Opposing PKA and Hog1 signals control the post transcriptional response to glucose availability in *Cryptococcus neoformans*

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Summary

The pathogenic fungus *Cryptococcus neoformans* must adapt to glucose-limited conditions in the lung and glucose replete conditions upon dissemination to the brain. We report that glucose controls ribosome biogenesis and translation by modulating mRNA decay through a balance of PKA and Hog1 signaling. Glucose signaling through PKA stabilized ribosomal protein (RP) mRNAs whereas glucose starvation destabilized RP transcripts through Hog1. Glucose starvation induced oxidative stress response genes, and treatment of glucose-fed cells with Reactive Oxygen Species (ROS) generating compounds repressed RP transcripts, both of which were dependent on Hog1. Stabilization of RP transcripts led to retention of polysomes in a *hog1* mutant, whereas stabilization of RP transcripts by cyclic AMP did not affect translation repression, suggesting that Hog1 alone signals translation repression. In sum, this work describes a novel antagonism between PKA and Hog1 controlling ribosome biogenesis via mRNA stability in response to glucose availability in this important human pathogen.

Abstract
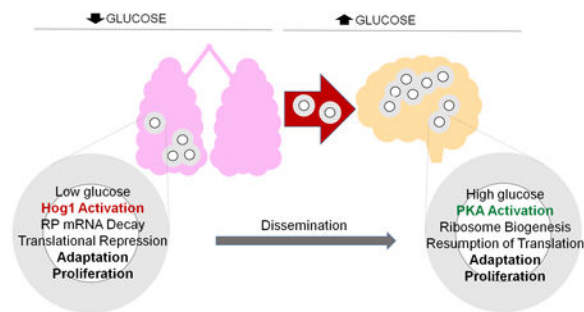
Introduction

Cryptococcus neoformans is a basidiomycetous, yeast-like, ubiquitous fungus that has emerged as an important etiological agent of meningoencephalitis in humans. Of the 19

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species that are characterized within the genus *Cryptococcus*, *C. neoformans* and *C. gatti* are pathogenic, causing disease in predominantly immunocompromised and immunocompetent individuals, respectively (Mitchell & Perfect, 1995, Pappas, 2013). *C. neoformans* var *grubii* (Serotype A), based on capsular serotyping, is known to cause the majority of opportunistic cryptococcal infections in HIV infected individuals in the US and Africa, as well as in patients with T cell deficiencies, hematologic malignancies and those undergoing immunosuppressive therapies (Mitchell & Perfect, 1995, Pappas, 2013, Evans, 1950). Available epidemiologic data reveal a substantial global burden of cryptococcal meningitis, with an estimated 600,000 deaths in sub-Saharan Africa, where limited availability and high cost of antifungal drugs hinder effective medical treatment (Park *et al.*, 2009).

Spores or desiccated yeast cells are the infectious propagules which, upon inhalation by susceptible hosts, get lodged in the pulmonary spaces to be eventually engulfed by alveolar macrophages. Depending upon the immune competence of the host and virulence of the strain, *C. neoformans* may be cleared from the lungs or remain latent within macrophages (Chretien *et al.*, 2002, Feldmesser *et al.*, 2000). Initial pulmonary infection may spread by hematogenous dissemination to other parts of the body with a tropism for the central nervous system leading to meningoencephalitis that is fatal if untreated.

