IDENTIFICATION OF CLOCK-CONTROLLED CPC-3 PHOSPHORYLATION SITES NECESSARY FOR RHYTHMIC P-EIF2α ACTIVITY IN NEUROSPORA CRASSA

A Thesis

by

JANA MARIE GOMEZ

Submitted to the Office of Graduate and Professional Studies of
Texas A&M University
in partial fulfilment of the requirements for the degree of

MASTER OF SCIENCE

Chair of Committee, Deborah Bell-Pedersen
Committee Members, Matthew Sachs
Daniel Ebbole
Head of Department, Thomas McKnight

MAY 2021

Major Subject: Microbiology

ABSTRACT

The circadian clock is an intrinsic time-keeping mechanism that is conserved between many species, ranging from cyanobacteria to mammals. Due to circadian clock conservation between organisms, understanding how the clock functions in a model organism can give us insights into the human circadian clock and how it impacts our health. It is known that the circadian clock regulates multiple biological pathways by controlling rhythmic gene expression at the transcriptional level. However, recent evidence in fungi and mammals suggested that translation is also regulated by the clock. In the clock model organism *Neurospora crassa*, the translation initiation factor eIF2α is rhythmically phosphorylated by the kinase CPC-3. The phosphorylation of eIF2α (P-eIF2α) peaks during the subjective daytime, and rhythmic P-eIF2α accumulation requires clock control of CPC-3 activity. My project involves investigating a potential mechanism of *N. crassa* CPC-3 activation that may be regulated by the clock. Specifically, I am examining three predicted CPC-3 autophosphorylation sites that may control its activity and that have been shown to have a significant time-of-day difference in constant dark conditions (T874, T879, and S238). I created two point mutations for each site, one that blocks phosphorylation at the site (converted to alanine) and one that mimics constant phosphorylation at the site (converted to glutamic acid). Mutations mimicking constant phosphorylation at T874 and S238 did not significantly alter the rhythms of P-eIF2α. However, removing phosphorylation and mimicking constant phosphorylation at T879 completely inhibited the phosphorylation of eIF2α. Furthermore, there is no P-

eIF2α present in these mutants when the cell is under amino acid starvation conditions. Based on these data, we concluded that phosphorylation of T879 abolishes CPC-3 activity. Further studies should aim to determine if the phosphorylation at this specific site is rhythmic, and if the phosphorylation is specifically under control of the clock.

CONTRIBUTORS AND FUNDING SOURCES

Contributors

This work was supported by a thesis committee consisting of Dr. Deborah Bell-Pedersen (advisor) and Dr. Matthew Sachs of the Department of Biology, and Dr. Daniel Ebbole of the Department of Agriculture and Life Sciences.

Zhaolan Ding contributed to the mass spectrometry for Chapter 2, mutant design for Chapter 3, and mutant testing of the T879 site for Chapter 4.

Dr. Kathrina Castillo and Manuel Ramirez contributed to the design of the 3-AT stress assay for Chapter 4.

All other work for the thesis was completed by the student independently.

Funding Sources

This work was funded by a grant to Deborah Bell-Pedersen from the National Institute of Health (NIH).

TABLE OF CONTENTS

LIST OF FIGURES

Page

LIST OF TABLES

Page

CHAPTER I

INTRODUCTION

The Circadian Clock

The circadian clock is an intrinsic time keeping mechanism that has been observed and studied in multiple organisms, including cyanobacteria, fungi, and mammals. The internal clock allows an organism to anticipate changes in the environment and adjust its physiology according to the time of day [1]. The core circadian oscillator is synchronized to a 24-hour day-night cycle through multiple environmental input pathways and regulates various output pathways. These environmental inputs entrain the clock, allowing it to regulate gene expression, metabolic homeostasis, and behavior of the organism at the appropriate time of the day [2-4]. Although environmental inputs help to regulate clock-controlled pathways, the circadian clock is an autonomous mechanism that can maintain a 24-hour cycle in the absence of environmental input [3]. In eukaryotic organisms, the circadian oscillator is a negative feedback loop that consists of positive elements that drive the expression of negative elements, which then feed back to inhibit the activity of the positive elements [5]. This reduces the expression of the negative elements and their turnover allows the cycle to start again the next day. The core oscillator sends time of day information through output pathways to control rhythmic gene expression, which in turn leads to physiological changes in the organism. Studying the intricacies of the circadian clock allows us to further understand metabolic regulation and diseases in higher organisms [2].

Neurospora as a Model Organism for Circadian Biology

Neurospora crassa is a haploid filamentous fungus that is a model organism for circadian biology due to the nature of its development. Rhythms are easily observed in asexual spore (conidia) production, which provides the opportunity to use genetic approaches to identify genes that are essential for clock function [6]. Furthermore, the sequenced and annotated genome of *N. crassa* is available, along with gene knockouts of nearly all known genes. These knockouts are maintained by the Fungal Genetics Stock Center [6, 7].

The *N. crassa* circadian oscillator consists of the positive elements WHITE COLLAR-1 (WC-1) and WHITE COLLAR-2 (WC-2), which heterodimerize to form the White Collar Complex (WCC), and a negative elements FREQUENCY (FRQ) and FREQUENCY-INTERACTING RNA HELICASE (FRH) [6]. During the subjective daytime, *frq* transcription is activated by the WCC by binding to the C-box of the *frq* promoter [8]. The FRQ protein is then translated and binds to FRH to create the FRQ/FRH complex (FFC). The FFC enters the nucleus and directs the kinases CKI and CKII to phosphorylate WCC [9]. This phosphorylation results in the inactivation of WCC, which inhibits *frq* transcription in a negative feedback loop during the late subjective daytime [6, 10]. Accumulated FRQ protein is also phosphorylated, targeting the protein for ubiquitination and degradation [6]. As the levels of FRQ protein fall, several phosphatases dephosphorylate existing WCC, and together with newly synthesized WCC activate *frq* transcription the next subjective day [6, 11]. This cycle continues in a continuous negative feedback loop, as shown in **Figure 1.1.**

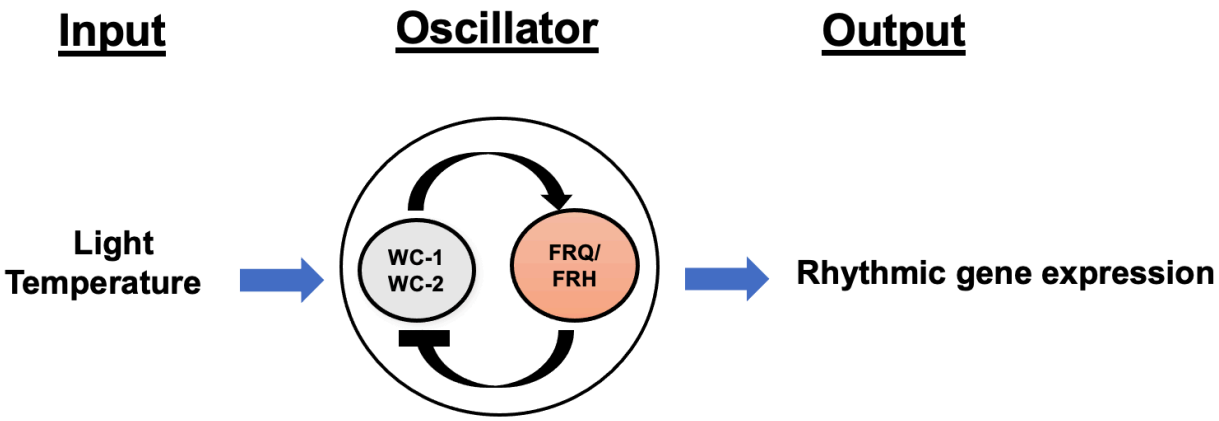

Figure 1.1: Diagram of the *N. crassa* circadian clock. See the text for details.

Post-Transcriptional Modifications Under Clock Control

Many studies in recent years have found that the circadian clock rhythmically regulates various post-transcriptional mechanisms [12-14]. Nearly half of rhythmically synthesized proteins in mammals and *N. crassa* do not have corresponding cycling mRNAs, indicating that protein turnover or translation itself may be rhythmically regulated [15, 16]. Translation can be regulated in a number of ways, including regulating the rate of mRNA synthesis, or by regulating factors needed for translation, such as initiation and elongation factors [17]. In support of clock regulation of mRNA translation, targets of the mTOR pathway have been shown to regulate entrainment of the clock by suppressing mRNA translation [18]. In addition, the levels and modifications of several translation initiation factors accumulate rhythmically in *N. crassa* and mammals [18, 19], including rhythmic accumulation of translation initiation factor eIF2α levels in mouse liver and

brain [20], and cycling phosphorylated eIF2α (P-eIF2α) levels in the mouse superchiasmatic nucleus [21]. Furthermore, the translation elongation factor 2 (eEF-2) in *N. crassa* was shown to be under control of the circadian clock through rhythmic activation of the p38 MAPK pathway and the downstream eEF-2 kinase RCK-2 [22]. These data support the idea that the circadian clock plays an active role in post-transcriptional regulation.

Regulation of Translation Initiation by the Clock

Recent studies have provided evidence that the initiation step of translation is under control of the clock [21, 23]. In eukaryotic organisms, translation initiation requires several initiation factors which are highly conserved across many species [24]. The eukaryotic initiation factor 2 (eIF2) is a heterotrimeric GTPase that is an essential factor in translation initiation [24, 25]. When GTP is present, eIF2 forms a stable complex with the initiator methionyl tRNA and delivers it to the P site of the ribosome [25]. The ribosome then recognizes the corresponding codon on the mRNA, thereby beginning translation. GTP is hydrolyzed upon the start of translation, and the guanine nucleotide exchange factor eIF2B converts eIF2 back to its GTP-bound form to start the process again [25, 26]. eIF2 activity is regulated via the phosphorylation of serine 51 (S51) of its alpha subunit [26]. This phosphorylation is triggered by uncharged tRNAs in the cell, which is indicative of amino acid starvation. The phosphorylation of the alpha subunit converts the stable initiation complex to a competitive inhibitor for eIF2B, thereby preventing the eIF2 initiation complex from binding to GTP [25-27]. This inhibits the translation of the majority of mRNAs in the cell, except for those needed during stress

conditions. Allocating cellular energy to translating only specific mRNAs, such as those encoding proteins involved in amino acid biosynthesis during amino acid depletion, is an energy cost-effective stress response strategy of the cells [28]. The phosphorylation of eIF2α has been shown to occur rhythmically, with peaks in the subjective daytime, and is under control of the core circadian oscillator in both mammals and *N. crassa* [21, 23]. In *N. crassa,* about 30% of available eIF2α is phosphorylated [23], leading to rhythmic translation of specific mRNAs (Castillo et al, in prep).

