.

Analysis of ribosome associated transcripts has shown that *rck2* is important for reduction of specific polysomal mRNAs following salt stress in *S. cerevisiae* (WARRINGER *et al.* 2010). TE analysis of *N. crassa* Δ*rck-2* revealed 98 genes to have a

reduction in TE following osmotic stress. This is significantly fewer than the number of genes (313) with reduced TE in wild-type following osmotic stress, which indicates a potential role of *rck-2* in the reduction in translation of mRNA following osmotic stress in *N. crassa*. FunCat analysis of these genes did not reveal any statistically significantly enriched categories. This indicates that *rck-2* is required for proper repression of genes involved the cell wall and polysaccharide metabolism.

RCK-2 has been demonstrated to be a kinase that is phosphorylated at the end of the OS pathway in response to salt stress. It then phosphorylates eEF-2, which represses translation. We hypothesized that this RCK-2-related change in translation may be impacting the synthesis of transcription factors important for the salt response and, as a result, impact the transcription of genes specifically involved in this response. To determine the effects of Δ*rck-2* on translation of these transcription factors we looked to see how translation efficiency changes in this strain in response to salt. Numerous potential and known transcription factors have been identified or characterized in *N. crassa* (CARRILLO *et al.* 2017). Our analysis revealed that 24 identified transcription factors have greater than a 1.5-fold increase in ΔTE in the Δ*rck-2* samples as compared to the wild type samples, while 31 have a greater than 1.5-fold decrease in TE as compared to wild type. One of these transcription factors, NCU07430, encodes MCM1, the homolog in *S. cerevisiae*, and is directly involved in rRNA synthesis and processing. These changes in the translation of various transcription factors may account for changes that are seen in the transcriptional salt response between wild type and Δ*rck-2*.

CHAPTER IV

THE CONSTRUCTION OF A BIOINFORMATICS PIPELINE FOR *Neurospora crassa*

Development of a bioinformatics pipeline for *Neurospora crassa*

RNA-seq and Ribo-seq are both well-developed methods with diverse toolkits that allows for analysis of data obtained from them (INGOLIA *et al.* 2009; WANG *et al.* 2009; INGOLIA *et al.* 2012; INGOLIA *et al.* 2013). RNA-seq and Ribo-seq have been previously performed on *N. crassa*, but the methodologies used for the analysis of the raw RNA-seq and Ribo-seq data have changed over time (WANG *et al.* 2009; INGOLIA *et al.* 2012). I have developed a system and pipeline that allows for the analysis of RNA-seq and Ribo-seq data that has been tailored for use on *N. crassa* next-generation sequencing (NGS) data.

Construction and deployment of the server used for NGS analysis

A system has been configured using Ubuntu 18.01 (Bionic Beaver Server, LTS) as the operating system for the base of the informatics core. The system is currently running a RAID 6 configuration for data backup and creates additional system backups using Backintime. This system has been built with the latest R version from CRAN and is designed to automatically update directly from CRAN when standard system updates are ran. RStudio has been installed to allow for a graphical user interface (GUI) when producing images, such as graphs, and to allow for interactive graphing through the use of Plot.ly. This allows us to see and modify graphs and images produced in R before exporting them to pdf, jpg, tif, or png format. Further, Plot.ly allows us to look at

specific genes or subsets of data on various plot types, such as scatter plots, without the need for data frame manipulation. Another benefit of RStudio is the ability to write scripts within the R environment and execute them without losing what was written. This allows for easy troubleshooting of scripts while performing analyses. Lastly, RStudio allows us to save environments so that data can be returned to even after the system has been restarted or a user has left the console. Java has been installed directly from Oracle and, like R, is designed to be automatically updated with system updates.

Anaconda 2.7 and 3.5 have been installed for use as the Python program repositories. The benefits of Anaconda for Python package distribution are many. Most notably is that it normalized languages across installation environments so that programs, algorithms, and scripts that utilize R, Python, or even Perl, can be utilized. Additional repositories have been added to Anaconda, one of which includes Bioconda (GRUNING *et al.* 2018). Bioconda allows for the installation of over 3,000 different packages from various languages, such as Python, R, Perl, and Java. Due to its flexibility and diversity in packages, it has become a staple in the bioinformatics world, garnering well over 6 million downloads (GRUNING *et al.* 2018). Bioconda works in concert with conda-forge to maintain and distribute packages through Anaconda. Together, these repositories allow for the installation of many biology-specific tools and algorithms, such as BWA (LI AND DURBIN 2009) or Bowtie (LANGMEAD *et al.* 2009).