C. neoformans possesses a number of virulence factors which include capsule, melanin and mannitol production, and multiple secreted enzymes such as protease, phospholipase C and others (Buchanan & Murphy, 1998). Along with these, another important feature in the pathogenesis of *C. neoformans* is its ability to withstand stresses and evade host immune system (Voelz & May, 2010, Brown *et al.*, 2007). Being an environmental microbe, *C. neoformans* encounters and survives exposure to a multitude of stresses during growth in the human host that include oxidative and nitrosative stress, high temperature, hypoxia, and nutrient deprivation (Brown *et al.*, 2007). Stress response pathways sense changes in the environment and signal reprogramming of gene expression on cellular processes promoting stress adaptation. Multiple studies over the years have revealed an involvement of several signaling mediators like Pkc1, Hog1, Pkh2 and calcineurin-calmodulin to regulate responsive mechanisms against diverse stresses encountered in the host (Brown *et al.*, 2007, Bahn *et al.*, 2006, Bloom *et al.*, 2013, Upadhya *et al.*, 2013, Chabrier-Rosello *et al.*, 2013). Although glucose is one of the foremost limiting factors the pathogen faces in the host pulmonary niche, *C. neoformans* is able to withstand this adverse environment, remain latent and later disseminate to the CNS which is comparatively more nutrient rich (Hu *et al.*, 2008). It is thus important to identify mediators of the adaptive response of *C. neoformans* to changes in nutrient availability.

Prompt and precise regulation of gene expression is required for cellular survival during adverse growth conditions. Nutrient limitation in *S. cerevisiae* has been reported to result in a global transcriptional and post-translational reprogramming to modulate ribosome biogenesis (Gasch & Werner-Washburne, 2002, Marion *et al.*, 2004, Lopez *et al.*, 2011). It has been shown that a majority of ribosomal protein genes (RP) undergo more than two-fold reduction in expression during amino acid and glucose starvation mainly as a mechanism to conserve energy (Warner, 1999, Gasch *et al.*, 2000, Natarajan *et al.*, 2001). Similar patterns of RP gene repression have been reported in transcriptional profiles of *Candida albicans* in

infected macrophages and murine infection studies with *C. neoformans* (Hu *et al.*, 2008, Lorenz *et al.*, 2004). Alterations in ribosomal gene expression and a role for mRNA turnover in response to nutritional changes have been studied in *S. cerevisiae*, but the importance of mRNA degradation and its downstream effects during starvation stress have not been investigated in the human pathogen, *C. neoformans* (Munchel *et al.*, 2011). The specific signaling mediators that engage the mRNA decay machinery in response to nutrient availability also remain unknown.

mRNA decay is a key post-transcriptional mechanism of gene regulation. It is a multistep process initiated by deadenylation or trimming of the 3′ poly (A) tail and is catalyzed by Ccr4p, the major deadenylase in *C. neoformans* (Chen & Shyu, 2011, Parker & Song, 2004). Deadenylation being the rate limiting step for mRNA decay is also instrumental in silencing translation, thereby playing an essential role in modulating gene expression (Wiederhold & Passmore, 2010, Goldstrohm *et al.*, 2006, Benoit *et al.*, 2005). Studies in *S. cerevisiae* and *C. neoformans* have revealed that degradation of RP transcripts accounted for a substantial part of adaptive response to temperature stress (Grigull *et al.*, 2004, Bloom *et al.*, 2013). These studies have paved a way for further investigations with the mRNA decay pathway and its involvement in general stress adaptation.

Here we have elucidated a novel regulation of RP gene expression with respect to glucose availability in *C. neoformans*. We report that glucose starvation is accompanied by oxidative stress which activates Hog1 to trigger the critical cellular response of RP transcript decay. We also show that an absence of RP degradation leads to a defect in translation repression, thereby affecting the adaptive cellular response during starvation stress. Following starvation, when cells encounter glucose rich environment, RP transcripts are induced and stabilized in a PKA dependent manner to resume protein synthesis enabling growth of the pathogen. Overall, our results indicate that the abundance of RP transcripts and in turn ribosome biogenesis is controlled at the level of mRNA stability by the dual and opposing actions of Hog1 and PKA.