A single kinase is responsible for phosphorylating eIF2α at the S51 site under constant conditions in *N. crassa* [23]. This serine/ threonine kinase is known as Cross-Pathway Control 3 (CPC-3) (NCU01187) [23, 26]. Not only is CPC-3 required for phosphorylation of eIF2α, but its activity is necessary to maintain rhythms of P-eIF2α phosphorylation [23]. Furthermore, evidence suggests that the activity of CPC-3, rather than the rhythmic accumulation of the protein, is required to maintain the rhythmic phosphorylation of eIF2α [23].

CPC-3 is structurally and functionally similar to the General Control Non-Derepressible 2 (GCN2) protein in *S. cerevisiae* [26]. GCN2 has been well studied for its role in amino acid starvation stress responses, so we can use studies of GCN2 to inform our understanding of CPC-3 activation. [23]. The activity of GCN2 is primarily regulated by the level of uncharged tRNAs in the cell and autophosphorylation is required for its activity [29]. Specifically, preventing phosphorylation at threonine 882 (T882) and threonine 887 (T887) of the GCN2 activation loop results in the loss of eIF2α

phosphorylation [29, 30]. However, it is not known if these conserved sites, or other phosphorylation sites, are necessary for rhythms in *N. crassa* CPC-3 activity, and hence this is the focus of this study. A summary of conserved domains and known autophosphorylation sites on GCN2, and predicted autophosphorylation sites on CPC-3 are noted in **Figure 1.2**.

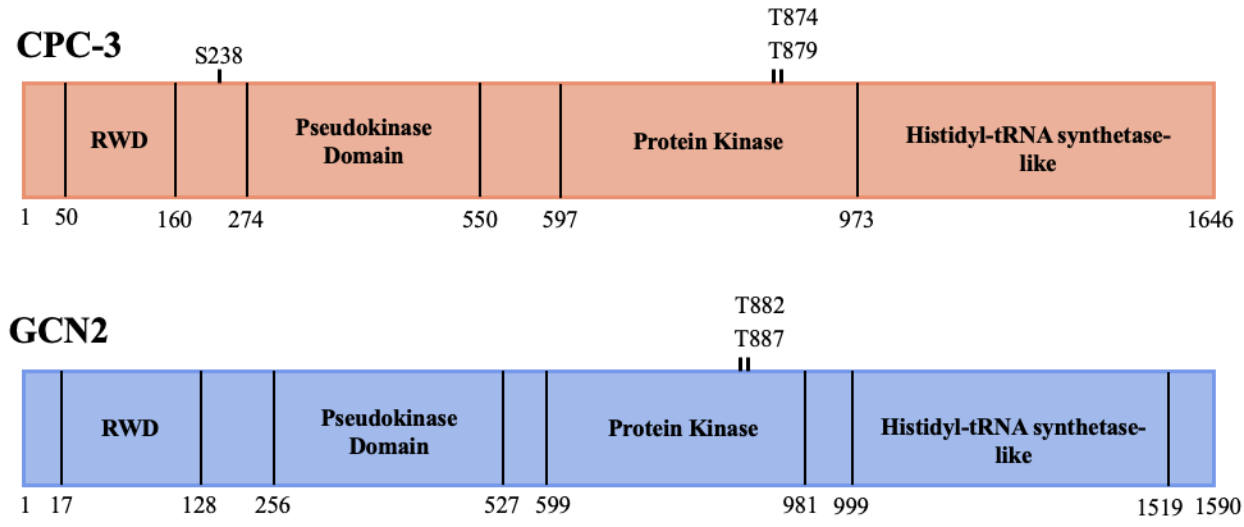

Figure 1.2: Conserved domains of CPC-3 kinase in *N. crassa* and GCN2 kinase in *S. cerevisiae.* These two kinases share a RWD domain and two protein kinase domains. Two of the predicted autophosphorylation sites of CPC-3 based on our mass spectrometry data are located in the second conserved protein kinase domain, in which the known autophosphorylation sites T882 and T887 of GCN2 are located [29, 31].

Objective

CPC-3 kinase is an important factor in a stress response that is triggered by low amino acid availability in the cell [26]. Our lab has shown that rhythmic eIF2α phosphorylation depends on rhythmic CPC-3 activity [23]. However, the exact mechanism of clock-dependent CPC-3 activation is not known. To investigate this, I tested the following hypotheses:

1. **The circadian clock drives rhythms in CPC-3 phospho-modification.**

   To determine if phosphorylation sites on CPC-3 kinase are involved in its activity, Ph.D. candidate Zhaolan Ding conducted post-translational mass spectrometry of the kinase at two different circadian time points, during the subjective nighttime (DD24) and the subjective daytime (DD36). Two phosphorylation sites showed significantly more phosphorylation during the subjective daytime threonine 874 (T874) and threonine 879 (T879), and one site showed significantly more phosphorylation during the subjective nighttime serine 238 (S238). These times correspond to when P-eIF2α levels are the lowest and highest in wild type (WT) conditions, respectively.

2. **Autophosphorylation sites on CPC-3 kinase regulate the rhythmic phosphorylation of eIF2α.**

   To determine if the phosphorylation of these sites is necessary for rhythmic phosphorylation of eIF2α, I created mutations that block phosphorylation at the sites (convert site to alanine) and that mimic constant phosphorylation (convert site to glutamic acid). Time course experiments were conducted to determine if the mutations affected the rhythmic phosphorylation of eIF2α.

T874E and S238E showed no difference in the rhythms of P-eIF2α. T879A and T897E mutants completely abolished the phosphorylation of eIF2α.

3. **Mutations on these phosphorylation sites affect the response of the cell to amino acid starvation.**

   To observe the effects of the mutations on the stress response of the cell, I measured the levels of P-eIF2α when the cultures were grown with 3-amino-1,2,4-triazole (3-AT) in the media. This compound impairs the histidine biosynthesis pathway, thereby inducing an amino acid-starved environment. Even in the presence of 3-AT, the T879 mutants showed no phosphorylation of eIF2α.

CHAPTER II

IDENTIFICATION OF RHYTHMICALLY PHOSPHORYLATED SITES OF CPC-3 KINASE

Introduction

The circadian clock regulates various biological processes that ultimately affect an organism's physiology and behavior [1]. In eukaryotic organisms, about 50% of the genome is regulated by the circadian clock at the level of mRNA accumulation [1, 3, 32]. In addition to transcriptional regulation, the circadian clock also regulates post-transcriptional mechanisms, including mRNA capping and splicing [12-15]. Furthermore, up to half of rhythmically produced proteins in mammals and *N. crassa* do not have corresponding cycling mRNAs, indicating that rhythmic protein accumulation is driven by post-transcriptional mechanisms [15, 16]. Evidence that the clock controls mRNA translation is growing. For instance, targets of the mTOR pathway have been shown to regulate the entrainment of the mammalian circadian clock by regulating mRNA translation [18]. It has also been shown that the levels of various translation initiation factors accumulate rhythmically, including rhythmic accumulation of eIF2α levels in the mouse liver and brain [20] and in cycling phosphorylation of eIF2α (P-eIF2α) in the mouse superchiasmatic nucleus [21]. In *N. crassa,* the activity of the translation elongation factor eEF-2 is controlled by the circadian clock through rhythmic activation of the p38 MAPK pathway and the downstream eEF-2 kinase RCK-2 [22].

The eukaryotic translation initiation factor 2 has been shown to be rhythmically phosphorylated in mammals [20, 21]. eIF2 is a heterotrimeric protein that is conserved in many eukaryotes and archaea, and is necessary for many stress response pathways in the cell [25, 33]. When the cell is specifically starved for amino acids, large amounts of uncharged tRNAs build up in the cell and bind to the eIF2α kinase, thereby activating it. The kinase then phosphorylates S51 of eIF2α (P-eIF2α), which converts eIF2 from the stable initiation complex to a competitive inhibitor of eIF2B [25-27]. In addition to an acute nutrient response, in constant conditions, the circadian clock controls the rhythmic accumulation of P-eIF2α levels, with peaks in the subjective daytime in both mammals and *N. crassa* [21, 23].

The kinase that is responsible for phosphorylating eIF2α is conserved across many eukaryotes. In *N. crassa,* the kinase is called Cross-Pathway Control 3 (CPC-3), and specifically phosphorylates S51 of eIF2α in response to high levels of uncharged tRNAs [23, 26]. Recent evidence has shown that not only is CPC-3 required for the phosphorylation of eIF2α, but its clock-controlled activity is necessary to maintain the rhythmic phosphorylation of eIF2α [23]. Furthermore, the rhythmic accumulation of CPC-3 is not required to maintain P-eIF2α rhythms, but rather the rhythmic activity of the kinase is required [23].

CPC-3 kinase is structurally and functionally the most similar to the General Control Non-Depressible 2 (GCN2) kinase in *S. cerevisiae* [23, 26]. The activity of GCN2 is primarily regulated by levels of uncharged tRNA levels. These high levels of uncharged

tRNAs bind to the histidyl-RS domain of the kinase and induce activity through autophosphorylation [29]. Preventing phosphorylation at the sites T882 and T887 of GCN2 in the activation loop prevents all phosphorylation of eIF2α in *S. cerevisiae* [29, 30]. However, if these conserved sites, or other phosphorylation sites, are necessary for rhythmic CPC-3 activity in *N. crassa* was not known and is the focus of this study.

To determine if potential autophosphorylation sites on CPC-3 are rhythmically phosphorylated under non stress conditions, post-translational modification (PTM) mass spectrometry with a focus on phosphorylation was performed on CPC-3 at two different times by Ph.D. candidate Zhaolan Ding. CPC-3 protein was isolated from cells grown in constant dark (DD) and harvested at two different times of day, the subjective daytime (DD36) and the subjective nighttime (DD28) when the levels of P-eIF2α peak and trough in wild-type cells, respectively. Three different phosphorylation sites were determined to have a significant time-of-day difference in phosphorylation levels. Peptides corresponding to Phospho-T874 and Phospho-T879 were twice as abundant in the subjective daytime (DD36) compared to the subjective nighttime (DD28). Furthermore, our mass spectrometry data showed that Phospho-S238 peptides were twice as abundant in the subjective nighttime (DD28) compared to the subjective daytime (DD36).