Alignment algorithms and their uses

Multiple tools have been installed that allow for the analysis of RNA-seq and Ribo-seq data. The aligners installed on this system are Bowtie (LANGMEAD *et al.* 2009),

Bowtie2 (LANGMEAD AND SALZBERG 2012), Tophat2 (TRAPNELL *et al.* 2009), HISAT2 (KIM *et al.* 2015), and STAR (DOBIN *et al.* 2013). All of these aligners have been installed from their respective online repositories. Of these aligners, STAR and HISAT2 are the most commonly used, with STAR seeing the most use.

Each of these aligners has different benefits and drawbacks with regards to the system resource requirements, time, and accuracy of the alignment algorithm (BARUZZO *et al.* 2017). Important to note, however, is that Tophat2 is built on Bowtie and Bowtie2, thus their alignment speeds and accuracies are tied to one another (TRAPNELL *et al.* 2009). STAR and HISAT2 have the fastest run times, while Tophat2 has the slowest (BARUZZO *et al.* 2017). To account for reduction in run times for STAR and HISAT2 alignments, both algorithms are very resource intensive and require a large amount of random access memory (RAM) to store alignment output files as they are produced, while Tophat2's RAM usage is not as high (TRAPNELL *et al.* 2009; DOBIN *et al.* 2013; KIM *et al.* 2015; BARUZZO *et al.* 2017). Of the aligners, STAR is the most accurate, with HISAT2 trailing very close behind, and Tophat2 having a much larger percentage of mismapped reads.

In addition to time, resource utilization, and accuracy, file outputs are different between the aligners. HISAT2 generates minimal output data unless specified, and the primary output alignment file produced is a SAM file, which requires refinement before it can be used in analysis (KIM *et al.* 2015). Tophat2 produces a BAM file of mapped reads and unmapped reads, and a text file detailing the run with regards to mapping and multi-mapping percentages (TRAPNELL *et al.* 2009). STAR is the most comprehensive,

producing ready-to-use BAM files, mapping and multi-mapping percentages, and a log file containing read counts per gene in the annotation used in the alignment, which is very useful for differential expression analysis (DOBIN *et al.* 2013).

Multiple tools have been deployed to handle alignment files (SAM/BAM). As mentioned, HISAT2 produces alignment files (SAM) that need refinement. Samtools allows us to do that refinement in that we can convert SAM files to BAM files and sort those BAM files for use in differential expression analysis and visualization of mapped reads. Further, Samtools allows us to index BAM files, which is necessary to view them on the Broad's Integrated Genome Viewer (IGV). IGV, which has been installed through the Debian, allows us to visualize alignment files, such as BAM files, BedGraphs, or BigWig files. This allows us to confirm knock-outs, look at general read distributions, and, in the case of Ribo-seq, observe if reads "pile-up" in specific regions of a gene.

In addition to Samtools, the UCSC Kentutils toolkit has been deployed (KUHN *et al.* 2013). This allows for the manipulation of alignment files to other visual formats, such as BedGraphs or BigWig files. Further, these utilities allow us to manipulate annotations files (gtf, gff, or bed). We can take one annotation file type and convert it into another. This is highly useful as some algorithms require one type of annotation file over another due to how the annotation information is stored in the file.

Read normalization and differential expression analysis

Multiple tools and algorithms are installed through the Debian and R (Bioconductor) that allow for differential expression analysis, normalized read counts (RPKM or CPM), and isoform prediction. The primary tools utilized on this system

include the Cufflinks Suite (TRAPNELL *et al.* 2012), CummeRbund (TRAPNELL *et al.* 2012), DESeq2 (LOVE *et al.* 2014), and EdgeR (LOVE *et al.* 2014). The Cufflinks Suite is the only differential expression tool installed using the Debian. The remaining four tools have been downloaded to R through RStudio using Bioconductor. Bioconductor is a repository of R-based biology-related tools (GENTLEMAN *et al.* 2004). Like Bioconda, Bioconductor has become the go-to source for biology-related and NGS-related tools and algorithms in the R language environment.

