SERINE THREONINE KINASE STK-10 IS NECESSARY FOR RHYTHMIC PHOSPHORYLATION OF EUKARYOTIC TRANSLATION INITIATION FACTOR EIF2α

A Thesis

by

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ABSTRACT

The circadian clock is a conserved endogenous time keeping mechanism that controls up to half of the eukaryotic genome at the level of transcript abundance. In addition to clock control of transcript abundance, our lab discovered that the *Neurospora crassa* clock controls mRNA translation through the rhythmic and inhibitory phosphorylation of eukaryotic translation initiation factor subunit, eIF2α. In wild type *N. crassa*, phosphorylated eIF2α (P-eIF2α) peaks during the day and troughs at night, resulting in increased mRNA translation at night. *N. crassa* CPC-3, the homolog of yeast and mammalian GCN2, is the kinase responsible for eIF2α phosphorylation. Several regulators are known to control GCN2 activity in yeast and mammalian cells. My goal is to determine if any of these regulators affect rhythmic activity of CPC-3 and if so, determine how that regulator's action is clock-controlled. To accomplish this goal, I assayed P-eIF2α levels in strains deleted for the putative regulators. I discovered that P-eIF2α levels remained rhythmic in Δ*reg1* and Δ*eIF4e3* cells. However, in strains deleted for *stk-10*, P-eIF2α levels were high and arrhythmic. STK-10 is a serine/threonine kinase that may be regulated by the nutrient sensing TOR pathway. Under nutrient rich conditions, TORC1 is active, phosphorylating and activating STK-10. Active STK-10 may then phosphorylate and inhibit CPC-3 kinase activity, leading to low levels of P-eIF2α and increased mRNA translation. Thus, the requirement of *N. crassa* STK-10 for rhythmic P-eIF2α levels suggests a model where at night, TORC1 activates STK-10, and STK-10 phosphorylates and inhibits CPC-3 kinase activity, under control of the clock. This would lead to low levels of P-eIF2α, and increased mRNA translation at night. Experiments are currently underway to test this model and to determine if rhythmic activity or abundance of STK-10 is responsible for the rhythmic activity of CPC-3.

DEDICATION

I would like to dedicate this work to my mother and sister who were able to support me financially and emotionally when I needed it most throughout my academic career. Thank you for your support.

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TABLE OF CONTENTS

Page

LIST OF FIGURES

Page

LIST OF TABLES

Page

CHAPTER I

INTRODUCTION

Endogenous Time Keeping Mechanism

The circadian clock is a conserved endogenous time keeping mechanism that generates daily rhythmic behaviors and activities in a wide range of organisms [1-3]. Organisms rely on their circadian clock to anticipate changes in their environment to allow them to prepare for the daily rhythms in environmental conditions and increase their fitness [2]. Understanding how circadian clocks operate may best be determined at the genetic level by performing mutagenic screens for potential clock proteins to observe if there are any alterations to the function of the organism's clock. Such screens have revealed clock genes within various model organisms such as *period (per)* in *Drosophila melanogaster* and *frequency (frq)* in *N. crassa* [3, 4]. While core clock genes of different organisms may not be conserved in sequence, they act in a conserved regulatory loop, with positive elements that drive the transcription of negative elements [2, 4]. These negative elements in turn are translated and feed back to inhibit the activity of the positive elements [2, 4]. Oscillations of these core clock genes typically occur with an ~24-hour period and the central oscillator drives not only transcription of the negative elements, but also of other genes designated clock-controlled genes (ccgs) which leads to vert rhythmic phenotypes [5, 6].

N. crassa as model organism for circadian clock study

Circadian rhythms have been studied extensively in the model organism *N. crassa* in which the FRQ/WHITE COLLAR oscillator (FWO) consists of a transcriptional/translational feedback loop

[7]. The positive elements of the FWO consist of WHITE COLLAR-1 and WHITE COLLAR-2 (WC-1 and WC-2) which heterodimerize to form the WHITE COLLAR complex (WCC) [8]. The negative elements of the FWO are FRQ and FRQ-interacting RNA Helicase (FRH) which heterodimerizes to form the FRQ/FRH complex (FFC) [7]. Oscillations of these core clock components occur as the WCC binds to the promoter of *frq* to activate its transcription. The WCC also binds to the promoters and activates transcription of downstream clock-controlled genes (ccgs) [9]. Throughout the day, FRQ protein accumulates and dimerizes with FRH to form the FFC which promotes the inhibitory phosphorylation of WCC that leads to a decrease in *frq* transcription [9, 10]. As FRQ protein degrades over time, WCC can once again be activated, and the clock-controlled transcription of *frq* and downstream cgs begins again the next day [9, 10].

Evidence of clock-regulated translation

Genome-wide experiments have shown that up to 50% of the eukaryotic transcriptome is clock controlled [11-13]. Furthermore, recent evidence demonstrates clock control of post-transcriptional events, including mRNA capping, splicing, polyadenylation, and de-adenylation [12, 14-19]. In fact, proteomic analysis in *N. crassa* and mammalian cells revealed that ~50% of rhythmic proteins are expressed from non-cycling mRNAs [11], suggesting that rhythmic protein accumulation arises from temporal protein degradation and/or mRNA translation. In support of clock control of translation, the levels and modification of several translation initiation factors, or upstream regulators, accumulate rhythmically in *N. crassa*, mammals, and *Drosophila*. [11, 20-22], including rhythmic accumulation and phosphorylation of translation initiation factor eIF2α in mouse liver and brain [23]. Furthermore, the activity of translation elongation factor eEF-2 is controlled by the *N. crassa* clock through rhythmic activation of the p38 MAPK pathway and the

downstream eEF-2 kinase RCK-2 [24]. However, the mechanisms and extent of clock regulation of translation initiation are poorly understood.

Initiation of translation in eukaryotes

In eukaryotic cells, translation initiation begins when GTP and the initiator methionyl tRNA (Met-tRNA) bind to eukaryotic translation initiation factor 2 (eIF2) to form the ternary complex that is then transferred to the 40S ribosomal subunit [25]. The binding of the 40S ribosomal subunit forms the pre-initiation complex (PIC) [26]. Utilizing eIF4F complex (eIF4FE, eIF4FG, and eIF4FA), the PIC will then bind to the 7-methyl guanine cap of the mRNA and scan the mRNA until reaching an appropriate start codon [27]. GTP bound to the γ subunit of eIF2 is hydrolyzed to GDP, the remaining initiation factors are released from the 40S ribosomal subunit, and the 60S ribosomal subunit joins to form the functional 80S ribosome [26]. When the α subunit of eIF2 is phosphorylated on the Ser51 residue, the GDP-bound eIF2 complex will then act as a competitive inhibitor of the guanine nucleotide exchange factor, eIF2B [27]. This inhibition of eIF2B prevents the transfer of GTP for GDP on the eIF2 complex, therefore inhibiting the next round of translation initiation. Four different kinases are responsible for phosphorylating eIF2α in mammals, each under different stimuli: HRI (heme-regulated inhibitor), PKR (protein kinase double-stranded RNA dependent kinase), PERK (PKR-like ER kinase), and GCN2 (general control non-derepressible-2) [28]. However, the GCN2 kinase is the only one that is conserved from fungi to mammals, and the *N. crassa* homolog of GCN2 is CPC-3 [16].