Results

Three phosphorylation sites of CPC-3 have a time-of-day difference. To determine if potential autophosphorylation sites of CPC-3 played a role in the rhythmic activation of CPC-3, we first wanted to determine if conserved phosphorylation sites with GCN2 had a significant time-of-day difference in phosphorylation levels. Protein extracts were isolated from wild-type *N. crassa* cells (FGSC#4200) grown in constant dark (DD) for 36 hours to represent the subjective daytime and 28 hours to represent the subjective nighttime. Protein extracts of CPC-3 were then sent to the proteomics core facility at UT Southwestern for post-translational modification (PTM) mass spectrometry with a focus on phosphorylation. Two sites in the protein kinase domain, P-T874 and P-T879, were twice as abundant in the subjective daytime (DD36) compared to the subjective daytime (DD28). These two sites are conserved with T882 and T887, which are known autophosphorylation sites in *S. cerevisiae* GCN2 (**Figure 2.1**). Another phosphorylation site, P-S238, was twice as abundant in the subjective nighttime (DD28) compared to the subjective daytime (DD36). However, this site is not conserved with any known autophosphorylation sites on *S. cerevisiae* GCN2.

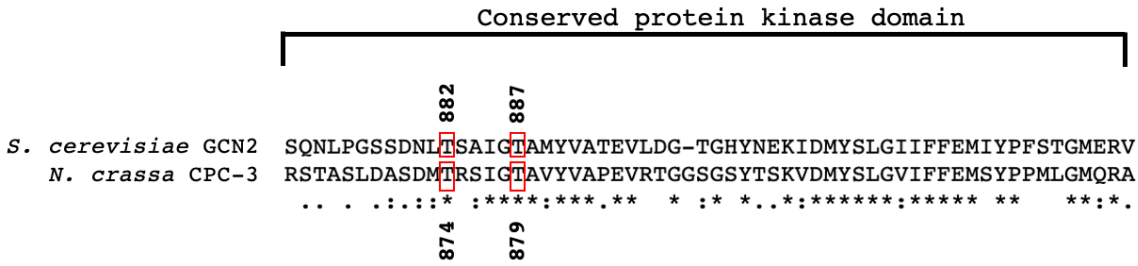

Figure 2.1: Sequence alignment of the conserved protein kinase domain of *S. cerevisiae* GCN2 and *N. crassa* CPC-3. Known autophosphorylation sites of GCN2 (T882 and T887) and predicted autophosphorylation sites of CPC-3 (T874 and T879) are highlighted in red. A "*" symbol indicated perfect alignment, a "." symbol indicates strong similarity, and a ":" symbol indicates weak similarity. Similarity was determined using Clustal Omega software.

Discussion

Translation initiation is a key regulatory process for eukaryotes, particularly under times of stress. CPC-3 kinase is the only known kinase to phosphorylate eIF2α when cells are starved for amino acids or grown in a circadian time course in *N. crassa.* Previous work has shown that the rhythmic activity of CPC-3 kinase is essential to maintain the rhythmic phosphorylation of eIF2α [23]. However, the exact mechanism of rhythmic activation of CPC-3 was not known. Since the conserved kinase GCN2 in *S. cerevisiae* is known to be regulated in part by autophosphorylation sites on the protein kinase domain, we wanted to investigate if potential autophosphorylation sites of CPC-3 have a time-of-day difference in phosphorylation levels. Post-translational modification (PTM) mass spectrometry data showed that the conserved autophosphorylation sites T874 and T879, as well as the non-conserved site S238 had a significant time-of-day-

difference in phosphorylation levels (**Figure 2.2**). Furthermore, T874 and T879 were found to be conserved with the autophosphorylation sites T882 and T887 of *S. cerevisiae* GCN2 (**Figure 2.1**). Due to the time-of-day difference in phosphorylation levels of these three sites, and due to the conservation with already known autophosphorylation sites, we pursued further study of these sites to determine if they were necessary for the rhythmic activation of CPC-3 kinase. A summary of the sites detected in our mass spectrometry data is summarized in **Table 2.1.**

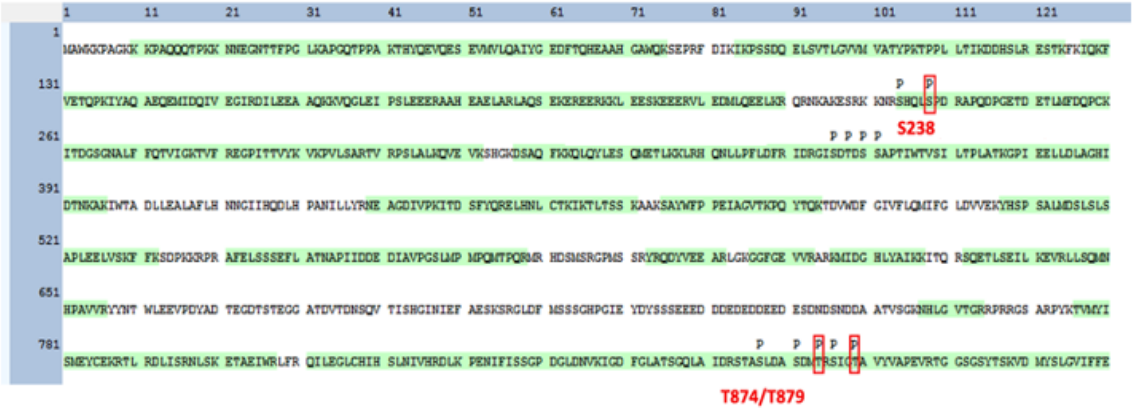

Figure 2.2: Post-translational modification mass spectrometry data for CPC-3 kinase. Samples of CPC-3 were obtained from cultures grown at DD36 (subjective daytime) and DD28 (subjective nighttime). Sites which had a significant time-of-day difference in the levels of phosphorylation (S238, T874, and T879) are highlighted in red. “P” indicates a phosphorylation site.

Table 2.1: Summary of phosphorylation sites detected by post-translational modification mass spectrometry. Abundance of the phosphorylated peptides at both times of day are indicated for each site.

Phosphorylation Site	Homolog in *S. cerevisiae*	Peptide Reads DD28	Peptide Reads DD40	Ratio of Peptides (DD28:DD36)	Peak Phosphorylation Time
T874	T882	1077424	1798161.25	21:49	Subjective daytime (DD36)
T879	T887	1077424	1798161.25	21:49	Subjective daytime (DD36)
S238	-	688925.125	243391.3906	11:5	Subjective nighttime (DD28)

Methods and Materials

Strains and Growth Conditions. Strains of *N. crassa* were grown as previously described [34]. *Ptcu1::cpc-3::v5* cell cultures were maintained on Vogel's minimal media (1X Vogel's, 2% glucose). Germinated conidia cultures were grown in constant light conditions (LL) for a minimum of 12 hours to synchronize the circadian clock in all cells of the culture in liquid V_2G (1X Vogel's, 2% glucose) media with 50mM of the copper chelator BCS to induce high expression. Samples for the subjective daytime (DD36) were shifted to the constant dark (DD) after 12 hours of light. Samples for the subjective nighttime (DD28) were shifted to constant dark after 20 hours of light. All samples were harvested after a total time of 48 hours of growth for each sample. Tissue samples were immediately frozen in liquid N_2.

Protein Extraction and PTM Mass Spectrometry. Protein was extracted from harvested tissue as previously described [35]. The extraction buffer was made with 100 mM Tris (pH 7.5), 1% SDS, 1X aprotinin, 1X leupeptin, 1X pepstatin, 10 mM NaF, 1 M β-glycerophosphate, 1 mM sodium ortho-vanadate and 1 mM PMSF. CPC-3 protein was then immobilized on magnetic Dynabeads (#10008D, Invitrogen) by immunoprecipitation with an anti-V5 antibody (#2109R, BioSS). The beads were then boiled with 2X Laemmli loading dye and run on a 8% SDS/PAGE gel and run using standard methods. The gel was stained with Coomassie Blue to visualize the proteins and the target bands were cut and sent to UT Southwestern Proteomics Core for PTM mass spectrometry. Peptides and modifications were identified using the Proteome Discoverer software (Thermo Fisher).

CHAPTER III

IMPACT OF PHOSPHORYLATION SITE MUTATIONS ON RHYTHMIC CPC-3 ACTIVITY

Introduction

An important conserved factor involved in eukaryotic translation is the eukaryotic initiation factor 2 (eIF2). This factor is a heterotrimeric protein with an alpha, beta, and gamma subunits that interact with GTP and the initiator methionyl tRNA (Met-tRNA) [25]. In order for translation to occur, eIF2 must form a pre-initiation complex with Met-tRNA and GTP, and deliver this tRNA to the P site of the ribosome [25, 26]. Once the Met-tRNA is delivered to the ribosome, GTP is exchanged for GDP by the guanine nucleotide exchange factor eIF2B, allowing a new GTP and Met-tRNA to bind to eIF2 to begin a new round of translation [25, 26]. However, if the cell is starved for amino acids, a large amount of uncharged tRNAs will accumulate in the cell, which is recognized by the eIF2 kinase CPC-3 [23, 26]. CPC-3 will then phosphorylate Ser 51 of eIF2's alpha subunit (P-eIF2α), which prevents the GTP/GDP exchange mediated by eIF2B and limits global translation in the cell [23, 26]. The phosphorylation of eIF2α has been shown to rhythmically accumulate in mammals and *N. crassa,* peaking in the subjective daytime in both organisms [21, 23]. However, the exact mechanism of this clock-controlled regulation was not known.