Results

Ccr4 mediates RP repression by accelerated decay of RP transcripts following carbon starvation

To determine the effect of carbon starvation on overall RP transcript abundance, we performed a Northern Blot time-course assay to analyze the steady state mRNA levels of our representative RP transcript, *RPL2* (CNAG_05232), in the wild type every 6 minutes over a 30 minute period following a shift from nutrient rich Yeast Peptone Dextrose (YPD-2% Dextrose) broth to minimal Yeast Nitrogen Base medium (YNB-0% Dextrose). We have previously shown that *RPL2* and *RPL27e* mRNA abundance and decay kinetics show similar profiles during temperature stress in *C. neoformans* (Bloom *et al.*, 2013). Studies in other systems have also demonstrated a tight co-regulation of RP transcripts in both synthesis and decay patterns in response to environmental stresses (Tavazoie *et al.*, 1999, Grigull *et al.*, 2004, Wang *et al.*, 2002, Mayer & Grummt, 2006). We observed a significant reduction in *RPL2* levels by 12 minutes (Fig. 1A, top panel) suggesting that carbon starvation results in a rapid repression of RP genes. Given the role of Ccr4 in repression of RP genes during

temperature stress in *C. neoformans* (Bloom *et al.*, 2013), we investigated the *RPL2* transcript steady state levels in a *ccr4* mutant post shift to YNB media. Our results demonstrate a more gradual and moderate change in RP transcript abundance in the mutant (Fig. 1A, bottom panel) suggesting that deadenylation-dependent mRNA decay mediated by Ccr4 is responsible for a large component of the RP transcript repression seen during starvation.

To verify that the reduced *RPL2* expression seen in the wild type was due to a change in the stability of the *RPL2* transcript, we monitored the starvation induced decay kinetics of RP transcripts in both wild type and *ccr4* mutant in a time course following transcriptional shutoff. Mid-log cells of both strains grown in either YPD or shifted to YNB were treated with 1, 10-phenanthroline to inhibit transcription. RNA was harvested over a one hour time course with intervals at every 15 minutes, and *RPL2* abundance measured by northern blot. We observed that wild type *RPL2* transcript degraded rapidly and drastically when shifted to YNB with the half life reducing from 33 minutes to 15 minutes (Fig. 1B, top panel) (P<0.0001). In contrast, the same transcripts were highly stabilized in *ccr4* mutant, both in YPD and YNB, with half lives of >60 minutes in both cases (Fig. 1B, bottom panel) (P<0.0001 compared to wild type *RPL2* in YPD, P<0.0001 compared to wild type *RPL2* in YNB). These data confirm that carbon starvation induces an accelerated deadenylation-dependent decay of RP transcripts mediated by Ccr4. We also performed a reciprocal stability experiment where mid-log wild type cells were shifted from glucose-rich YPD medium to YNB supplemented with glucose (0.002%), at a concentration that models host nutrient deficient environment of lungs and macrophages (Lorenz *et al.*, 2004, Williams & Del Poeta, 2011, Hu *et al.*, 2008, Kronstad *et al.*, 2012). Our result from Fig. 1C again demonstrated a shorter half-life of *RPL2* in low glucose medium, (P<0.0001 compared to wild type *RPL2* in YPD) suggesting that carbon deficiency induced an accelerated decay of RP transcripts in *C. neoformans*.

Reduction in polysome abundance on glucose withdrawal

Since deadenylation occurs on mRNAs associated with ribosomes and is a regulatory factor for translation, we investigated the translation state of the wild type and *ccr4* mutant in both glucose replete and depleted conditions by polysome profile analysis (Chen & Shyu, 2011, Garneau *et al.*, 2007). The profiles of both wild type and the mutant in YPD media revealed a normal distribution of ribosomes with expected peak of 40S ribosome subunit, 60S subunit, an 80S monosome peak followed by multiple polysome peaks (Fig. 2A and B, top panels). Following a shift to glucose deficient media for 15 minutes and 30 minutes, we observed a significant redistribution of ribosomes from polysomes to the ribosomal subunit peaks in the wild type with a marked increase in the 60S peak and a concomitant decrease in the polysome pool leading to an almost absence of polysomes at the 30 minute time point (Fig. 2A middle and bottom panels, respectively). Interestingly, polysome profiles from starved cultures of the *ccr4* mutant revealed an accumulation of 60S subunit as well, but a comparatively modest change in the polysome abundance compared to the wild type, both at 15 minutes and 30 minutes after starvation (Fig. 2B, middle and bottom panels respectively). Our findings suggest that glucose withdrawal results in translation repression and a

significant loss of polysomes. Additionally, a defect in mRNA degradation leads to retention of polysomes during starvation.