Regulation of GCN2 kinase

The roles of GCN2 have been studied in various organisms; in plants it was identified as an essential component for growth in stress conditions, while studies in mice suggested that it may enhance certain cancer treatments that involve amino acid starvation. [31, 32]. Given the diverse ways that GCN2 can affect the cellular environment, it's imperative that its activity be tightly regulated. For example, in *Saccharomyces cerevisiae*, GCN2 was first found to be activated in a signaling pathway involved in sensing amino acid starvation, which is signaled with a buildup of uncharged tRNAs [29, 33, 34]. Furthermore, in *S. cerevisiae* studies have uncovered the potential for translation elongation factors eEF1A and eEF3 to play a role in keeping GCN2 inactive in environments of sufficient nutrients [35, 36]. In addition to GCN2-regulated amino acid starvation responses, the TOR (target of rapamycin) pathway also senses amino acid depletion through the Tor containing protein complex 1 (TORC1) protein kinase. TORC1 directly phosphorylates and activates ribosomal protein S6 kinase (S6K) in mammals and Sch9 in yeast which leads to an increase of protein synthesis [37, 38]. Studies have shown that GCN2 can be activated by the inactivity of TORC1 in a mechanism that leads to the dephosphorylation of GCN2 on Ser-577 [39, 40]. Experiments in yeast suggested that dephosphorylation of Ser-577 may be sufficient for the ability of GCN2 to bind to deacylated tRNAs, as an S577A substitution led to an increased affinity for GCN2 and uncharged tRNAs. [41].

The role of CPC-3 in circadian regulation of translation initiation

Previous findings from our lab demonstrated the significant role CPC-3 has in the circadian regulation of translation initiation in *N. crassa*. It was discovered that eIF2α was not

phosphorylated when the *cpc-3* gene was deleted from the genome; however, complementing the knockout strain with a wild type copy of *cpc-3* restored rhythmic phosphorylation of eIF2α [42]. Furthermore, it was also demonstrated that the rhythmic activation, and not rhythmic CPC-3 abundance, of CPC-3 was responsible for the rhythmic accumulation of P-eIF2α levels [42]. From these data we can conclude that global translation initiation is, at least in part, regulated by the clock through CPC-3; however, how CPC-3 activity is controlled by the clock was not known, and is the focus of this study [42].

Objective

Clock control of transcription regulatory pathways has been widely studied in different model systems, but rhythmic translation mechanisms have only recently been investigated. Our lab is studying how rhythmic translation initiation is regulated in *N. crassa* through rhythmic phosphorylation of eIF2α by CPC-3 kinase. Using knowledge about the regulation of the CPC-3 homolog, GCN2, I propose that similar regulatory mechanisms exist for CPC-3 and may be the mechanism by which the clock impinges on CPC-3 activity.

CHAPTER II

SCH9 HOMOLOG STK-10 IS REQUIRED FOR RHYTHMIC PHOSPHORYLATION OF EIF2α

Introduction

The basis for circadian rhythms can be found at the molecular level through oscillations in clock genes, which were originally uncovered when organisms containing mutations in these genes led to an observable arrhythmic phenotype once placed in constant light (LL) or dark (DD) conditions [43, 44]. Clock genes are responsible for maintaining the endogenous rhythms associated with circadian cycles and are involved in regulating downstream gene expression [45]. Indeed, up to half of the eukaryotic genome is regulated by the circadian clock at the level of mRNA transcript abundance [46, 47]. Proteomic studies have also shown that up to 40% of observed clock-regulated proteins were not accounted for by cycling mRNA transcripts [46, 48]. These studies suggested that rhythms in post-transcriptional and post-translational processes are responsible for these observed rhythmic protein levels. Moreover, studies from our lab have demonstrated that translation is regulated by the clock in *N. crassa* through the eukaryotic elongation factor eEF-2 and through rhythmic phosphorylation of eukaryotic initiation factor, eIF2 [42, 49]. In regard to regulating translation initiation, we know that CPC-3 kinase is rhythmically activated to phosphorylate eIF2α during the subjective day, yet there is limited understanding of the mechanism of this activation. Based on what's known about GCN2 regulators, we can postulate that they may also act similarly in *N. crassa* to control CPC-3 activity. We will test CPC-3 activity in *N. crassa* homologs of GCN2 regulators, eEF1a, eIF4e3, PPH-1, REG1, KOG1, and STK-10 by examining P-eIF2α levels throughout a circadian time course in DD. This will allow us to

determine which regulators have a potential role in the circadian regulation of CPC-3 activity. Elucidation of circadian mechanisms may allow for potential therapies against circadian-related disorders, such as certain forms of cancers and metabolic disease [2].

GCN2 in yeast and mammals was found to be regulated through various pathways including through translation elongation factors such as eukaryotic elongation factor, eEF1A [50]. This elongation factor is responsible for bringing charged tRNAs to the acceptor site in ribosomes and acts as an inhibitor of GCN2. Specifically, when purified eEF1A was present with GCN2 *in vitro*, it resulted in lower efficiency of GCN2 to phosphorylate its substrate, eIF2α. [50]. Additionally, dysfunctional translation initiation factors have been shown to cause defects in growth [51, 39]. For example, studies revealed that the initiation factor responsible for binding the mRNA 5' cap, eIF4E, is needed for normal growth in cells. This was observed when reduced expression of eIF4E in HeLa cells led to a decrease in global translation [50]. The eIF4E family consists of three members: eIF4E1, eIF4E2, and eIF4E3, the latter of which may be involved in inhibitory mechanisms of eIF4E1, the prominent eIF4E family member involved with 5' cap-binding. [51]. Studies have shown that eIF4E3 may also act as a tumor suppressor [51, 52]. Due to the interactions of eIF4E3 in translation initiation, it is possible that it may play a role in regulating CPC-3 in *N. crassa*.

Three different phosphorylation sites on GCN2 are involved in regulating its activation: Ser-577, Thr-882, and Thr-887, the latter two of which are auto-phosphorylation sites that are required for activation of GCN2 [53]. When Ser-577 is phosphorylated, GCN2 activity is inhibited, and it is believed that inactivity of TOR (target of rapamycin) is involved in Ser-577 dephosphorylation [39, 55]. One of the phosphatases thought to be involved in dephosphorylating Ser-577 is the serine/threonine protein phosphatase PP2A [56]. This heterotrimeric phosphatase consists of a

structural A subunit, one of at least 3 distinct regulatory B subunits, and one catalytic C subunit. [57]. In *N. crassa*, the catalytic subunit of PP2A is encoded by the *pph-1* gene. Another phosphatase, protein phosphatase type 1 (PP1), in yeast is encoded by the essential gene *glc7* and its homolog in mammals was shown to regulate protein synthesis and glycogen metabolism [58-60]. A regulatory subunit of PP1, REG1, was found to interact with GLC7 and this interaction is necessary for glucose repression [60]. Another study showed that REG1 acts as a negative regulator of Snf1, which is itself an activator of GCN2 [61]. Taken together, the data support that REG1 is an inhibitor of GCN2 activity. In *N. crassa*, the homolog of the REG1 subunit is encoded by the *pph-12* gene [62].

As previously mentioned, cross talk between the TOR pathway and GCN2 occurs during times of nutrient deprivation or under general stress conditions [37-40]. TOR is a conserved serine/threonine kinase complex consisting of two multiprotein complexes in most eukaryotes, TORC1 and TORC2. Of these, TORC1 was found to be involved in the rapamycin sensitive pathways of TOR-mediated signaling [37]. Fluctuations in hormone levels, nutrients, and growth factors can all lead to the activation of TORC1 [63, 64]. One of the substrates of TORC1 is the serine/threonine kinase S6K1 in mammals, the homolog of which is Sch9 in yeast cells [63]. Studies have shown that phosphorylation of S6K1/Sch9 leads to an increase in translation while defects in this gene results in deficits in transcription and translation [37, 65-67]. In *S. cerevisiae*, TORC1 consists of interactions of either TOR1 or TOR2 with KOG1, LST8, and Tco89 [68-70]. However, the interaction between TOR and KOG1 appears to be responsible for the sensitivity of TORC1 to rapamycin as rapamycin may inhibit KOG1 to successfully present substrate to the catalytic domain of TOR [71]. The *N. crassa* homolog of yeast Sch9 is the STK-10 kinase, and the homolog of yeast KOG1 is also named KOG1 in *N. crassa*.