Previous work has shown that the rhythmic activity of CPC-3 kinase in *N. crassa* is needed for the rhythmic accumulation of P-eIF2α, rather than rhythmic levels of CPC-3

kinase [23]. There are many different activation mechanisms that may be under clock control in this case. For instance, in *S. cerevisiae,* evidence has shown that the drug rapamycin, which inhibits the Target of Rapamycin (TOR) pathway, regulates the phosphorylation of eIF2α by activating GCN2 [36]. Since the mammalian TOR pathway (mTOR) has been shown to regulate intrinsic clock components [37], it is possible that the TOR pathway may also have some control over the rhythmic phosphorylation of eIF2α in *N. crassa.*

The potential mechanism of activation that is pursued in this study is the rhythmic phosphorylation of sites in the pseudo kinase and kinase domains of CPC-3. In the CPC-3 homolog GCN2, auto-phosphorylation sites are necessary for the activation of the kinase [29]. This knowledge, along with our mass spectrometry data, led me to hypothesize that T874, T879, and/or S238 rhythmically control CPC-3 activity in *N. crassa* via phosphorylation. To test this hypothesis, I designed single point mutations at each of these phosphorylation sites. Without changing any surrounding amino acids, I converted each site to an alanine (to prevent phosphorylation at the site) and to glutamic acid (to mimic constant phosphorylation at the site). Once these mutations were synthesized and integration confirmed in *N. crassa*, I ran time course experiments to determine if these mutations affected the rhythmic phosphorylation of eIF2α. Mutating T874 and S238 to glutamic acid (T874E and S238E) did not alter the rhythms of P-eIF2α. However, mutating T879 to both alanine and glutamic acid (T879A and T879E) resulted in a complete loss of CPC-3 activity.

Results

T874E and S238E Mutations Did Not Abolish P-eIF2α Rhythms. To determine if the potential CPC-3 phosphorylation sites affected the rhythmic phosphorylation of eIF2α, I designed mutant strains for each site that either abolish phosphorylation at the site (convert to alanine) or to mimic constant phosphorylation at the site (convert to glutamic acid). I designed these mutations using a two-way overlapping PCR method. Once the mutations were integrated into the genome, I grew both WT and mutant cells in a two-day circadian time course experiment (DD8 to DD52). Protein was then extracted from these samples and the levels of P-eIF2α and total eIF2α were visualized by western blots. In these time course experiments, mimicked constant phosphorylation at T874 (T874E) and at S238 (S238E) did not alter the rhythms of P-eIF2α compared to WT (**Figure 3.1A, Figure 3.2A**). In all time course experiments, there was some variation in the levels of total eIF2α over time, but in no instance were the total eIF2α levels defined as rhythmic in our analysis. This indicated that the rhythms observed in P-eIF2α were due to rhythms in the phosphorylation rather than rhythms of the entire protein (**Figure 3.1B. Figure 3.2B**). Furthermore, the phase, amplitude, and period of the T874E mutant time course experiments were not significantly altered compared to WT (**Table 3.1).** Similarly, the phase and amplitude of the S238E mutant time course experiments did not change compared to WT (**Table 3.2**). However, a 4 h shorter period was observed in the S238E mutant strain compared to WT. Based on these results, we concluded that mimicking constant phosphorylation at T874 and S238 of CPC-3 does not abolish rhythmic CPC-3 activity. However, there is potential for these

sites to have some impact on CPC-3 activity when mutated together or when mutated to an alanine to block phosphorylation at the site.

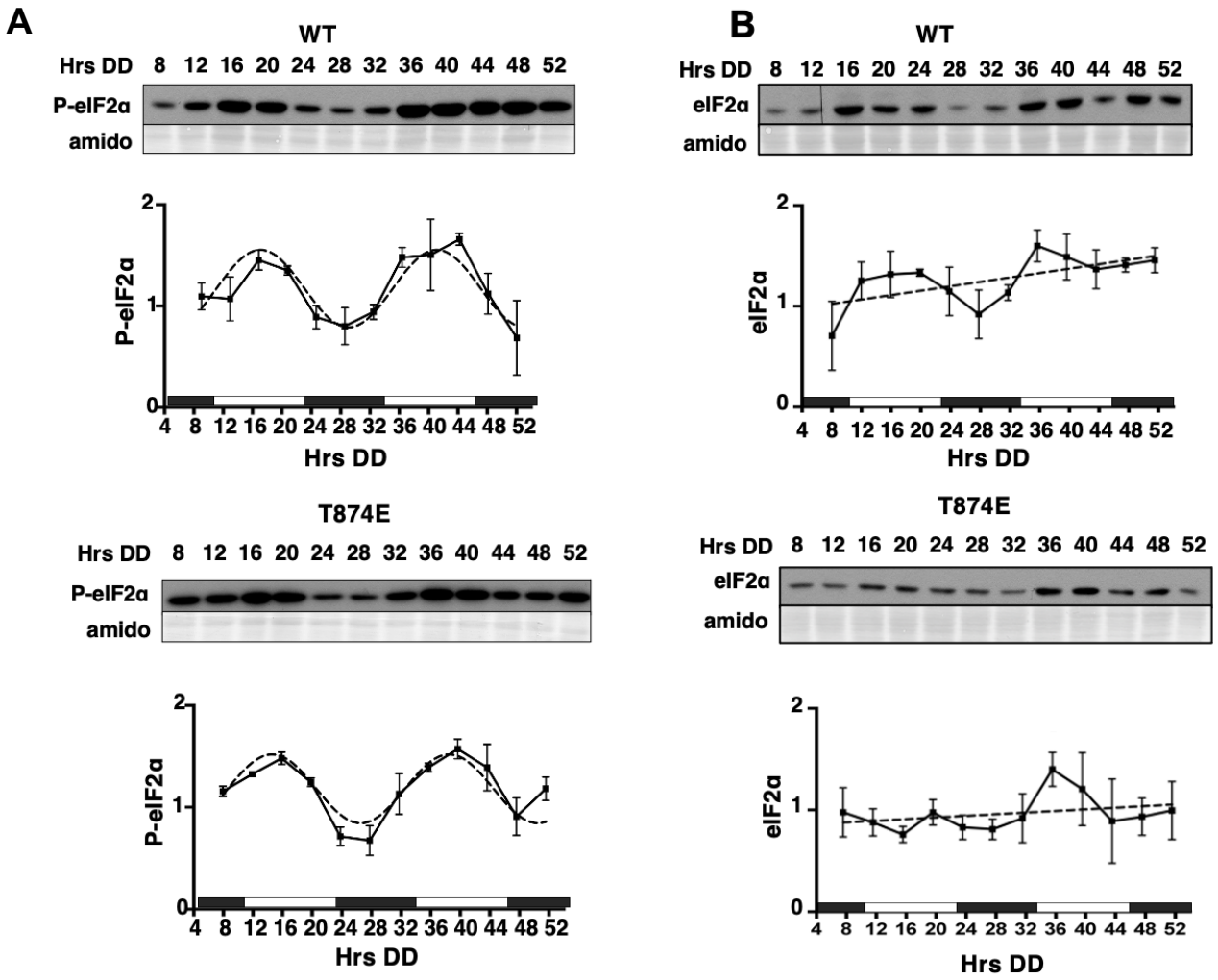

Figure 3.1 Mimicked constant phosphorylation at T874 of CPC-3 does not abolish P-eIF2α rhythms. Representative western blots of protein extracts from WT and T874E strains grown over a circadian time course. Blots were probed for P-eIF2α (A, left panel) and for total eIF2α (B, right panel). Amido black-stained protein is shown as a loading control. Plots of the data (mean ± SEM; n=3) on the left show levels of P-eIF2α (A) and on the right show total eIF2α levels (B). The signal was normalized to the total protein levels (solid black line). Rhythmicity in P-eIF2α levels (A) was determined using F-tests

of the fit of the data to a sine wave (dotted black line; $p < 0.001$) and arrhythmicity in total eIF2α levels (B) was determined using F-tests of the fit of the data to a straight line (dotted black line; $p < 0.05$). F-tests were performed in the Prism software from GraphPad. The black and white bars bellow the plots designate subjective daytime (white) and subjective nighttime (black).

Strain	Phase	Amplitude	Period
WT	2.24 ± 1.3	0.3 ± 0.06	24.51 ± 1.37
T874E	0.63 ± 0.86	0.26 ± 0.03	24.32 ± 0.89

Table 3.1 Phase, amplitude, and period comparison in T874E time course experiments. The mean values ± SEM were calculated using Prism software. There is no significant difference between the T874E and WT phase, amplitude, or period based on the means and the range of error. Statistical significance was determined using student t-test ($p<0.05$).

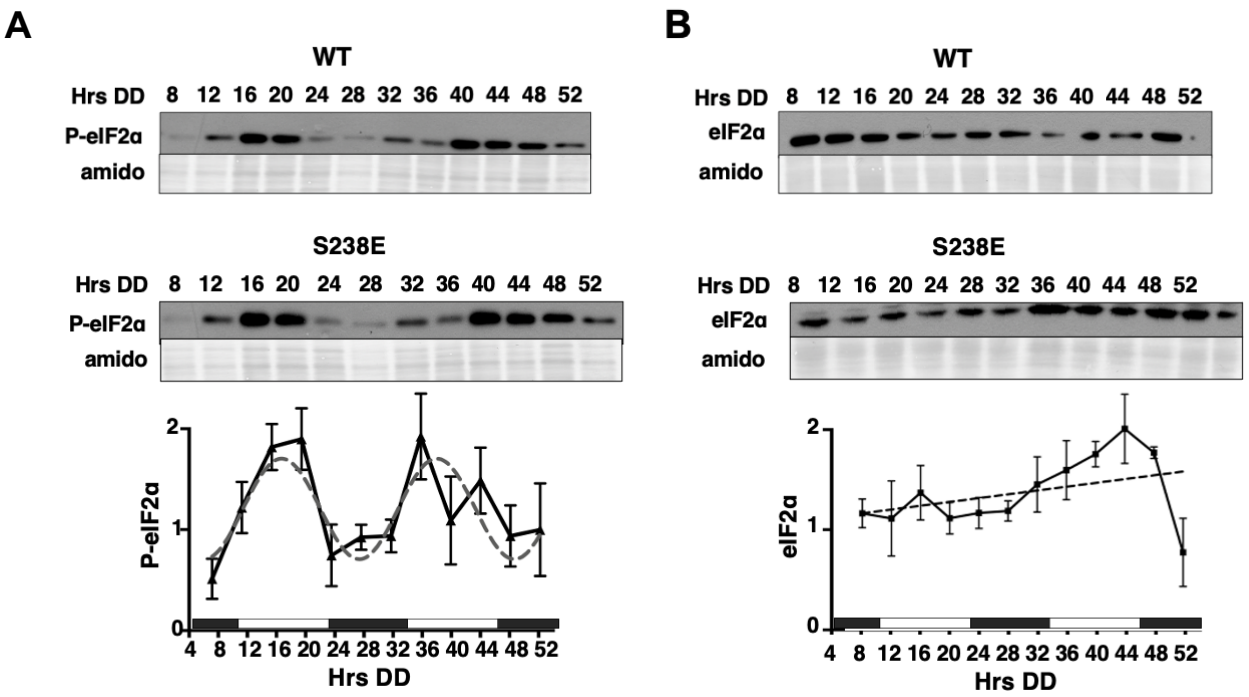

Figure 3.2 Mimicked constant phosphorylation at S238 of CPC-3 does not abolish P-eIF2α rhythms. Representative western blots of protein extracts from WT and S238E strains grown over a circadian time course. Blots were probed for P-eIF2α (A, left panel) and for total eIF2α (B, right panel). Amido black-stained protein is shown as a loading control. Plots of the data (mean ± SEM; n=3) on the left show levels of P-eIF2α (A) and on the right show total eIF2α levels (B). The signal was normalized to the total protein levels (solid black line). Rhythmicity in P-eIF2α levels (A) was determined using F-tests of the fit of the data to a sine wave (dotted black line; $p < 0.001$) and arrhythmicity in total eIF2α levels (B) was determined using F-tests of the fit of the data to a straight line (dotted black line; $p < 0.05$). F-tests were performed in the Prism software from GraphPad. The black and white bars bellow the plots designate subjective daytime (white) and subjective nighttime (black).