RP transcript degradation is specific to the absence of glucose signaling

C. neoformans has a functional gluconeogenetic pathway and can utilize glycerol and acetate among others as an alternative carbon source for growth (Price *et al.*, 2011, Hu *et al.*, 2008). To investigate if the RP transcript degradation was a response to the absence of glucose specifically or to lack of a carbon source in general, we performed a stability time-course experiment where mid-log wild type cells were shifted from YNB+2% dextrose to YNB supplemented with 2% glycerol as an alternative carbon source. The half-life of *RPL2* was calculated to be 35 minutes in YNB+2% dextrose (Fig. 3A), similar to half-life of *RPL2* in another dextrose containing media YPD (P value=0.5262). The *RPL2* transcript underwent an accelerated degradation when cells were shifted to the glycerol containing media with a half life of 17 minutes (Fig. 3A), (P<0.0002 compared to *RPL2* half-life in YNB-2% dextrose), suggesting that the RP transcript decay response was specific to glucose depletion, and that an alternative carbon source was unable to suppress this response. There are two major ways in which glucose can signal to the mRNA decay machinery. Glucose can be sensed directly and signal through the cAMP-dependent PKA pathway, or the cells can sense a change in metabolic state through ATP/AMP ratios. To address if RP transcript degradation was specific to the absence of glucose as a metabolite, we performed a stability time course assay to look at *RPL2* decay kinetics in wild type cells shifted from YNB+2% dextrose to YNB supplemented with a non-metabolizable glucose analogue, 2-Deoxy-D-glucose (2-DG) that would provide the glucose signal, but not result in ATP production (Panepinto & Williamson, 2006). In the presence of 2-DG, *RPL2* transcripts degraded with a normal half-life of 33 minutes (Fig. 3B) (P value=0.4577 compared to *RPL2* half life in YNB+2% dextrose), which suggested that activation of glucose signaling even in the absence of glucose itself had a stabilizing effect on RP transcripts.

Activation of cAMP/PKA pathway stabilizes RP transcripts

Once we established that activation of glucose signaling stabilized RP transcripts, we next investigated the role of the cAMP/PKA pathway, which is a reputed key player in the cell's ability to sense and adapt to glucose availability (Choi *et al.*, 2015, Santangelo, 2006). cAMP/PKA is known to regulate RP gene expression subject to nutrient availability, but the contribution of this pathway to post-transcriptional regulation of RP gene expression in *C. neoformans* has not been investigated (Zurita-Martinez & Cardenas, 2005, Klein & Struhl, 1994, Martin *et al.*, 2004). Studies in mammalian cells revealed that cAMP is instrumental in stabilizing selective mRNAs which prompted us to investigate whether cAMP/PKA exerted any effect on RP transcript stability in *C. neoformans* (Chen et al., 1993, Hod & Hanson, 1988). To answer this, we performed a stability time course experiment where mid-log wild type cells were shifted from YPD to YNB supplemented with 10 mM cAMP to activate the pathway even in the absence of glucose. Addition of cAMP stabilized *RPL2* transcript, with a half life of >60 minutes, (Fig. 3C) (P<0.0001, compared to *RPL2* half-life in YNB), confirming a role for PKA as a positive regulator of mRNA stability. We further wanted to examine whether this increase in RP transcript abundance due to cAMP activation had any effect on the ribosome population. Polysome analysis of mid-log wild type cells that were