Results

Regulators KOG1, eEF1A, and PP2A catalytic subunit are essential in *N. crassa*

The deletion strains Δ*kog1::hph*, *Δeef1a::hph*, and Δ*pph-1::hph* were obtained as heterokaryons from the Fungal Genetics Stock Center (FGSC) (**Table 2.1**). While these strains were confirmed to contain a knockout of the indicated gene (Jana Gomez, unpublished), as heterokaryons, they also carried a wild type copy of that gene. To isolate homokaryotic knockout strain, I crossed each heterokaryon knockout strain with wild type *N. crassa* 74-OR23-1V of appropriate mating type, either DBP984 (mating type A) or DBP945 (mating type a). After crossing, picking and germinating single spores, I screened the progeny by growing them on media containing hygromycin and then by PCR for the progeny that grew best on the selective medium to examine if they have knockout or WT genotypes. However, none of the viable progeny were confirmed to carry the knockouts of *kog1, eef1a,* and *pph-1* genes (**Table 2.2**). This suggests that these three genes are necessary for growth in *N. crassa* and therefore, we were unable to test the role of these genes in promoting CPC-3 activity rhythms. However, three more knockout strains Δ*pph-12*, Δ*eif4e3*, and Δ*stk-10* were confirmed to be homokaryons and thus were tested for cycling P-eIF2α levels.

Table 2.1: *N. crassa* strains used in this study

Genotype	Note(s)	DBP#
Wild type, *mat A*	FGSC#4200	985
Wild type, *mat a*	FGSC#2489	984
Δ*kog1::hph, mat a*	NCU00621 FGSC#16121	3421
Δ*eef1a::hph, mat a*	NCU07437 FGSC#13223	3423
Δ*pph-1::hph, mat a*	NCU06630 FGSC#23717	3389
Δpph-*12::hph, mat a*	NCU09310 FGSC#21131	3386
Δ*eif4e3::hph, mat A*	NCU09546 FGSC#23514	3388
Δ*stk-10::hph, mat a*	NCU03200 FGSC#14226	3422
ras^{bd}*, mat A*	FGSC#1858	369
Δ*stk-10::hph,* ras^{bd}*, mat a*	Generated in this study	3721
frq::luc::bar, mat A	(see DBP#)	1563
Δ*stk-10::hph,* *frq::luc::bar, mat a*	Generated in this study	3720

Table. 2.2: Summary of the genetic crosses between the heterokaryon and WT

Strains crossed	Spores Picked	Spores Germinated (%)	% Hygromycin-resistant Progeny
Δ*kog1, mat a* Wild type, *mat A*	72	59	0
Δ*eef1a, mat a* Wild type, *mat A*	72	57	0
Δ*pph-12, mat a* Wild type, *mat A*	72	47	0

Rhythms in P-eIF2α were observed in WT, Δ*pph-12*, and Δ*eif4e* cells but not in Δ*stk-10* cells

To determine if regulators of GCN2 also regulate the rhythmic activity of CPC-3, Δ*pph-12,* Δ*eif4e3*, and Δ*stk-10* strains, were examined in a circadian time course to assay P-eIF2α levels. Protein was extracted from *N. crassa* mycelia grown in liquid V2G media in DD and harvested every 4 hours over a two-days. This circadian time course was used to evaluate total eIF2α as well as P-eIF2α levels over circadian time. As expected, in WT cells the levels of P-eIF2α cycled daily, peaking in the subjective day (DD16 and DD40), and troughing in the subjective night (DD28) (**Figure 2.1A**) [28]. P-eIF2α levels were also rhythmic in Δ*pph-12* cells (**Figure 2.1B**) and Δ*eif4e3* cells (**Figure 2.1C**). However, in Δ*stk-10* cells, the levels of P-eIF2α appeared to be arrhythmic

with no apparent peak or trough (**Figure 2.1D**). Furthermore, to confirm that the observed rhythms of P-eIF2α levels in WT cells were not due to cycling of eIF2α, protein samples from the time courses for both WT and Δ*stk-10* were probed using anti total eIF2α antibodies. For both WT (**Figure 2.2A**) and Δ*stk-10* cells (**Figure 2.2B**), the eIF2α levels were relatively constant throughout the circadian time course, supporting that rhythmic P-eIF2α levels observed in WT cells (**Figure 2.2A**) were not due to cycling eIF2α levels [28].

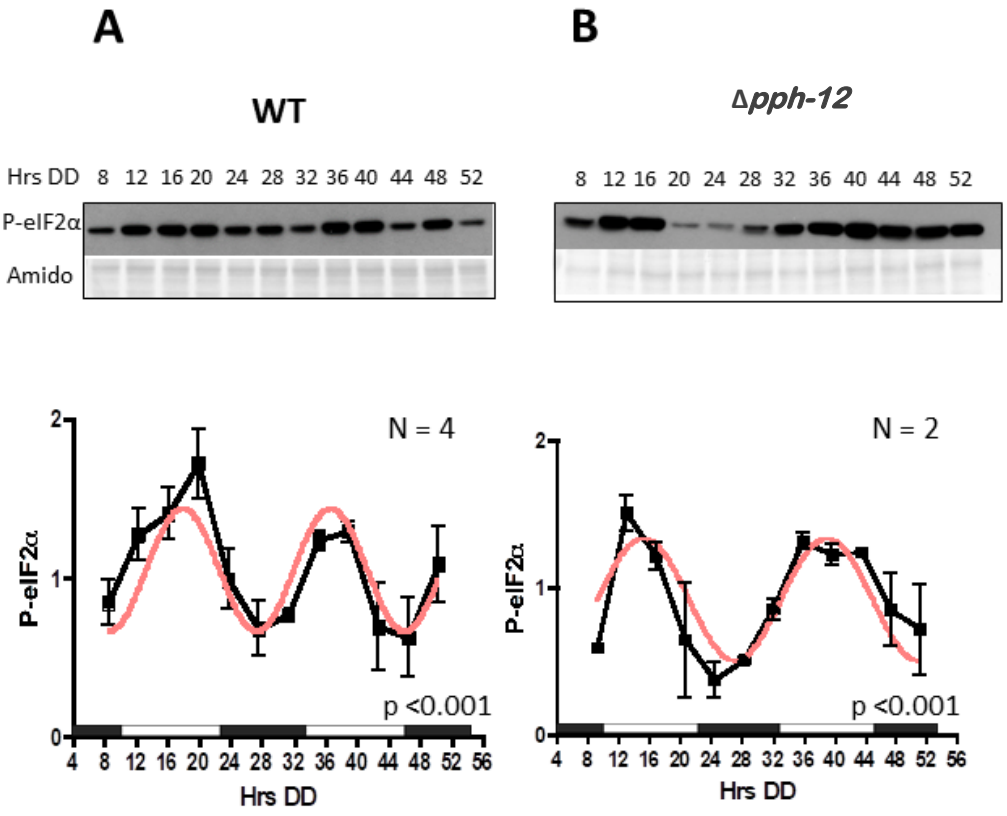

Figure 2.1. **Rhythms in P-eIF2α levels require *stk-10***. Representative western blots of WT (A), Δ*pph-12* (B), Δ*eif4e3* (C) and Δ*stk-10* (D) cells grown in a circadian time course and probed with anti-P-eIF2α antibody. Total protein loaded is shown with the amido black-stained membranes. Plots of the data (mean ± SEM) show the average P-eIF2α signal normalized to total protein (solid black line). Rhythmicity of P-eIF2α in cells of WT (A), Δ*pph-12* (B), and Δ*eif4e* (C) was determined using F tests of fit of the data to a sine wave (solid red line; P <0.001). P-eIF2α levels in Δ*stk-10* (D) cells were arrhythmic as indicated by a better fit of the data to a line (solid red line). The subjective day and night are represented by the white and black bars, respectively, on the bottom of the plots.