Strain	Phase	Amplitude	Period*
WT	1.59 ± 0.82	0.50 ± 0.06	25.09 ± 0.88
S238E	4.13 ± 1.46	0.39 ± 0.1	20.81 ± 1.32

Table 3.2 Phase, amplitude, and period comparison in S238E time course experiments. The mean values ± SEM were calculated using Prism software. There is no significant difference between the S238E and WT phase and amplitude. A significant change in the period of the S238E mutant was observed. Statistical significance was determined using student t-test ($p<0.05$)*.

T879A and T879E Mutations Completely Abolish CPC-3 Activity. As with the previous potential autophosphorylation sites, I developed mutants that converted T879 into alanine (to block phosphorylation) and to glutamic acid (to mimic constant phosphorylation). These two mutants were developed and analyzed with the help of Ph.D. candidate Zhaolan Ding. When grown in constant light (LL) for 24 hours, both T879A and T879E CPC-3 mutations show no detectable P-eIF2α (**Figure 3.3**).

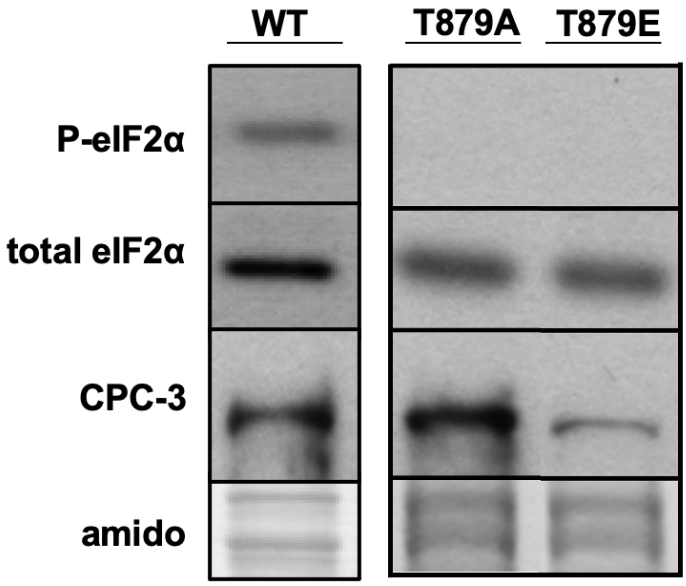

Figure 3.3 T879A and T879E mutants prevent the activation of CPC-3 kinase. Representative western blots of protein extracts from WT, T879A, and T879E cells. P-eIF2α, total eIF2α, and CPC-3 levels were detected. Membrane was stained with amido black as a protein loading control.

Furthermore, both CPC-3 and total eIF2α levels were still detectable in the mutant strains, indicating that these mutations altered the phosphorylation of eIF2α, but did not abolish the levels of eIF2α or CPC-3 (**Figure 3.3**). However, the levels of CPC-3 kinase were significantly higher in the T879A mutant compared to both WT and the T879E mutant.

Discussion

Previous experiments have shown that rhythmic CPC-3 activity is required for the rhythmic phosphorylation of eIF2α [23]. However, the exact mechanism of rhythmic activation of CPC-3 was not known. Based on our mass spectrometry data, we

hypothesized that three potential phosphorylation sites of CPC-3 regulated its rhythmic activity. Mimicking constant phosphorylation (mutation to glutamic acid) at T874 and S238 of CPC-3 did not affect the rhythmic phosphorylation of eIF2α. Furthermore, the T874E mutant did not significantly affect the phase, amplitude, or period of the WT P-eIF2α rhythm. However, a significant change was observed in the period of the S238E mutant compared to the wild type. Based on this data, we concluded that dephosphorylation of T874 and S238 of CPC-3 is not required for the rhythmic activation of CPC-3. However, this does not rule out the sites as potential CPC-3 activation modulators. For instance, mutating either of these sites to alanine to prevent phosphorylation may alter the rhythms of P-eIF2α. Numerous attempts to generate the alanine mutations were made; however, they were not successful. This suggests that the phosphorylation at T874 and S238 may be essential for growth in *N. crassa.* This phenotype would be surprising, as the T874A analog in *S. cerevisiae* (T882A) is viable in yeast [29].

Furthermore, these sites, with or without P-T879, may all need to be phosphorylated or dephosphorylated to observe an effect on CPC-3 activity. This idea is supported by the small change in S238E phase compared to WT (**Table 3.2**). Similar results have been observed in *S. cerevisiae,* in which phosphorylating or dephosphorylating both T882 and T887 of GCN2 showed a larger change in CPC-3 activity compared to the single mutations [29]. Future studies of these two sites should focus on analyzing mutants that block phosphorylation at the single sites and mutations that alter both sites.

Although mimicking constant phosphorylation at T874 and S238 of CPC-3 did not significantly alter the rhythmic phosphorylation of eIF2α, mimicking constant phosphorylation and inhibiting phosphorylation of T879 completely abolished CPC-3 activity. No detectable P-eIF2α was observed in either of these mutations in LL. Therefore, we can conclude that phosphorylation and dephosphorylation at this site is necessary for CPC-3 activity in LL. This assay should be repeated in DD conditions to confirm that the rhythms of P-eIF2α are abolished in these mutants. Similarly, the corresponding site in *S. cerevisiae,* T887, has been shown to diminish *in vitro* kinase activity of GCN2 when the phosphorylation of this site is blocked [29]. However, it is still unknown if this site is rhythmically auto-phosphorylated by CPC-3, or if this site is rhythmically dephosphorylated by a clock-controlled phosphatase. To determine if this site is rhythmically phosphorylated, an antibody should be developed for this site and a full time course experiment should be performed in a WT strain and in a clock mutant strain. This experiment will determine if this site is rhythmically phosphorylated and if it is under control of the circadian clock. Furthermore, it is unclear why the T879A mutant had much higher levels of CPC-3 compared to both the WT and the T879E mutant. This site may be important for mechanisms other than CPC-3 activity, such as the translational efficiency of CPC-3 and the stability of the protein. Studies investigating how blocking phosphorylation at T879 affects CPC-3 protein accumulation and turnover may provide insight on this result.

Methods and Materials

Strains and Growth Conditions. Strains of *N. crassa* were grown as previously described [34]. All mutant strains were maintained on Vogel's minimal media (1X Vogel's, 2% glucose).

Development of Point Mutations. Point mutations of potential CPC-3 kinase auto-phosphorylation sites were developed using a two-way PCR single site mutagenesis method (**Figure 3.1**). Genomic DNA from WT *N. crassa* (FGSC#4200) was used as a template. Primers were designed with a single amino acid change at the potential auto-phosphorylation site (**Table 3.1**). To create the *cpc-3*^{T874A} mutation, P1 and P2 were used to create a 1,010 bp fragment and P3 and P4 were used to create a 840 bp fragment. These two fragments contained an overlapping region of the *cpc-3* ORF with the single amino acid mutation and with a new *BssHII* restriction site. These fragments were stitched together with P1 and P4 to create a 1,850 bp fragment. To create the *cpc-3*^{T874E} mutation, P1 and P2 were used to create a 1,013 bp fragment and P3 and P4 were used to create a 854 bp fragment. These two fragments contained an overlapping region of the *cpc-3* ORF with the single amino acid mutation and with a new *AfeI* restriction site. These fragments were stitched together with P1 and P4 to create a 1867 bp fragment.

To create the *cpc-3*^{T879A} mutation, P1 and P2 were used to create a 1,032 bp fragment and P3 and P4 were used to create a 833 bp fragment. These two fragments contained an overlapping region of the *cpc-3* ORF with the single amino acid mutation and with a

new *KasI* restriction site. These fragments were stitched together with P1 and P4 to create a 1,865 bp fragment. To create the *cpc-3*^{T879E} mutation, P1 and P2 were used to create a 1,035 bp fragment and P3 and P4 were used to create a 882 bp fragment. These two fragments contained an overlapping region of the *cpc-3* ORF with the single amino acid mutation and with a new BstZ17I-HF restriction site. These fragments were stitched together with P1 and P4 to create a 1,917 bp fragment.

To create the *cpc-3*^{S238A} mutation, primers P1 and P2 were used to amplify a 1,100 bp fragment and primers P3 and P4 were used to amplify a 898 bp fragment. These two fragments contained an overlapping region of the *cpc-3* ORF with the single amino acid mutation, which introduced a new *NheI* restriction site. These fragments were stitched together with primers P1 and P4 to amplify a 1,998 bp fragment. To create the *cpc-3*^{S238E} mutation, P1 and P2 were used to create a 1,100 bp fragment and P3 and P4 were used to create a 898 bp fragment. These two fragments contained an overlapping region of the *cpc-3* ORF with the single amino acid mutation and with a new *XhoI* restriction site. These fragments were stitched together with P1 and P4 to create a 1,998 bp fragment.