shifted from YPD to cAMP-supplemented minimal media revealed that there was a marked reduction in the polysome pool with a concurrent increase in 60S subunit (Fig. 3D, bottom panel) a profile similar to wild type cells during starvation (Fig. 3D, top panel). Data from above experiments convey that exogenously added cAMP activated the PKA pathway rendering a stabilizing effect on RP transcripts, but that stabilization does not relieve translation repression or lead to retention of polysomes. We know that eukaryotic mRNA decay is a multi-step process that is initiated by deadenylation of poly-A tail followed by the sequential process of decapping and exonucleolytic cleavage at 5′-end in the P-bodies or by the exosome at the 3′-end after the transcript is dissociated from ribosome. Any defect in these later steps of mRNA decay would hence result in stabilized deadenylated transcripts. Our data from experiments with cAMP led us to hypothesize that stabilization of *RPL2* transcripts occurs in the mRNA degradation pathway after the point of deadenylation because the mRNAs remain stable, but the ribosomes have dissociated.

We further hypothesized that if RP transcripts were stabilized by activation of PKA, then inactivating the same, even in the presence of glucose, would lead to a starvation response resulting in rapid decay of *RPL2* mRNA. To address this question, we performed a stability time course assay and Northern blot analyses to measure *RPL2* decay kinetics in *gpa1* mutant lacking the Gα subunit that is an activator of PKA and a *pka1* mutant that lacks a PKA catalytic subunit (Alspaugh *et al.*, 1997, D'Souza *et al.*, 2001). We did not observe any difference in the rate of *RPL2* degradation in either of the mutants as compared to wild type, the half-lives of *RPL2* being 31 minutes in both *gpa1* and *pka1* mutants during their growth in YPD (Fig. 3E middle and right panels respectively), (P value=.5183, compared to wild type *RPL2* half-life in YPD). This suggested that although activating PKA exhibited a stabilizing effect on RP transcripts, inactivation of the pathway did not elicit an opposing effect, suggesting that another signaling pathway is providing a destabilizing signal in the absence of glucose.

Positive regulation of mRNA stability via PKA is important during refeeding

Given the role of cAMP/PKA in induction of RP genes during nutrient replete condition in *S. cerevisiae*, we next wanted to determine if similar phenomenon occurred in *C. neoformans* following starvation (Zurita-Martinez & Cardenas, 2005, Neumansilberberg *et al.*, 1995, Kraakman *et al.*, 1999). Mid-log wild type and *pka1* mutant cells were starved for 2 hours in YNB, followed by re-addition of dextrose for an hour. Cells were harvested from mid-log, starved and refed cultures (every 15 minutes) and RNA was extracted to perform Northern Blot and probed for *RPL2* steady state levels. We observed a significant decrease in *RPL2* levels following starvation in the wild type, which gradually increased upon refeeding with glucose and reached almost pre-starved levels by the end of 1 hour (Fig. 4A). In contrast, transcript abundance did not increase as efficiently in *pka1* mutant with *RPL2* reaching only about 60% of wild type level, (Fig. 4A) suggesting that this increase in *RPL2* mRNA is dependent on the functionally active PKA pathway. Given our earlier observation that cAMP activation of PKA led to a stabilizing effect on *RPL2* transcripts (Fig. 3D), we next wanted to investigate whether the increase in *RPL2* abundance during refeeding phase was due to a pro-stabilizing effect by this pathway on RP transcripts. To address this, mid-log wild type and *pka1* cells were shifted from YPD to YNB for 2 hours

and supplemented with dextrose for 30 minutes to induce RP transcripts. Once RP genes were induced, 1, 10-phenanthroline was added to halt further transcription and cells were harvested every 15 minutes for an hour to analyze RP decay kinetics during the refeeding stage. During refeeding, *RPL2* mRNA was highly stabilized in the wild type with a half life of >60 minutes in contrast to the significantly less stable *RPL2* transcript in the *pka1* mutant (Fig. 4B) (P<0.001), thereby confirming a role for PKA1 in positively regulating RP mRNA stability during glucose refeeding.