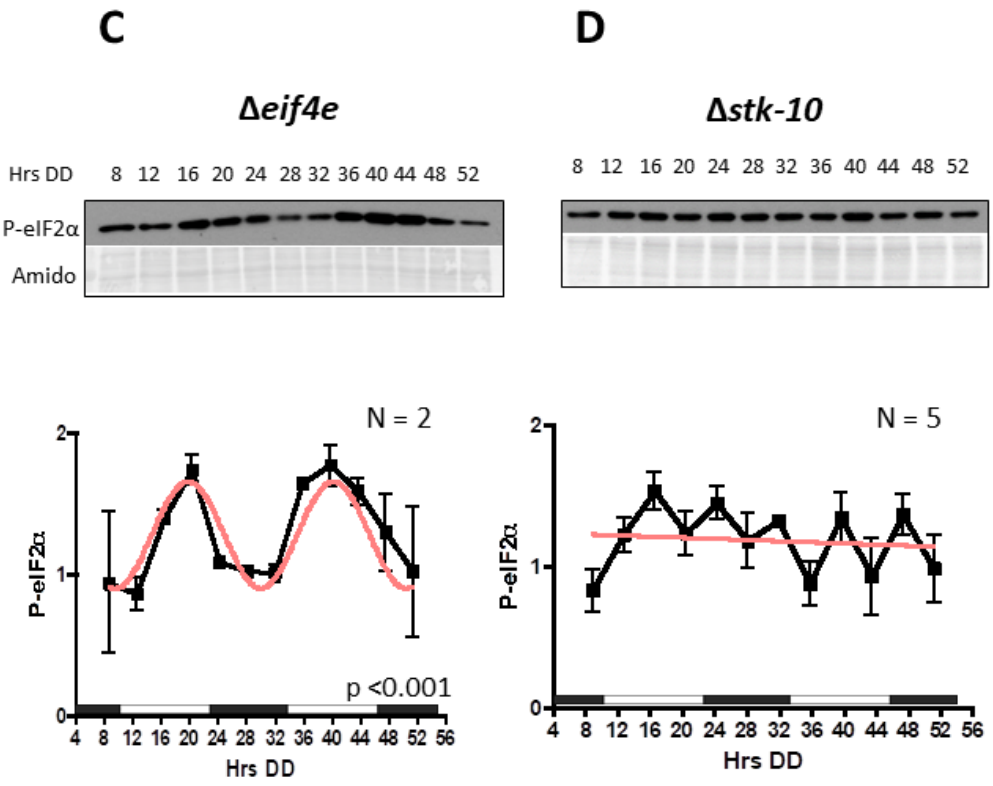

Figure 2.1 Contnued.

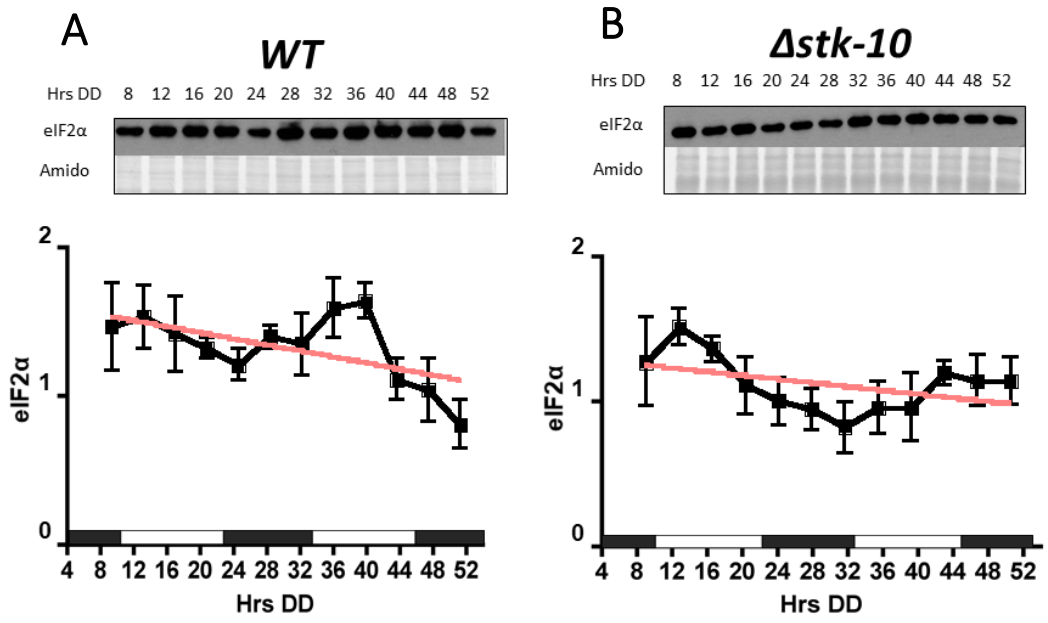

Figure 2.2. **Total eIF2α levels are not clock-controlled**. Western blots of protein isolated from cultures harvested over a circadian cycle and probed with anti-total eIF2α antibody. The data are plotted below (mean ± SEM; n = 3) with day and night bars below the plotted data. In both WT (A) and Δ*stk-10* (B) strains, eIF2α levels were arrhythmic in F tests of fit of the data to a line.

P-eIF2α levels are higher at night in Δ*stk-10* cells

While P-eIF2α levels appeared to be at constant higher levels in the Δ*stk-10* strain compared to WT (**Figure 2.2**), it is difficult to make a direct abundance comparison since these proteins were run on different gels. Thus, protein from both *Δstk-10* and WT cells were loaded onto a western gel side by side to observe differences in P-eIF2α levels between the two strains. Protein samples from DD28 and DD40 timepoints were used. These time points represent the trough (DD28) and peak (DD40) of P-eIF2α levels in WT cells. As expected, there was a time-of-day difference in WT cells between the subjective day and night (**Figure 2.3**); however; this time-of-day difference was not observed in Δ*stk-10* cells. Furthermore, the levels of P-eIF2α were high during the

subjective night (DD28) in Δ*stk-10* cells compared to WT (**Figure 2.3**), suggesting that STK-10 inhibits CPC-3 activity.