The Cufflinks Suite has been deployed to allow for normalized read counts (FPKMs) through the command line (TRAPNELL *et al.* 2012). The Cuffdiff command in the Cufflinks Suite allows for the determination of both FPKMs and differential expression. The use of a second R-based program, CummeRbund (TRAPNELL *et al.* 2012), allows for the utilization of Cufflinks output data to determine statistically significantly differentially expressed genes and to plot basic graphs of those data. The Cufflinks Suite, however, is not the most accurate differential expression algorithm currently available (SEYEDNASROLLAH *et al.* 2015). That being said, Cufflinks requires relatively little input as compared to DESeq2 or EdgeR, as read count tables or SummarizedExperiments (a R object generated from an annotation file and BAM file containing read counts) are not required for analysis.

Similarly to Cufflinks and CummeRbund, EdgeR (ROBINSON *et al.* 2010) allows for both differential expression analysis and the output of normalized read counts in the form of RPKMs or CPMs. This algorithm utilizes read count tables (as reads per gene) to perform its normalization, meaning additional grooming of the data is required before

analysis can be performed. Despite requiring more work to complete, EdgeR is substantially more accurate in differential expression analysis than Cufflinks/CummeRbund (SEYEDNASROLLAH *et al.* 2015) and, as a result, is more commonly used in our studies.

In contrast, DESeq2 (LOVE *et al.* 2014) allows for a robust differential expression analysis through normalized read counts using read count tables, but does not generate the normalized read counts (such as FPKMs or RPKMs) that Cufflinks or EdgeR produce as an output file. Similarly to EdgeR, DESeq2 requires read count tables, but has the ability to utilized SummarizedExperiments in R to generate said table directly from input BAM files. The accuracy and performance of DESeq2 is very similar to EdgeR (SEYEDNASROLLAH *et al.* 2015), and, as a result, this algorithm is also commonly used in our analyses.

Galaxy deployment and Ribo-seq analyses

A docker installation of Galaxy has been deployed. The Galaxy instance is a close copy of RiboGalaxy and utilizes their docker Galaxy package (MICHEL *et al.* 2016). In this Galaxy instance there are many tools that allow for in-depth analysis of Ribo-seq data. RiboTools allows for phasing and frame analysis, codon density analysis, and stop codon readthrough event detection (LEGENDRE *et al.* 2015). Ribocount determines read counts for all annotated genes. Riboplot gives A-site maps for specific annotated genes (MICHEL *et al.* 2016). GWIPs-viz allows for A-site mapping of Ribo-seq data that was either treated with MNase (3’ A-site mapping) or RNase 1 (5’ A-site mapping) (MICHEL *et al.* 2016). Further, aligners, such as Bowtie and Tophat2, and

differential expression analysis algorithms, such as DESeq2 and RiboSeqR, have been deployed on this Galaxy instance.

CHAPTER V

CONCLUSIONS

The results presented in this dissertation provide insight into the light and salt response of *Neurospora crassa*. I have demonstrated that 11 known, light-responsive transcription factors whose promoters are directly bound by WCC are involved in the transcript-level light-response of numerous pathways, including (i) rRNA processing, (ii) rRNA synthesis, (iii) ribosome biogenesis, (iv) chromatin remodeling, (v) homeostasis of metal ions, (vi) metabolism of vitamins, cofactors, and prosthetic groups, and (vii) synthesis of vitamins, cofactors, and prosthetic groups. Further, the roles of NCU00275 and NCU09615 in the transcriptional light response have been explored, showing that these transcription factors are necessary for transcript-level changes seen in many gene categories and in genes of the core clock in response to light.

The transcript and translation-level salt response of *N. crassa* has also been explored through RNA-seq and Ribo-seq analysis. I have demonstrated the major transcript and translation level changes that occur in response to salt in wild type *N. crassa* and how these findings relate to current *Saccharomyces cerevisiae* data. Salt stress causes a decrease in transcript levels of genes involved in general transport, cation homeostasis, sugar metabolism, and polysaccharide metabolism, while causing an increase in genes involved in rRNA synthesis and processing, ribosome biogenesis, and sugar metabolism. The translation-level impacts of salt stress were on categories of genes including those involved in ribosome biogenesis, rRNA processing and synthesis, and ribosomal proteins. These categories saw an increase in TE in response to salt stress.

Salt stress also caused decreases in TE in genes involved in the cell wall, budding, cell polarity, and filament formation, and polysaccharide metabolism.