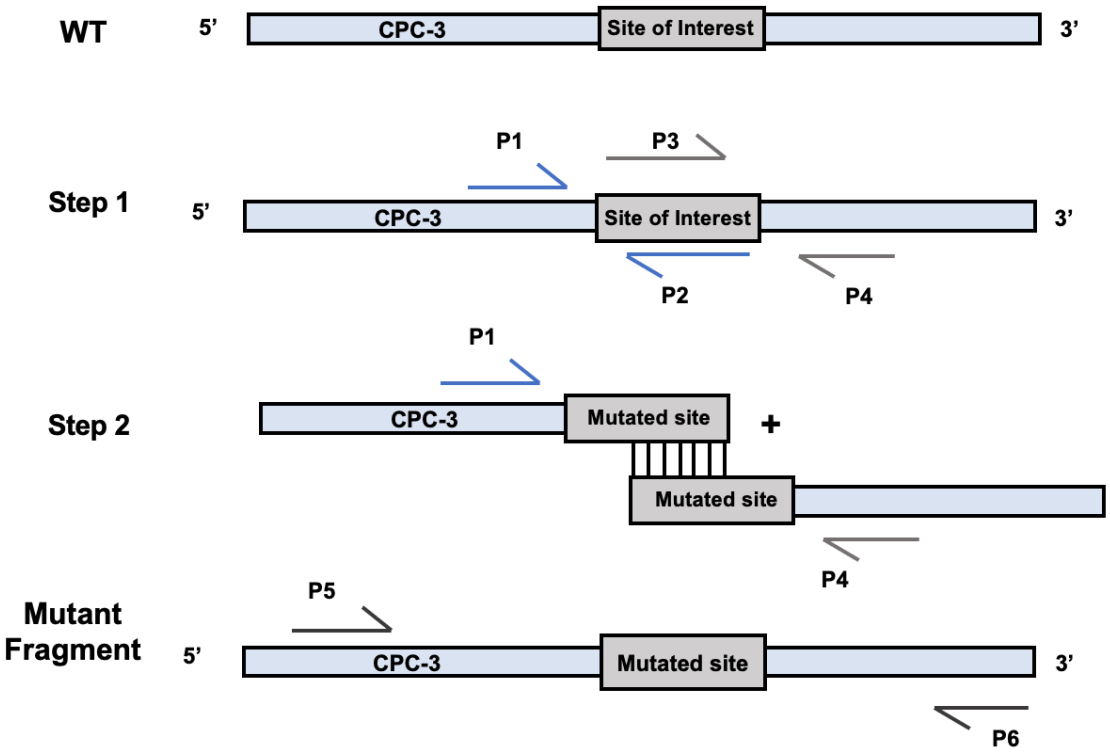

Figure 3.4: Representation of the generation of CPC-3 point mutations via two-way overlapping PCR. There are two main steps in this process. Step 1 involves two PCR reactions. The first uses P1 as the forward primer and P2 as the reverse primer. The second reaction uses P3 as the forward primer and P4 as the reverse primer. P2 and P3 have homologous sequences that are the same as WT CPC-3, except for the desired point mutation. Step two utilizes P1 as the forward primer and P4 as the reverse primer to stitch together the two fragments that were generated in step 1.

The PCR fragments were then ligated into the PCR blunt plasmid using the Zero Blunt PCR Cloning Kit (#K270020, Thermo-Fisher) and were transformed into DH5-alpha *Escherichia coli* cells. The plasmid was then extracted and confirmed to have the single site mutation via PCR with P5 and P6 (**Table 3.3)** and restriction digest **(Table 3.4)**. The plasmid was linearized with *EcoRI* and co-transformed into wild-type *N. crassa* cells

(FGSC#4200) with the hygromycin resistant pDBP301 plasmid into the *Δmus-52::bar* strain (FGSC#9719). Transformants were screened using the restriction sites that were generated along with the single amino acid mutations. A positive transformant was then crossed with WT cells (FGSC#4200) to obtain DBP# 3784 (*cpc-3*^{T879A} and DBP# 3714 (*cpc-3*^{T879E}) homokaryons. Progeny from the crosses were screened by PCR with P5 and P6 (**Table 3.3**) and the corresponding restriction enzyme (**Table 3.4)**.

Time Course Experiments. Circadian time course experiments were performed according to previously described methods [35]. Mycelial mats were grown in Volgel's media (2% glucose, 0.5% arginine, pH 6.0). The mats were then transferred to constant light at 30°C with shaking for a minimum of 4 hours to synchronize the clock to dusk for all cells. Flasks were periodically shifted into DD at 25°C according to the indicated times [35]. Harvested tissue was frozen immediately in liquid N_2.

Protein Extraction and Western Blotting. Protein was extracted from the harvested tissue as previously described [38] with changes to the extraction buffer. The extraction buffer was made with 100 mM Tris (pH 7.5), 1% SDS, 1X aprotinin, 1X leupeptin, 10 mM NaF, 1M β-glycerophosphate, 1X pepstatin, 1mM sodium ortho-vanadate and 1 mM PMSF. Protein concentration was determined via Nanodrop. 100µg of each protein sample was separated on a 10% SDS-PAGE gel and blotted to a Immobilon-P PVDF transfer membrane (#IPVH00010, Millipore-Sigma) according to the standard methods. The levels of P-eIF2α were detected with a rabbit monoclonal Anti-EIF2S1 antibody (#32157 AbCam) diluted 1:5000 in 5% Bovine Serum Albumin (BSA),

1X TBS, and 0.1% Tween. The secondary antibody was an anti-rabbit IgG HRP (#170-6515, Bio-Rad) diluted 1:10000. The levels of total eIF2α were detected with a rabbit polyclonal Anti-EIF2S1 antibody (#9722, Cell Signaling) diluted 1:5000 in 5% BSA, 1X TBS, and 0.1% Tween. An anti-mouse IgG HRP secondary antibody (#170-6515, Bio-Rad) diluted 1:10000 in 5% BSA, 1X TBS, and 0.1% Tween was used. Proteins were detected with chemiluminescence SuperSignal West Pic Substrate (#34577, Thermo-Fischer). Protein levels were measured using NIH ImageJ software system and were normalized to the protein loading. Protein loading was detected using an amino-black stain.

Statistical Analysis. Time course protein levels were fit to either a sine wave or a line using Prism from GraphPad software as previously described [35]. P-values indicate the probability that a sine wave best fits the time course data. Error bars on graphs indicate the standard error of the mean (SEM) from at least 3 experimental replicates. Significance between the WT and mutant period, amplitude, and phase values was determined with a student T-test.

Table 3.3 : PCR primers used to develop point mutations of CPC-3. P1 and P2 were used for the first PCR reaction. P3 and P4 were used for the second PCR reaction. To stick these two fragments together, P1 and P4 were used to generate the entire fragment.

Mutant	Primer Name	Sequence
T882A	P1	5'-GATGACGAGGACATTGCTGTG-3'
T882A	P2	5'-GCCAATGCTGCGCGCCATGTCGCTTGCATCCAAGC-3'
T882A	P3	5'-GACATGGCGCGCAGCATTGGCACAGCAGTTTATG-3'
T882A	P3	5'-GACGATGAACTGTCGCATGAG-3'
T882E	P1	5'-GATGACGAGGACATTGCTGTG-3'
T882E	P2	5'-GCCAATGGAGCGCTCCATGTCGCTTGCATCCAAGC-3'
T882E	P3	5'-GACATGGAGCGCTCCATTGGCACAGCAGTTTATGTC-3'
T882E	P4	5'-GCGTCTGAAGTGACAATGTC-3'
T882A and T882E	P5	5'-CTGGTCTCCAAGTTCTTCAAG-3'
T882A and T882E	P6	5'-GTCGCAATCTCATCCAGAAC-3'
T887A	P1	5'-GATGACGAGGACATTGCTGTG-3'
T887A	P2	5'-ATAAACTGCGGCGCCAATGCTCCTGGTCATGTC-3'
T887A	P3	5'-CATTGGCGCCGCAGTTTATGTCGC-3'
T887A	P4	5'-GTCTAAGGCGTCTGAAGTGAC-3'

Table 3.3 Continued

Mutant	Primer Name	Sequence
T887E	P1	5’-GATGACGAGGACATTGCTGTG-3’
T887E	P2	5’-GACGTATACTGCTTCGCCAATGCTCCTGGTCATGTC-3’
T887E	P3	5’-GAGCATTGGCGAAGCAGTATACGTC-3’
T887E	P4	5’-CAAGGTCTAAGGCGTCTGAAGTGAC-3’
T887A and T887E	P5	5’- CCAAGTTCTTCAAGTCCGACC-3’
T887A and T887E	P6	5’-GTCGCAATCTCATCCAGAAC-3’
S238A	P1	5’-CACTCACTTGTCCACTTCAC-3’
S238A	P2	5’-GTCAGGGGCTAGCTGATGGCTCCTGTTCT-3’
S238A	P3	5’-CAGCTAGCCCCTGACCGTGCTCCGCAAGAT-3’
S238A	P4	5’-GGTCGGACTTGAAGAACTTG-3’
S238E	P1	5’-CACTCACTTGTCCACTTCAC-3’
S238E	P2	5’-GTCAGGCTCGAGTTGATGGCTCCTGTTCTT-3’
S238E	P3	5’-CAACTCGAGCCTGACCGTGCTCCGCAAGAT-3’
S238E	P4	5’-GGTCGGACTTGAAGAACTTG-3’
S238A and S238E	P5	5’-GAATGGATGCTGTTGTCGTGG-3’
S238A and S238E	P6	5’-CCTCCTCCAGCCATGTATTG-3’

Table 3.4 Restriction sites introduced into each point mutation of CPC-3. Restriction sites were developed without altering the amino acids surrounding the mutation. The site of interest is bolded for emphasis.