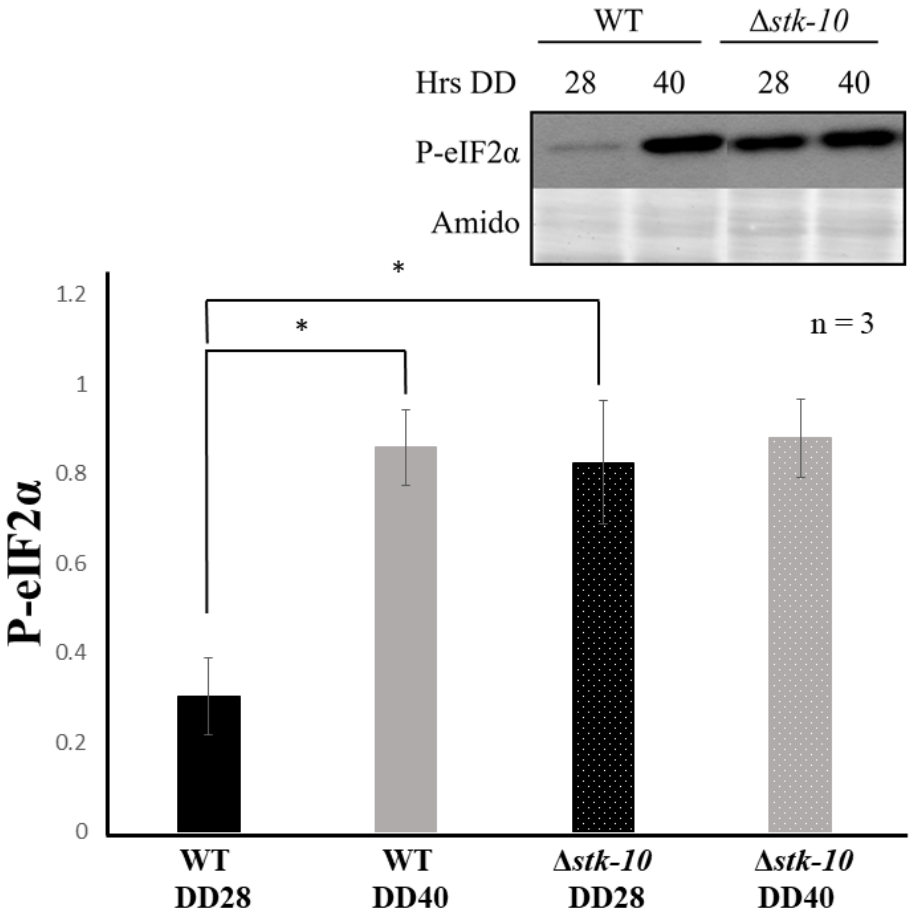

Figure 2.3. P-eIF2α levels are higher at night in Δ*stk-10* cells. P-eIF2α levels during the subjective night (black bars) and subjective day (gray bars) (mean ± SEM; n = 3). P-eIF2α peaked in the subjective day (DD40) compared to the subjective night (DD 24) in WT cells (solid bars) ($P < 0.05$). The representative western blot from one of the replicates is shown above with labeled P-eIF2α and the amido-stained membrane below. In Δ*stk-10* cells (dotted bars), the time-of-day difference in P-eIF2α levels was abolished. Furthermore, P-eIF2α levels were higher at night in *Δstk-10* cells compared to WT ($P < 0.05$).

Core clock function is not altered in Δ*stk-10* cells

To determine if the absence of *stk-10* from the led to arrhythmic P-eIF2α levels, affecting clock, Δ*stk-10* was crossed with an *N. crassa* strain containing the *ras*^{bd} mutation. The "band" allele reduces growth rate and clarifies rhythmic banding of asexual spore production controlled by the circadian clock [72]. Race tube assays of both *ras*^{bd} and Δ*stk-10, ras*^{bd} strains were analyzed to detect differences in period or growth rate (**Figure 2.4A**). The rhythmic banding of the conidia was apparent in both strains with periods of about 22 hours for each strain, suggesting normal clock control of conidiation rhythms in the STK-10 deletion strain. (**Figure 2.4B**). However, there was a significant decrease in the growth rate of the Δ*stk-10, ras*^{bd} strain compared to the *ras*^{bd} strain (**Figure 2.4C**). A second way to monitor clock function is to examine FRQ-LUC rhythms in *in vivo* luciferase assays. This provides a real-time assessment of FRQ cycling where period, amplitude, and phase can be measured. To obtain a Δ*stk-10* strain containing the FRQ::LUC construct, I crossed a WT strain containing the FRQ::LUC translational fusion (DBP1563) with the Δ*stk-10* strain (DBP3422). After germinating spores from this cross, I selected for luciferase positive progenies that produced a bioluminescent signal when placed in media containing luciferin. The progenies that were luciferase positive were then hygromycin and PCR-screened to see if they also carried the *Δstk-10::hph* genotype. Out of 6 germinated spores, one was LUC-positive and also contained the desired *Δstk-10* mutation (DBP3720). Bioluminescence from the WT *frq::luc::bar* and Δ*stk-10::hph, frq::luc::bar* were assayed for FRQ::LUC rhythms in DD (**Figure 2.4D &E**). WT and Δ*stk-10* cells had similar rhythmic FRQ::LUC levels. Altogether, these data suggested that the clock is functioning normally in the Δ*stk-10* knockout strain.

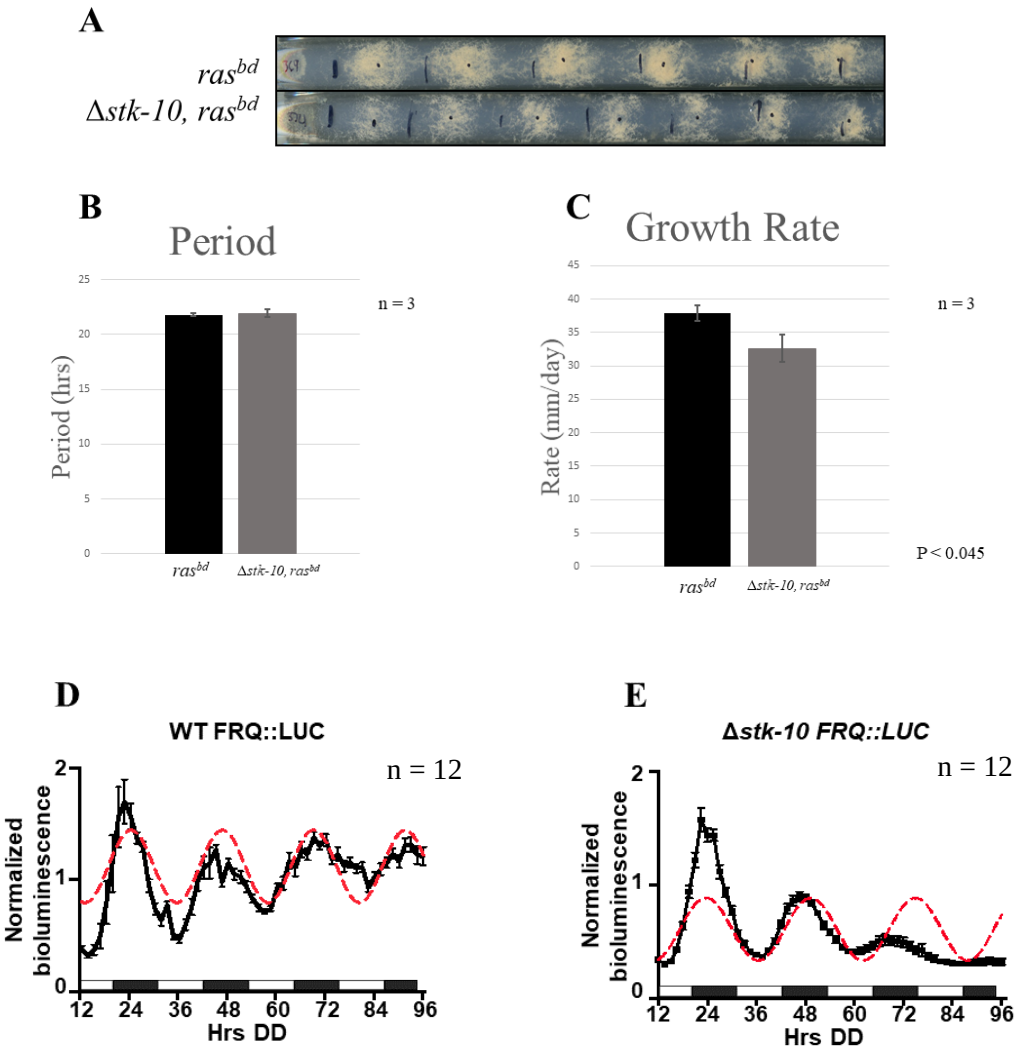

Figure 2.4. Core clock functions in Δ*stk-10* are not altered. Race tube assay of *ras*^{bd} and Δ*stk-10, ras*^{bd} strains examined over multiple days (A). The center of each conidiation band was marked for periodicity analysis, and a line was drawn at the growth front every 24 hours once the tubes were placed in DD to track growth rate. The average period (B) was 21.81 and 21.92 h for the *ras*^{bd} and Δ*stk-10, ras*^{bd} strains, respectively. The average growth rate (C) was significantly decreased in *Δstk-10, ras*^{bd} cells (32.63 mm/day ± 2.08 mm/day, compared to *ras*^{bd} (37.83mm/day ± 1.17 mm/day) (P<0.045). Normalized bioluminescence from luciferase activity is plotted for a FRQ::LUC translational fusion in a WT (D) and Δ*stk-10* (E) cells. These cells were grown in LL for 24 hours at 30°C and then shifted to DD at 25°C and bioluminescence was measured every 90 minutes over the course of 7 days. Only data from 12-96 hours were plotted (mean ± SEM, n=12) with day and night bars shown below the plotted data. Rhythmicity of FRQ::LUC in cells was determined using F tests of fit of the data to a sine wave (dashed red line; P <0.001).