The roles of RCK-2 in transcription and translation, both with and without salt stress, have been explored. RCK-2 has been demonstrated to be involved in transcript level changes in response to salt in that it is required for proper down-regulation of transcript levels of numerous genes following salt stress. Further, RCK-2 is important for translation-level changes in response to salt stress. Numerous genes involved in ribosome biogenesis, rRNA synthesis and processing, and the cell wall are all translationally up-regulated (by TE) in Δ*rck-2*, indicating that RCK-2 is necessary for repressing these genes in response to salt stress. Interestingly, I found that RCK-2 is not involved in basal transcript levels in the absence of salt, but is involved in the reduction of translation of genes involved in respiration and the electron transport chain.

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APPENDIX A

SUPPLEMENTARY TABLE INDEX

Table 1: Index of all supplementary tables and their contents.

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Table S14	FunCat data for genes that show at least one log2-fold up-regulation in wild type after exposure to salt.
Table S15	FunCat analysis of all genes that show at least one log2-fold down-regulation of mRNA levels in response to salt in wild type.

Table S16	Calculated CPM and differential expression data from EdgeR for all genes that show at least one log2-fold up or down-regulation in mRNA levels in the rck-2 deletion strain in response to salt.
Table S17	FunCat analysis of all genes that show up-regulated of mRNA levels after exposure to salt in the rck-2 deletion strain.
Table S18	FunCat of all genes that show at least one log2-fold down-regulation of mRNA levels in the rck-2 deletion strain in response to salt.
Table S19	Calculated CPM and differential expression data for all genes that show at least one log2-fold increase by Ribo-seq in response to salt in wild type.
Table S20	FunCat analysis for all genes that show up-regulation by Ribo-seq in response to salt in wild type.
Table S21	FunCat analysis of all genes that show at least one log2-fold down-regulation by Ribo-seq in response to salt in wild type.
Table S22	Calculated CPM and differential expression data from EdgeR for all genes that show at least one log2-fold up or down-regulation in response to salt in the rck-2 deletion strain.
Table S23	FunCat analysis of all genes that showed at least one log2-fold down-regulation by Ribo-seq in response to salt in the rck-2 deletion strain.
Table S24	Normalized read counts (FPKM) and calculated TE ratios for all genes that show either 1.5 log2-fold increase or decrease in TE after exposure to salt in wild type.
Table S25	FunCat analysis of all genes that showed a > 1.5 log2-fold reduction in TE after exposure to salt in wild type.
Table S26	Normalized read counts (FPKM) and calculated TE ratios for all genes that show either 1.5 log2-fold increase or decrease in TE after exposure to salt in Δrck-2.
Table S27	Normalized read counts (FPKM) and calculated TE ratios for all genes that have an increase or decrease in TE of 1.5-fold and FPKM>1 for RNA-seq and ribo-seq after exposure to salt in Δrck-2.
Table S28	FunCat analysis of all genes that showed an increase in TE of 1.5-fold and FPKM>1 for RNA-seq and ribo-seq after exposure to salt in Δrck-2.
Table S29	FunCat analysis of all genes that showed a decrease in TE of 1.5-fold and FPKM>1 for RNA-seq and ribo-seq after exposure to salt in Δrck-2.
Table S30	FPKM and TE data for all genes which have an increase or decrease in TE of greater than 1.5 and FPKMs>1 for RNA-seq and ribo-seq in response to salt in wild-type.
Table S31	FunCat analysis of all genes that have an increase in response to salt expression as determined by TE ratios of greater than 1.5 for RNA-seq and ribo-seq FPKMs >1 in wild-type.

Table S32	FunCat analysis of all genes which have a decrease in TE of 1.5-fold for FPKM>1 for RNA-seq and ribo-seq for wild-type in the presence versus

	absence of salt.
Table S33	FPKM and TE data for all genes which have an increase or decrease in TE of 1.5-fold for FPKM>1 for RNA-seq and ribo-seq for rck-2 in the presence versus absence of salt.
Table S34	FunCat analysis for all genes which have an increase in TE of 1.5-fold for FPKM>1 for RNA-seq and ribo-seq for rck-2 in the presence versus absence of salt.
Table S35	FunCat analysis of all genes that have a decrease in TE of 1.5-fold for FPKM>1 for RNA-seq and ribo-seq for rck-2 in the presence versus absence of salt.
Table S36	FPKM and TE data for all genes which have an increase or decrease in (rck-2 TE)/(wt TE) of 1.5-fold for FPKM>1 for RNA-seq and ribo-seq in both rck-2 and wild-type with no exposure to salt.
Table S37	FunCat analysis of all genes which have an increase in (rck-2 TE)/(wt TE) of 1.5-fold for FPKM>1 for RNA-seq and ribo-seq in both rck-2 and wild-type with no exposure to salt.