Mutation	**Original Sequence**	**New Sequence**	**Restriction Site Introduced**
T882A	ATG**ACC**AGGAGC	ATG**GCG**CGCAGC	BssHII GCGCGC
T882E	ATG**ACC**AGGAGC	ATG**GAG**CGCTCC	AfeI AGCGCT
T887A	GGC**ACA**GCAGTTTAT	GGCGCCGCAGTTTAT	KasI GGCGCC
T887E	GGC**ACA**GCAGTTTAT	GGC**GAA**GCAGTATAC	BstZ17I-HF GTATAC
S238A	CAATTA**TCC**CCTGAC	CAGCTA**GCC**CCTGAC	NheI GCTAGC
S238E	CAATTA**TCC**CCTGAC	CAACTC**GAG**CCTGAC	XhoI CTCGAG

CHAPTER IV

IMPACT OF PHOSPHORYLATION SITE MUTATIONS ON THE INTEGRATED STRESS RESPONSE

Introduction

In eukaryotes, there are many biochemical mechanisms in place to combat environmental or internal stresses, particularly by inhibiting global translation via the phosphorylation of eIF2α. These pathways are often referred to as the Integrated Stress Response (ISR) [33]. Multiple kinases are involved in this stress response, including PKR (protein kinase double stranded RNA dependent) and HRI (heme regulated inhibitor) [39]. In *N. crassa*, the ISR kinase CPC-3 is activated in response to high levels of uncharged tRNAs, which accumulate during times of amino acid starvation. Studies of the structural homolog of CPC-3 in yeast (GCN2) have shown that a protein known as GCN1 directly binds to GCN2 in response to high levels of uncharged tRNAs and promotes its activity [26]. Deletions of GCN1 in *N. crassa* have shown to inhibit CPC-3 activity. However, this inhibition can be overcome by constitutively activating CPC-3 [23]. These data suggested that while the recruitment of GCN1 activity in response to uncharged tRNAs aids in the ISR, it is not the sole activator of CPC-3 in this pathway.

Since the potential autophosphorylation sites T874, T879, and S238 are predicted to influence CPC-3 activity, it is likely that they also affect the cell's ability to overcome stress. To test how mutations in these sites behaved when the cell was under amino acid starvation stress, I inoculated the mutant strains in media with added 3-amino-

1,2,4-triazole (3-AT). This compound induces a stress response in the cell by inhibiting the production of the amino acid histidine. In the T879A and T879E mutations, there was no CPC-3 activity with or without the presence of 3-AT. This indicates that the T879 is necessary for the cell's response to amino acid starvation and control of CPC-3 activity.

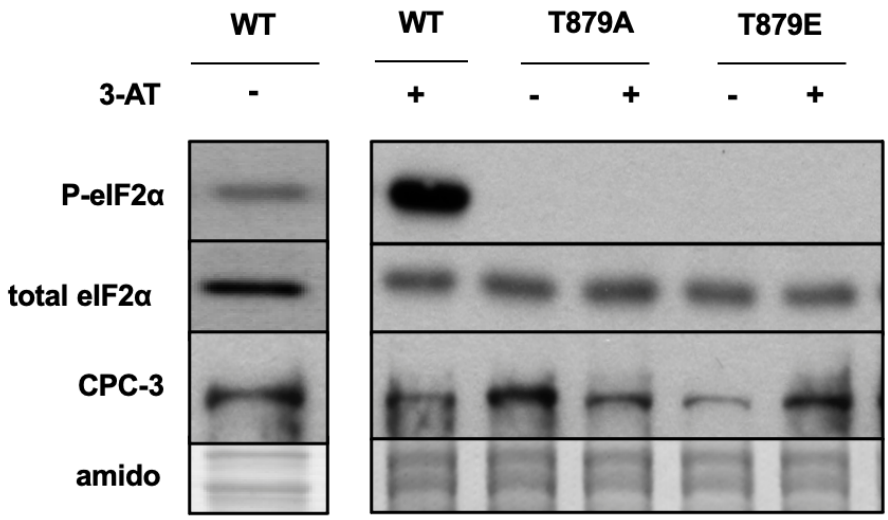

Figure 4.1 T879A and T879E prevent CPC-3 activation under amino acid starvation conditions. Germinated conidia were grown at 25°C in LL for 24 hours with shaking. P-eIF2α was undetectable in the T879A and T879E mutants with or without the presence of 3-AT.

Results

To determine if the phosphorylation site mutants affected the cell's ability to respond to amino acid starvation, each culture was grown with 3-amino-1,2,4-triazole (3-AT), which starves the cells of the amino acid histidine. Similar to normal growth conditions, both mutations at T879 (T879A and T879E) showed no detectable P-eIF2α in response to 3-AT (**Figure 4.1**). Furthermore, both CPC-3 and total eIF2α were present in the mutant strains, which indicates that these mutants are affecting the activity of CPC-3 rather than the levels of the kinase or the levels of total eIF2α. Based on these results, we can conclude that T879 phosphorylation is essential for the phosphorylation of eIF2α whether the cells are amino acid-depleted or not. Further tests are needed to determine if the T874E and S238E mutations have any effect on CPC-3 activity under these stress conditions.

Discussion

The Integrated Stress Response (ISR) is a key regulatory system in eukaryotes that regulates many pathways during stress conditions. Specifically, these pathways phosphorylate Ser51 of eIF2α, which inhibits global translation [33]. In *N. crassa,* we can induce a stress response in the cell by inhibiting histidine biosynthesis with a compound called 3-AT. Similar to unstressed conditions, both T879A and T879E mutants did not show P-eIf2α signals, indicating that CPC-3 activity is abolished. Based on these results, we can conclude that the phosphorylation and dephosphorylation of T879 is essential for CPC-3 activity when the cell is under normal or stress conditions. Moreover, we can speculate that T879 control of CPC-3 is able to overcome the natural

stress response when high levels of uncharged tRNAs accumulate in the cell. Since GCN1 is primarily involved in sensing high levels of uncharged tRNAs in the cell and activates CPC-3 as a response [26], we can hypothesize that the phosphorylation state of T879 is inhibiting GCN1 binding to CPC-3. Future studies could determine if mutating T879 prevents GCN1 binding by performing co-immunoprecipitation assays of CPC-3 and GCN1.

Despite the result that T874E and S238E mutations do not affect the rhythms of CPC-3 activity (Chapter 3), several attempts were made to determine if these mutants affect the phosphorylation of eIF2α under stress conditions. However, the results were inconsistent and therefore need to be repeated. In any case, we can speculate that these mutations may affect the time-of-day difference in P-eIF2α levels that were observed in the previous time course experiments only when stress levels are high. If this is the case, these two phosphorylation sites may be regulators of CPC-3 activity under amino acid starvation conditions.

Materials and Methods

Strains and Growth Conditions. Strains of *N. crassa* were grown as previously described [34]. All WT and mutant strains were maintained on Vogel's minimal media (1X Vogel's, 2% glucose).

3-AT Assay. Since the T879 mutations showed no detectable P-eIF2α in WT conditions, these mutants were not assayed at specific times of the day. Germinated conidia were grown at 25 degrees Celsius in liquid V2G media (2% glucose, 1X Vogel's) for 24 hours (LL24) with constant shaking. 1 hour before harvest, 9mM of 3-AT was added to the conidia. Immediately after harvest, tissue samples were frozen in liquid N_2.

Protein Extraction and Western Blotting. Protein was extracted from the harvested tissue as previously described [38] with changes to the extraction buffer. The extraction buffer was made with 100 mM Tris (pH 7.5), 1% SDS, 1X aprotinin, 1X leupeptin, 10 mM NaF, 1M β-glycerophosphate, 1X pepstatin, 1mM sodium ortho-vanadate and 1 mM PMSF. Protein concentration was determined via Nanodrop. 100µg of each protein sample was separated on a 10% SDS-PAGE gel and blotted to a Immobilon-P PVDF transfer membrane (#IPVH00010, Millipore-Sigma) according to the standard methods. The levels of P-eIF2α were detected with a rabbit monoclonal Anti-EIF2S1 antibody (#32157 AbCam) diluted 1:5000 in 5% Bovine Serum Albumin (BSA), 1X TBS, and 0.1% Tween. The secondary antibody was an anti-rabbit IgG HRP (#170-6515, Bio-Rad) diluted 1:10000. The levels of total eIF2α were detected with a rabbit polyclonal Anti-EIF2S1 antibody (#9722, Cell Signaling) diluted 1:5000 in 5% BSA, 1X TBS, and 0.1% Tween. An anti-mouse IgG HRP secondary antibody (#170-6515, Bio-Rad) diluted 1:10000 in 5% BSA, 1X TBS, and 0.1% Tween was also used. CPC-3 protein was detected using a rabbit polyclonal antibody (Genscript) diluted 1:1000 in 7.5% Milk, 1X TBS, and 0.1% Tween. A secondary anti-rabbit IgG antibody was used and diluted 1:10000 in 7.5% Milk, 1X TBS, and 0.1% Tween. Proteins were detected

with chemiluminescence SuperSignal West Pic Substrate (#34577, Thermo-Fischer). Protein levels were measured using NIH ImageJ software system and were normalized to the protein loading. Protein loading was detected using an amino-black stain.

CHAPTER V

CONCLUSION

Summary

The circadian clock is an intrinsic time-keeping mechanism that controls many biological pathways and systems based on environmental cues. It is widely known that the circadian clock is a key component in transcriptional and post-transcriptional control [12-14], but recent studies have provided evidence of clock-controlled translation and protein turnover in *N. crassa* and mammals [17-21]. One of these translational mechanisms is the rhythmic phosphorylation of the eukaryotic initiation factor 2 (eIF2) on its alpha subunit [23]. In WT conditions, this initiation factor is essential to form the pre-initiation complex [24, 25]. However, under stress conditions, kinases involved in the Integrated Stress Response (ISR) phosphorylate Ser 51 of eIF2α and as a result, block global translation [23, 33]. Since this response is conserved between many eukaryotes, including mammals, understanding how the clock controls the phosphorylation of eIF2α is important to understanding translational control. In *N. crassa,* a single kinase called CPC-3 is known to rhythmically phosphorylate eIF2α under control of the circadian clock [23]. Furthermore, recent studies from our lab have shown that it is the rhythmic activity of CPC-3 rather than the levels of the kinase that controls this rhythmicity [23]. However, the exact mechanism of rhythmic CPC-3 activation was not known.

We predicted that the activity of CPC-3 may be regulated similarly to GCN2 in *S. cerevisiae.* GCN2 kinase relies two autophosphorylation sites (T882 and T887) to control its activity [29, 30]. Based on post-translational modification mass spectrometry, we identified three potential sites of CPC-3 (T874, T879, and S238) that had a significant time of day difference in their phosphorylation levels. I then designed and created mutations that block phosphorylation at the specific site (converted site to alanine) and that mimic constant phosphorylation at the site (converted site to glutamic acid). Time course experiments showed that mimicking constant phosphorylation at T874 and S238 (T874E and S238E) did not alter the rhythmic accumulation of P-eIF2α. However, both mutations at T879 (T879A and T879E) completely abolished the phosphorylation of eIF2α. Similarly, when the cells were starved for the amino acid histidine, the T879 mutations still prevented CPC-3 activity.