Discussion

Circadian control of CPC-3 activity is critical for rhythmic translation regulation, and my work here has examined the role of several potential CPC-3 regulators in transmitting that rhythmic signal. The Δ*kog1*, Δ*eef1a*, and Δ*pph-1* strains were determined to be inviable as a homokaryon. In *S. cerevisiae*, homolog or double-paralog knockouts of these respective genes were inviable as well, providing more evidence that these are conserved necessary genes [73, 74]. Out of the screened knockout strains of potential CPC-3 regulators, Δ*eif4e3* (**Figure 2.1B**) and Δ*pph-12* cells (**Figure 2.1C**) remained rhythmic for P-eIF2α with similar peaks and troughs compared to the wild type (**Figure 2.1A**). However, P-eIF2α rhythms in the Δ*stk-10* strain (**Figure 2.1D**) were abolished. These data suggested that the eIF4e3 and REG1 regulators in *N. crassa* do not affect the ability of CPC-3 to rhythmically phosphorylate eIF2α under our experimental conditions. Importantly, STK-10 does play a role in regulation of CPC-3 as the normal rhythmic P-eIF2α levels observed in WT cells were no longer apparent. To directly compare P-eIF2α levels in the Δ*stk-10* strain compared to the WT strain, protein from DD16 and DD24 time points, corresponding to the peak and trough of P-eIF2α, were co-loaded on a western gel. Average levels for each time point revealed that while the WT strain maintains a time-of-day difference between these two time points, the knockout strain had no significant time-of-day difference (**Figure 2.3**). Furthermore, the Δ*stk-10* strain had higher levels of P-eIF2α in the subjective night compared to the WT, when levels are expected to be at their trough in the circadian cycle [42]. Overall, the data suggested that STK-10 may act as either a direct or indirect inhibitor of CPC-3 *in vivo* and that when STK-10 is absent from cells CPC-3 phosphorylates eIF2α unchecked, leading to higher and arrhythmic levels of P-eIF2α. Further studies are needed to determine if STK-10 directly acts on CPC-3 to phosphorylate and inactivate it, or if another mechanism of inactivation occurs indirectly

whereby STK-10 phosphorylates an upstream target to cause the subsequent inactivation of CPC-3. From these collected data and previously published findings, we can construct a model in which at night STK-10 may become active to phosphorylate CPC-3, inhibiting it. This would lead to low levels of P-eIF2α, observed under normal conditions, and allows for global translation initiation to proceed uninhibited. During the day, STK-10 may become inactive, allowing CPC-3 to phosphorylate eIF2α and inhibit global translation initiation.

In addition, we also know that a functional clock is necessary for CPC-3 to rhythmically phosphorylate eIF2α, as demonstrated by arrhythmic levels of P-eIF2α in a Δ*frq* strain [42]. This leaves open the possibility that STK-10 may also impact core clock function, thereby disrupting the rhythmic activity of CPC-3 normally seen in the WT strain. However, when looking at the race tube assay for WT and Δ*stk-10* strains (**Figure 2.4A**), both strains had normal periods of ~22 hours (**Figure 2.4B**). Additionally, the luciferase assay reporting FRQ::LUC levels in both WT (**Figure 2.4D**) and *Δstk*-10 strains (**Figure 2.4E**) revealed rhythmic levels that peak at about 20 hours DD and trough at about 32 hours DD, with continued rhythms throughout the time course. However, there is a noticeable dampening of the rhythms in the *Δstk*-10 strain starting before the 72-hour timepoint. This result may implicate that deleting *stk-10* from the genome has a late effect on FRQ, possibly increasing degradation as time goes on, or it may just be a result of an unhealthy mutant strain as observed with the significant decrease in growth rate compared to the WT (**Figure 2.4C**). Taken together, these data show that the clock is still functional in the knockout strain as it maintains the same period of rhythmic conidiation as the WT, on the race tube, and that there was a strong cycling in FRQ::LUC levels, similar to WT levels. Due to our findings suggesting that the Δ*stk-10* deletion does not affect clock function, we can also conclude that the observed arrhythmic levels of P-eIF2α are not due to aberration of core oscillator function. In light of this finding, future

experimentation is needed with a complemented Δ*stk-10* strain containing a WT copy of the *stk-10* gene. If during a circadian time course this complemented strain has rescued rhythmic levels of P-eIF2α, we can then conclude with certainty that STK-10 is responsible for rhythmic CPC-3 activity and ensure that no other unknown mutations in the Δ*stk-10* strain are responsible for the observed arrhythmic CPC-3 activity.

In addition to GCN2 playing an important role in stress-related cellular responses, the TOR signaling pathway is also involved in regulating stress responses in eukaryotes [39, 63-65]. One of the kinases involved in this pathway is the *S. cerevisiase* serine/threonine kinase Sch9, which is the homolog of STK-10 [66, 67]. Although TOR signaling has been extensively studied in yeast, plants, and mammals, not much is known how the TOR pathway operates in *N. crassa* [67, 68]. A study in yeast has shown that TORC1, one of 2 protein complexes of TOR, directly phosphorylated Sch9 at 6 different amino acid sites in the C terminus and that Sch9 activity is dependent on phosphorylation by TORC1 [66, 67]. Consistent with clock control of TORC1 activity in mammalian cells [54], we can hypothesize that in *N. crassa*, TOR may activate STK-10 rhythmically, or that STK-10 protein abundance may cycle throughout the day resulting in rhythmic CPC-3 activity. Future studies looking at STK-10 protein levels are needed to test this hypothesis as well as performing mass spectroscopy in WT versus Δ*stk-10* cells to determine if STK-10 is necessary for CPC-3 phosphorylation and co-immunoprecipitation to determine if STK-10 interacts with CPC-3.

Conclusion

The STK-10 kinase is likely required for CPC-3 activity rhythms, pending confirmation by *Δstk-10* complementation. Since the TOR pathway is known to regulate the STK-10 homolog Sch9, it is reasonable to think that clock control of the TOR pathway may lead to rhythmic activation of STK-10 and CPC-3 activity rhythms. We can hypothesize a model in which activation of the TOR pathway at night may lead to activation of STK-10 through phosphorylation by TORC1. STK-10 may then be able to phosphorylate CPC-3, inhibiting it, and resulting in low levels of P-eIF2α. During the day, deactivation of TOR may lead to decreased activation of STK-10. This may lead to cessation of the inhibitory phosphorylation of CPC-3 and allow it to phosphorylate eIF2α. These mechanisms may promote rhythmic in CPC-3 activity in *N. crassa*. Results from this study may be able to not only reveal how STK-10 is involved in regulating rhythmic CPC-3 activity, but it might also demonstrate how TOR is involved in this circadian regulation of global translation initiation.