Common nutrient and stress signaling cascades do not mediate the RP decay response to glucose starvation

Once we established a pro-stabilizing effect of PKA on RP transcripts during glucose availability, we focused our interest on other well characterized nutrient sensing and stress responsive pathways for a possible role in accelerating RP mRNA decay during glucose starvation. We hypothesize that if RP decay during glucose depletion was mediated by any of these cascades, then inactivating the pathway would lead to a stabilization of RP transcripts under starvation conditions. Earlier studies with *S. cerevisiae* have revealed a role for TOR signaling in regulating RP gene expression and ribosome biogenesis in response to nutrients (Marion *et al.*, 2004, Lopez *et al.*, 2011, Powers & Walter, 1999). To determine if TOR signaling mediated RP decay in response to glucose starvation, mid-log wild type cells growing in YPD were pre-treated with rapamycin (inhibitor of TOR kinases) for twenty minutes before shifting them to minimal media containing rapamycin and a stability time course was performed for an hour. Northern blot analyses revealed that inhibiting TOR did not stabilize *RPL2* and its half life was 15 minutes (Fig. 5A, left panel). This acceleration was similar to that observed in wild type during starvation, (P=0.9813). To exclude the role of TOR more convincingly, we performed a reciprocal experiment where mid-log wild type cells were treated with rapamycin for twenty minutes during its growth in glucose-rich YPD before shutting off-transcription by adding 1,10-phenanthroline. (Fig. 5A, right panel) demonstrates that simply inactivating TOR did not have any effect on the *RPL2* degradation and transcript half-lives were the same both in rapamycin treated and control cells (P=0.6731), thereby excluding a role for TOR in regulating *RPL2* stability in *C. neoformans*. Glucose depletion and an altered AMP:ATP ratio is known to activate the AMP activated kinase, SNF1, that regulates a multitude of processes including stress response and mRNA metabolism (Hu *et al.*, 2008, Braun *et al.*, 2014). We therefore created a *snf1* knockout mutant and assayed *RPL2* stability as above. The *snf1* mutant also exhibited a wild type response to starvation with the half life of *RPL2* calculated as 15 minutes (Fig. 5C, P=0.7832), ruling out this regulator in starvation induced RP transcript degradation in *C. neoformans*. Given the role for Pkh2 signaling in RP mRNA decay during host-temperature adaptation in *C. neoformans* (Bloom *et al.*, 2013), we performed similar experiment on *pkh2-02* mutant to investigate for a possible interaction between Pkh2 signaling and RP transcript decay during glucose deficiency. *RPL2* degraded rapidly with a half life of 16 minutes in the *pkh2-02* mutant, (Fig. 5D) (P=0.6642, compared to *RPL2* half-life of wild type in YNB) ruling out Pkh2 as a mediator of RP transcript decay.

Hog1 signals accelerated RP transcript degradation during starvation stress

We know Hog1 plays a pivotal role in conferring cellular adaptive responses during multiple environmental stresses (Bahn *et al.*, 2006, Giles *et al.*, 2006). To determine a possible role of Hog1 signaling in RP gene expression in *C. neoformans* during glucose deprivation, we performed steady state time course experiments where both wild type and *hog1* mutant mid-log cells were shifted from YPD to YNB (Bahn *et al.*, 2005). Northern blot analyses of *RPL2* expression revealed relatively higher levels of steady state mRNA in the mutant compared to wild type (Fig. 6A). In accordance with this result, stability time course assay with *hog1* mutant cells demonstrated a relative stabilization of the *RPL2* transcript in the *hog1* mutant with a longer half life (>60 minutes) (Fig. 6B), (P<.001 compared to wild type). This suggested that Hog1 provides the signal for accelerated RP degradation during glucose starvation in *C. neoformans*, and RP transcripts were stabilized in its absence. To determine the effect of RP transcript stabilization on ribosome abundance, we performed polysome profile analysis on mid-log *hog1* mutant cells in YPD and 15 minutes and 30 minutes post shift to YNB. Results showed that in normal growth conditions, *hog1* mutant had a typical profile with high levels of polysomes (Fig. 6C left panel). Interestingly, polysomes were retained when cells were incubated in YNB media at both time points (Fig. 6C, middle panel and right panel), with comparatively higher abundance of polysomes in *hog1* mutant compared to wild type. Taken together, our data indicate that Hog1 positively regulates RP mRNA decay in the absence of glucose and is also involved in modulating ribosome biogenesis and translation repression during starvation stress.