Future Directions

We discovered that the activity of CPC-3 requires T879. Altering T879 to glutamic acid or alanine residues blocks kinase activity and suggested that cycling of this residue between phospho- and non-phospho states is required for kinase function. However, it is still not known what causes this phosphorylation or whether it is under control of the core circadian clock. First, future studies should aim to identify if T879 of CPC-3 is rhythmically phosphorylated, and if it is under the control of the clock. To do this, a P-T879 specific antibody would need to be produced. Time course experiments could then be performed on WT and a clock mutant strain. If this site is phosphorylated under control of the clock, the next step would be to determine the mechanism of T879

phosphorylation. One potential mechanism could be that this site is rhythmically auto-phosphorylated in a clock-controlled manner. Another potential mechanism is that either a clock-controlled kinase or a clock-controlled phosphatase is controlling the rhythmic phosphorylation at this site. Determining what other proteins are potentially involved in this process will bring additional insights into the stress response pathway in *N. crassa*.

Since neither single mutant of T874 or S238 showed an obvious phenotype in rhythmic kinase activity, mutants that prevent phosphorylation at both sites (T874A, S238A) and mutants that mimic constant phosphorylation at both sites (T874E, S238E) should be developed and analyzed in a time-course experiment. It is possible that mutations at these two sites cannot influence rhythmic CPC-3 activity on their own, but may influence the activity if both are affected. This trend was also seen in *S. cerevisiae,* as mutants that affected both the T882 and T887 autophosphorylation sites had a greater effect on *in vitro* kinase activity and growth rate compared to the single point mutations [29].

Understanding how the stress response in *N. crassa* is regulated by the circadian clock is an important model for studying circadian regulation in mammals, as eIF2 and CPC-3 kinase are conserved. Furthermore, eIF2α regulation and circadian regulation has been shown to be misregulated in many mammalian disorders, such as cancer [40, 41]. Understanding how CPC-3 regulates the rhythmic phosphorylation of eIF2α in *N. crassa* will provide insight into how the circadian clock affects translational regulation in higher eukaryotes.

REFERENCES

1. Panda, S., J.B. Hogenesch, and S.A. Kay, *Circadian rhythms from flies to human.* Nature, 2002. **417**(6886): p. 329-335.

2. Green, C.B., J.S. Takahashi, and J. Bass, *The Meter of Metabolism.* Cell, 2008. **134**(5): p. 728-742.

3. Roenneberg, T. and M. Merrow, *The Circadian Clock and Human Health.* Current Biology, 2016. **26**(10): p. R432-R443.

4. Bell-Pedersen, D., et al., *Circadian rhythms from multiple oscillators: lessons from diverse organisms.* Nature Reviews Genetics, 2005. **6**(7): p. 544-556.

5. Dunlap, J.C., *Molecular Bases for Circadian Clocks.* Cell, 1999. **96**(2): p. 271-290.

6. Hurley, J., J.J. Loros, and J.C. Dunlap, *Dissecting the mechanisms of the clock in Neurospora.* Methods Enzymol, 2015. **551**: p. 29-52.

7. McCluskey, K., A. Wiest, and M. Plamann, *The Fungal Genetics Stock Center: a repository for 50 years of fungal genetics research.* J Biosci, 2010. **35**(1): p. 119-26.

8. Cha, J., M. Zhou, and Y. Liu, *Mechanism of the Neurospora circadian clock, a FREQUENCY-centric view.* Biochemistry, 2015. **54**(2): p. 150-6.

9. He, Q., et al., *CKI and CKII mediate the FREQUENCY-dependent phosphorylation of the WHITE COLLAR complex to close the Neurospora circadian negative feedback loop.* Genes Dev, 2006. **20**(18): p. 2552-65.

10. Dunlap, J.C. and J.J. Loros, *The neurospora circadian system.* J Biol Rhythms, 2004. **19**(5): p. 414-24.

11. Schafmeier, T., et al., *Transcriptional feedback of Neurospora circadian clock gene by phosphorylation-dependent inactivation of its transcription factor.* Cell, 2005. **122**(2): p. 235-46.

12. Koike, N., et al., *Transcriptional architecture and chromatin landscape of the core circadian clock in mammals.* Science, 2012. **338**(6105): p. 349-54.

13. Menet, J.S., et al., *Nascent-Seq reveals novel features of mouse circadian transcriptional regulation.* Elife, 2012. **1**: p. e00011.

14. Preußner, M. and F. Heyd, *Post-transcriptional control of the mammalian circadian clock: implications for health and disease.* Pflugers Arch, 2016. **468**(6): p. 983-91.

15. Hurley, J.M., et al., *Circadian Proteomic Analysis Uncovers Mechanisms of Post-Transcriptional Regulation in Metabolic Pathways.* Cell Syst, 2018. **7**(6): p. 613-626.e5.

16. Reddy, A.B., et al., *Circadian orchestration of the hepatic proteome.* Curr Biol, 2006. **16**(11): p. 1107-15.

17. Hershey, J.W., N. Sonenberg, and M.B. Mathews, *Principles of translational control: an overview.* Cold Spring Harb Perspect Biol, 2012. **4**(12).

18. Cao, R., et al., *Translational control of entrainment and synchrony of the suprachiasmatic circadian clock by mTOR/4E-BP1 signaling.* Neuron, 2013. **79**(4): p. 712-24.

19. Jouffe, C., et al., *The circadian clock coordinates ribosome biogenesis.* PLoS Biol, 2013. **11**(1): p. e1001455.

20. Wang, R., et al., *Circadian control of stress granules by oscillating EIF2α.* Cell Death Dis, 2019. **10**(3): p. 215.

21. Pathak, S.S., et al., *The eIF2α Kinase GCN2 Modulates Period and Rhythmicity of the Circadian Clock by Translational Control of Atf4.* Neuron, 2019. **104**(4): p. 724-735.e6.

22. Caster, S.Z., et al., *Circadian clock regulation of mRNA translation through eukaryotic elongation factor eEF-2.* Proc Natl Acad Sci U S A, 2016. **113**(34): p. 9605-10.

23. Karki, S., et al., *Circadian clock control of eIF2α phosphorylation is necessary for rhythmic translation initiation.* Proceedings of the National Academy of Sciences, 2020: p. 201918459.

24. Aylett, C.H. and N. Ban, *Eukaryotic aspects of translation initiation brought into focus.* Philos Trans R Soc Lond B Biol Sci, 2017. **372**(1716).

25. Stolboushkina, E.A. and M.B. Garber, *Eukaryotic type translation initiation factor 2: structure-functional aspects.* Biochemistry (Mosc), 2011. **76**(3): p. 283-94.

26. Castilho, B.A., et al., *Keeping the eIF2 alpha kinase Gcn2 in check.* Biochim Biophys Acta, 2014. **1843**(9): p. 1948-68.

27. Krishnamoorthy, T., et al., *Tight binding of the phosphorylated alpha subunit of initiation factor 2 (eIF2alpha) to the regulatory subunits of guanine nucleotide exchange factor eIF2B is required for inhibition of translation initiation.* Mol Cell Biol, 2001. **21**(15): p. 5018-30.

28. Ross, J.A., K.R. Bressler, and N. Thakor, *Eukaryotic Initiation Factor 5B (eIF5B) Cooperates with eIF1A and eIF5 to Facilitate uORF2-Mediated Repression of ATF4 Translation.* Int J Mol Sci, 2018. **19**(12).

29. Romano, P.R., et al., *Autophosphorylation in the activation loop is required for full kinase activity in vivo of human and yeast eukaryotic initiation factor 2alpha kinases PKR and GCN2.* Mol Cell Biol, 1998. **18**(4): p. 2282-97.

30. Qiu, H., et al., *Mutations that bypass tRNA binding activate the intrinsically defective kinase domain in GCN2.* Genes Dev, 2002. **16**(10): p. 1271-80.

31. Sattlegger, E., A.G. Hinnebusch, and I.B. Barthelmess, *cpc-3, the Neurospora crassa homologue of yeast GCN2, encodes a polypeptide with juxtaposed eIF2alpha kinase and histidyl-tRNA synthetase-related domains required for general amino acid control.* J Biol Chem, 1998. **273**(32): p. 20404-16.

32. Zhang, R., et al., *A circadian gene expression atlas in mammals: implications for biology and medicine.* Proc Natl Acad Sci U S A, 2014. **111**(45): p. 16219-24.

33. Pakos-Zebrucka, K., et al., *The integrated stress response.* EMBO Rep, 2016. **17**(10): p. 1374-1395.

34. Rowland H. Davis, F.J.d.S., *Genetic and Microbiological Research Techniques for Neurospora crassa.* Methods Enzymol, 1970. **17**: p. 79-143.

35. Lamb, T.M., et al., *Direct Transcriptional Control of a p38 MAPK Pathway by the Circadian Clock in Neurospora crassa.* PLOS ONE, 2011. **6**(11): p. e27149.

36. Cherkasova, V.A. and A.G. Hinnebusch, *Translational control by TOR and TAP42 through dephosphorylation of eIF2alpha kinase GCN2.* Genes Dev, 2003. **17**(7): p. 859-72.

37. Ramanathan, C., et al., *mTOR signaling regulates central and peripheral circadian clock function.* PLoS Genet, 2018. **14**(5): p. e1007369.

38. Jones, C.A., S.E. Greer-Phillips, and K.A. Borkovich, *The Response Regulator RRG-1 Functions Upstream of a Mitogen-activated Protein Kinase Pathway Impacting Asexual Development, Female Fertility, Osmotic Stress, and Fungicide Resistance in Neurospora crassa.* Mol Cell Bio, 2007. **18**(6): p. 2123-2136.

39. Donnelly, N., et al., *The eIF2α kinases: their structures and functions.* Cell Mol Life Sci, 2013. **70**(19): p. 3493-511.

40. Fu, L. and N.M. Kettner, *The circadian clock in cancer development and therapy.* Prog Mol Biol Transl Sci, 2013. **119**: p. 221-82.

41. Lobo, M.V., et al., *Levels, phosphorylation status and cellular localization of translational factor eIF2 in gastrointestinal carcinomas.* Histochem J, 2000. **32**(3): p. 139-50.