Materials and Methods

Strains and growth conditions

N. crassa strain growth conditions and crossing protocols were as previously described [75]. Strains containing the hygromycin resistance cassette, *hph*, were maintained on Vogel's minimal medium [75], that also contained 200 µg/mL of hygromycin B (#80055-286, VWR, Radner, PA). The medium used for the race tube assays monitoring rhythmic growth in strains containing the ras^{bd} mutation was comprised of 1X Vogel's salts, 0.1% D-glucose, 0.17% L-arginine, and 50 µg/mL biotin medium as previously described [76]. Wild-type *N. crassa* FGSC#2489 (mat A, 74-

OR23-IV) or FGSC#4200 (mat a, 74-OR23-IV), FGSC#16121 (mat a, Δ*kog1::hph*), FGSC#13223 (mat , Δ*eef1a::hph*), FGSC#23717 (mat a, Δ*pph-1::hph*), FGSC#21131 (mat a, Δ*pph-12::hph*), FGSC#23514 (mat A, Δ*eif4e3::hph*), and FGSC#14226 (mat a, Δ*stk-10::hph*) were obtained from the Fungal Genetics Stock Center (FGSC, Kansas State University). Primers that were used to validate the strains received are listed in **Table 2.1.** To assay FRQ::LUC protein levels, FGSC#14226 strain was crossed to a strain containing the translation fusion FRQ::LUC that was also linked to bar [77]. Progeny that were resistant to hygromycin and basta were screened for luciferase activity, resulting in a FRQ::LUC Δ*stk-10* (DBP3720) strain. To generate a ras^{bd} mutation in the Δ*stk-10* background, FGSC#14226 was crossed with DBP369. Progeny from the cross that conferred hygromycin resistance were validated for having the Δ*stk-10* deletion using STK-10F and STK10 primers. These strains were then inoculated on race tube media to select for progeny that displayed the banding phenotype. The *stk-10::v5::hph* strain was generated by a 3-way PCR with 1.2 kb of the *stk-10* ORF (primers STK-10V5 AF and STK-10V5 AR), 1.8 kb 10X glycine linker-V5-hygromycin-B resistance gene (*hph*) (primers STK-10V5 BF and STK-10V5 BR), and 1.3 kb of the 3' end of *stk-10* (primers STK-10V5 CF and STK-10V5 CR). PCR was then used to verify endogenous integration of the construct into the *stk-10* locus using primers STK-10V5 OF and STK-10V5 OR. Expression of STK-10::V5 was validated by western blot using anti-V5 antibody (R960-25, Invitrogen, Carlsbad CA).

Circadian time courses for analysis of P-eIF2α or total eIF2α levels were carried out according to previously described methods [78]. In short, mycelial mats of each strain was grown in flasks of Vogel's minimal medium, 2% glucose, 0.5% arginine, with a pH of 6 while shaking in constant light (LL) at 25°C for at least 4 hours. Flasks were then shifted at staggered times into DD at 25°C and then harvested after the appropriate amount of time in DD. The tissue collected was

immediately flash frozen in liquid nitrogen and stored at -80°C until ready to be crushed for protein extraction.

Protein extraction and western blotting

Protein extracts were obtained by previously published methods [79], but the extraction buffer was supplemented with 100 mM Tris pH 7.0, 1% SDS, 10 mM NaF, 1mM PMSF, 1 mM sodium ortho-vanadate, 1 mM β-glycerophosphate, 1X aprotinin (#A1153, Sigma-Aldrich), 1X leupeptin hemisulfate salt (#L2884, Sigma-Aldrich), and 1X pepstatin A (#P5318, Sigma-Aldrich). Concentration of protein in extracts was determined by the Bradford assay (#500-0112, Bio-Rad Laboratories, Hercules, CA). A volume containing 50 µg of protein was subjected to 10% SDS-PAGE gel and then blotted to Immobilon-P nitrocellulose membranes (#IPVH00010, Millipore, Billerica, MA) utilizing standard western blotting methods.

To assay levels of P-eIF2α, rabbit monoclonal Anti-EIF2S1 antibody was used (phosphoS51, #32157 Abcam, Cambridge UK) diluted in a 1:5000 ratio in 5% Bovine Serum Albumin (BSA), 1X TBS, 0.1% Tween, and secondary antibody of goat anti-rabbit IgG HRP (#170-6515, Bio-Rad) diluted in a 1:10000 ratio, in 5% BSA, 1X TBS, and 0.1% Tween. Total eIF2α levels were detected using rabbit polyclonal anti-EIF2S1 antibody (#47508, Abcam) diluted 1:5000, and secondary antibody goat anti-rabbit IgG HRP (#170-6515, Bio-Rad) diluted in a 1:10000 ratio. Protein blotted on membranes were detected via chemi-luminescence SuperSignal West Pico Substrate (#34077, Thermo Scientific). Protein levels were quantitated utilizing NIH ImageJ [80] software and normalized to total protein loaded using amido-stained protein.

Luciferase assays

To measure bioluminescence from strains containing luciferase translational fusions, $1X10^5$ conidia were inoculated into 96-welled microtiter plates containing a medium of 150 µl of 1X Vogel's salts, 0.01% glucose, 0.03% arginine, 0.1 M quinic acid, 1.5% agar, and 25 µM firefly luciferin (LUNCA-300; Gold Biotechnology, St. Louis, MO), pH = 6. After inoculation of conidia, the microtiter plate was incubated at 30°C LL for 24 hours and then transferred to 25°C DD to record bioluminescence levels using the EnVision Xcite Multilabel Reader (PerkinElmer, Life Sciences, Boston, MA). Bioluminescence was recorded every 90 minutes over the course of a week. Luciferase data was normalized to the mean before statistical analysis of rhythms.

Statistical analysis

Protein abundance or luciferase fluorescence readings from time courses were fit to either a sine wave or a line as described [81]. P-values given to plots with rhythmic data represent that the probability for a sine wave best fitting to the data. The student T-test was used to compare statistical significance between period, growth rate, and time of day differences between P-eIF2α in WT DD28 and DD40 cells. Error bars in all graphs, unless otherwise noted, represent SEM from at least 3 independent samples.

Table 2.3: Primers used in this study

Primer name	Used for	Primer sequence
STK-10 F	Validating knockout from FGSC, validate presence of knockout in cross progeny	5’ CTCGATCTGTGGTACGTATC 3’
STK-10 R	Validating knockout from FGSC, validate presence of knockout in cross progeny	5’ GTGTTGAAGGCTCTCATGTG 3’
STK-10V5 AF	*stk-10::v5::hph*	5’ TCAGGTGTATCAGGTTCGGAAG 3’
STK-10V5 AR	*stk-10::v5::hph*	5’ TCCGCCGCCTCCAGGGTCGAAGTTGGTTCCAC 3’
STK-10V5 BF	*stk-10::v5::hph*	5’ AACTTCGACCCTGGAGGCGGCGGAGGCGGTAA 3’
STK-10V5 BR	*stk-10::v5::hph*	5’ GGCGTAGGCCATACTTCCGAGCTCGGATCCAT 3’
STK-10V5 CF	*stk-10::v5::hph*	5’ GAGCTCGGAAGTATGGCCTACGCCGCACAG 3’
STK-10V5 CR	*stk-10::v5::hph*	5’ TCTGATATAGACTAGCTTCCTCC 3’
STK-10V5 OF	Validation of endogenous integration of *stk-10::v5::hph*	5’ GGTAGACTCGGACATGCTTGT 3’
STK-10V5 OR	Validation of endogenous integration of *stk-10::v5::hph*	5’ AGTACATGCTAGACACTGGCG 3’

CHAPTER III

SUMMARY

Proteomic studies in eukaryotes revealed that up to 50% of rhythmic proteins arise from non-cycling mRNAs, implying that rhythms in post-transcriptional and post-translational modifications contribute to observed rhythms in proteins [46-48, 82]. Studies from our lab have shown that certain translation elongation and initiation factors are regulated by the circadian clock [24, 42]. Phosphorylation and inactivation of translation initiation factor eIF2α by CPC-3 kinase is clock-controlled in *N. crassa* [42]. GCN2, the homolog of CPC-3 in *S. cerevisiae*, has been shown to be regulated by uncharged tRNAs, translation elongation factors, and TOR-regulated pathways [83]. During stress, GCN2 phosphorylates eIF2α, and dysregulation of this phosphorylation has been implicated in neurodegenerative, cardiac, and metabolic diseases in humans [84-87]. While mechanisms have been uncovered in *S. cerevisiae* GCN2 as to how it operates during stress, data from these adverse conditions may not be representative of how GCN2/CPC-3 function under favorable physiological conditions.