Studies in yeast and mammalian cells have shown that nutrient starvation can additionally lead to oxidative stress in cells (Varela *et al.*, 1995, Suzuki *et al.*, 2011). To determine whether glucose starvation was accompanied by oxidative stress in *C. neoformans*, we measured steady state mRNA levels of oxidative stress induced genes *CAT3* (CNAG_00575) and *SOD2* (CNAG_04388) which encode for catalase and superoxide dismutase, respectively (Giles *et al.*, 2006, Cox *et al.*, 2003). Wild type and *hog1* mid-log cells were shifted from YPD to YNB and harvested at designated time points over two hours. RNA was extracted and northern blot performed to reveal a rapid but transient induction of both *CAT3* and *SOD2* transcripts by 15 minutes which then reduced to minimal levels by the end of 1 hour in the wild type (Fig. 6D, top panel). On the contrary, we observed only basal levels *SOD2* which did not elevate during starvation, along with a total absence of *CAT3* induction in *hog1* mutant (Fig. 6D, bottom panel). We hypothesized that if our earlier observation of RP transcript repression and decay was an effect of starvation induced oxidative stress, then treatment with Reactive Oxygen Species (ROS) generating agents would also show similar response. In our study, treatment of wild type cells with two ROS generating compounds antimycin and hydrogen peroxide also caused a Hog1 dependent repression of the *RPL2* mRNA (Fig. 6E and 6F), consistent with the hypothesis that glucose starvation is accompanied by oxidative stress that leads to Hog1 mediated regulation of RP genes. In order to verify our hypothesis, we next wanted to determine if adding anti-oxidants in glucose depleted medium prevented RP repression and so we performed reciprocal experiments using multiple anti-oxidants to determine any compensatory mechanism that prevented accelerated degradation of *RPL2* transcripts. Stability experiments were performed with wild type cells that were pre-treated with tocopherol (Raspor *et al.*, 2005),

EUK-134 (Batinic-Haberle *et al.*, 2010) and mito-Tempo (He *et al.*, 2014) for 30 minutes before shifting them to glucose deficient conditions to investigate for *RPL2* decay kinetics. Results demonstrated rapid decay of *RPL2* with significantly shorter half-lives in all the cases suggesting that pre-treatment with anti-oxidants singly and in combination failed to abrogate the oxidative stress induced upon starvation. Result demonstrated drastically reduced levels of *RPL2* transcript (Supplementary Fig. S1, bottom panel) which suggested that pre-treatment of cells with a mixture of anti-oxidants failed to prime them against the effects of oxidative stress. We also probed for *CAT3* and *SOD2* transcripts in the same samples to reveal transient induction of both the genes verifying that pre-treatment of cells with anti-oxidants was insufficient to negate the effect of starvation induced oxidative stress and thus failed to stabilize *RPL2* transcript (Supplementary Fig. S1, top panel). We hypothesize that failure to prevent the induction of oxidative stress despite using the anti-oxidants was presumably due to the inability of the anti-oxidants to permeate the cells or reach an effective intracellular concentration. Alternatively, carbon starvation may activate Hog1 independent of ROS production. The specific mechanism by which oxidative stress activates Hog1 has not yet been defined.