In my study, I discovered that STK-10 kinase acts as an inhibitor of CPC-3 kinase activity, demonstrated by a Δ*stk-10* strain leading to higher and arrhythmic levels of P-eIF2α throughout a 2-day time course. Because STK-10 activity may affect the stability of core clock proteins, I examined core clock functions, and found that the mutation does not affect the clock. However, I need to complement this knockout strain with a WT copy of *stk-10* in order to ensure that no other mutations in the Δ*stk-10* strain are responsible for this observed arrhythmic activity in CPC-3.

Mammalian S6K and yeast Sch9, the homologs of STK-10 in *N. crass*a, are direct substrates for TORC1 protein complex. TORC1 and TORC2 make up the TOR complex and are involved in the conserved TOR-regulated stress response pathways. TORC1 is the rapamycin-sensitive complex

and is responsive to the availability of cellular nutrients [37]. In *S. cerevisiae*, phosphorylation of Sch9 is absent when cells are treated with rapamycin, or when cells are starved for carbon or nitrogen starvation [66]. Based on my findings and previously published data our current model is that during the day, a stressful cellular environment leads to inactivation of TORC1, thereby reducing the phosphorylation of STK-10. In this inactive state, STK-10 may be unable to phosphorylate CPC-3, preventing its inhibition and allowing for phosphorylation of the eIF2α subunit, inhibiting global translation initiation (**Figure 3.1**). At night, with sufficient nutrients, TORC1 can phosphorylate and activate STK-10 which then allows for inhibitory phosphorylation of CPC-3. At this time of the day, eIF2α remains largely unphosphorylated and global translation initiation can proceed.

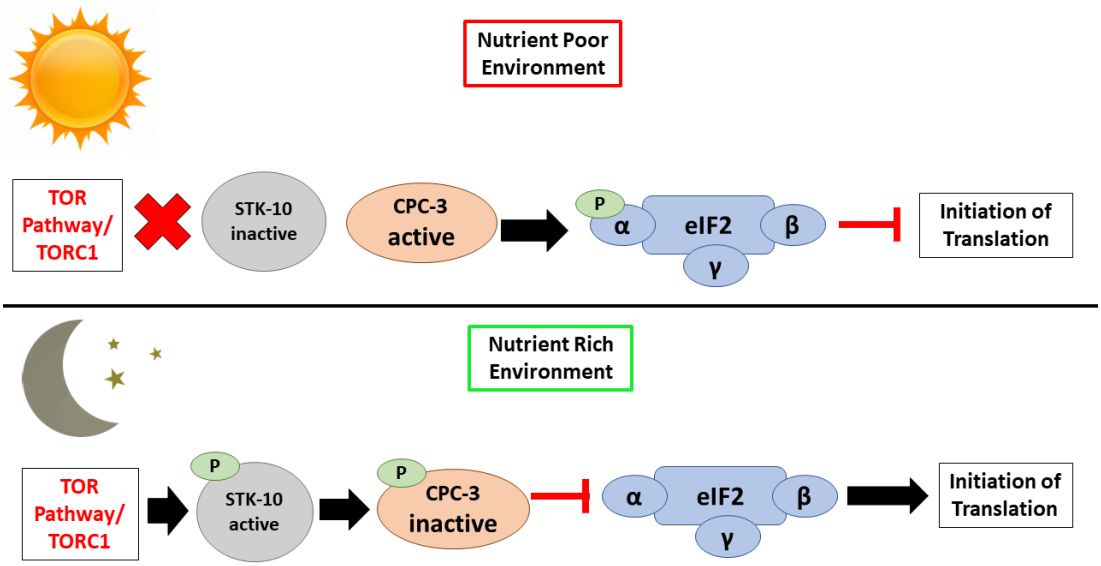

Figure 3.1. Proposed model of how TOR-regulated pathways may be involved in the regulation of CPC-3 through STK-10 kinase.

FUTURE STUDIES

My data supports that STK-10 is required for CPC-3 activity to rhythmically phosphorylate eIF2α. This led me to hypothesize that either rhythmic activation or rhythmic STK-10 abundance is responsible for rhythmic CPC-3 activity. RNA-seq and ribo-seq data (Kathrina Castillo, unpublished) revealed that *stk-10* mRNA levels and ribosome occupancy cycle, peaking during the subjective night. Also, mass spectrometry data has shown that STK-10 protein cycles throughout a 2-day time course peaking in the circadian night [11]. These data suggested that the cycling of STK-10 protein levels may be responsible for rhythms in CPC-3 activity. To test this hypothesis, I have created an STK-10::V5 strain to monitor STK-10 protein rhythms in a circadian time course. Based on the genomic and proteomic datasets, I predict that STK-10::V5 protein levels would peak during the subjective night when P-eIF2α levels are at the lowest. To determine if rhythms in STK-10 levels are necessary for rhythmic CPC-3 activity, *stk-10* could be constitutively activated using the copper regulatable *tcu-1* promoter [88] and the levels of P-eIF2α measured.

In addition, it is known that nutrient starvation activates GCN2 [83]. I am currently examining the activity of STK-10 under amino acid starvation conditions. To test this, I will use the histidine biosynthesis inhibitor, 3-amino-1, 2, 4-triazole (3-AT), added 30 minutes before harvest to growing WT and *Δstk-10* cells in a circadian time course. I will also collect cells at DD28 and DD40, corresponding to a trough and peak, respectively, of P-eIF2α in WT cells under normal conditions, in order to observe potential time of day differences. 3-AT was shown to abolish the time-of-day difference in CPC-3 activity and overall increase its activity in WT cells [42]. With this in mind, I would expect that if STK-10 is necessary for the amino acid starvation response of CPC-3, 3-AT-treated Δ*stk-10* cells would have P-eIF2α levels similar to the 3-AT-untreated cells.

Similarly, rapamycin treatment of WT cells was also shown to induce CPC-3 activity (Anji Karki, unpublished). Therefore, I plan to replicate the 3-AT previously described using rapamycin in lieu of 3-AT in order to see if STK-10 is needed to facilitate TOR-regulated stress responses via CPC-3. I would expect that if STK-10 is needed, rapamycin-treated Δ*stk-10* cells would have similar levels of P-eIF2α when compared to the untreated cells.

Lastly, in *S. cerevisiae* GCN2, when Ser-577 is dephosphorylated it led to greater GCN2 activation, and conversely greater phosphorylation at this site leads to deactivation of GCN2 activity [83]. I propose that a similar serine site may exist on CPC-3 which STK-10 can phosphorylate, leading to deactivation of CPC-3. Preliminary PTM mass spectrometry data compiled by Zhaolan Ding revealed that Ser-238 of CPC-3 had higher phosphorylation levels in the subjective night, suggesting that this may be a site for STK-10 phosphorylation. To test this idea, I will perform post-translational modification (PTM) mass spectrometry on purified CPC-3::V5 from WT and Δ*stk-10* cells to examine if there is a serine site on CPC-3 that is not being phosphorylated in the mutant strain. This may provide evidence that STK-10 directly acts on CPC-3 to phosphorylate and inhibit it.

There is mounting evidence to suggest that regulation of GCN2/eIF2α is important in maintaining proper cellular functions as dysregulation of these two proteins have been shown to lead to a variety of diseases such as metabolic disease and cancer [83-87]. The circadian clock regulates the expression of genes, nutrient metabolism, DNA repair, transcription and translation; disruptions to these rhythmic events have been shown to promote a tumorigenic environment [87]. Knowing that eIF2α plays a critical role in regulating translation, is itself regulated by the clock, and that improper eIF2α activity leads to disease, it may serve as a target for therapeutics. Given the

conserved nature of GCN2 and eIF2α, elucidating mechanisms underlying clock-regulated translation in *N. crassa* may also provide insight in how this regulation occurs in mammals.

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