Discussion

The concentration of glucose is nearly 3-20 times lower in the airway surface liquid (ASL) and lower respiratory tract airways, than the plasma glucose content which is normally maintained at 5 mM L^{-1} (Garnett *et al.*, 2012). This is also in contrast to the comparatively elevated levels of glucose (2.5-4 mML^{-1} or 0.18% glucose) in the human cerebrospinal fluid (CSF) which is 50-70% of blood glucose levels (Leen *et al.*, 2012, Pastuszczak *et al.*, 2013). A key factor that differentiates glucose content between lungs and the brain compartments is the presence of multiple glucose transporters in the latter and a substantial decrease of the same in lungs (Wood & Trayhurn, 2003, Garnett *et al.*, 2012). *C. neoformans* pathogenesis initiates within the human lung and culminates in the brain; both of the compartments are varied in glucose availability. HIV infected individuals have been reported to show a very high burden of *C. neoformans* in their CSF (Bicanic *et al.*, 2009) suggesting that the glucose rich environment favors proliferation of cryptococcal cells in the brain tissue and subarachnoid spaces. Thus *C. neoformans* in the course of its pathogenesis transitions from a glucose-deprived niche in the lungs and macrophages and moves to a glucose rich environment in the CNS. Our work is a novel approach to decipher the adaptive mechanisms required by the pathogen to mediate this transition and promote its survival when it encounters environments with variable glucose availability. Our data has led to a hypothetical model of how the antagonism of Hog1 and PKA could promote host adaptation in the glucose-deficient environment of the lung, and promote proliferation in the CNS where glucose is available (Fig. 7).

We demonstrate that on exposure to glucose-limiting conditions, (Fig. 1C), that models the glucose concentration of lungs and the macrophage intracellular environment, *C. neoformans* undergoes an acute and prompt response where RP transcripts are rapidly degraded. We believe that this is a cellular response to rid the cell of one of the most abundant classes of transcripts to conserve energy. This is supported by our findings from polysome profiles of starved cells where polysome abundance was reduced in wild type in

contrast to a *ccr4* mutant. Ribosome biogenesis and protein synthesis are high energy driven processes, and inhibiting them in starved cells is an efficient way of conserving energy to utilize it for other stress adaptive mechanisms (Warner, 1999, Warner *et al.*, 2001, Causton *et al.*, 2001). We also determined that this regulation of RP mRNA was mediated by Hog1 signaling. Hog1 has been well documented as a global response mediator to a number of environmental cues along with its importance in virulence; a *hog1* mutant was reported to be attenuated in virulence in an animal model of cryptococcal infection (Bahn *et al.*, 2006). Transcriptome profiling in hydrogen peroxide treated *C. neoformans* cells have also shown a down-regulation of RP genes (Upadhya *et al.*, 2013). Our findings now suggest that a defect in Hog1 further leads to impaired RP mRNA decay, and stabilization of the RP transcripts results in retention of polysomes, thereby disrupting stress adaptive mechanisms. We also provide the first evidence of the role of Hog1 in translation repression in *C. neoformans* through modulation of RP transcript stability during a nutritional down-shift.

Once *C. neoformans* faces glucose-rich conditions, as it does upon entry into the CNS, it is required to adapt to the increase in the nutrient levels and elicit appropriate responses to utilize these resources and proliferate. In order to resume and sustain protein synthesis, induction of RP genes are of prime importance and previous findings from gene expression studies supports this hypothesis. Transcriptional profiles of *C. neoformans* cells obtained from CNS of infected rabbits have shown a significant upregulation of ribosomal protein genes, transporters including hexose transporter, and numerous genes involved in carbohydrate metabolism (Steen *et al.*, 2003). We also know that transcription of RP genes and other ribosome biogenesis factors are regulated primarily by PKA and TOR pathways (Zurita-Martinez & Cardenas, 2005, Klein & Struhl, 1994, Martin *et al.*, 2004). The significance of a PKA mediated response during growth of *C. neoformans* in CNS can be reinforced from studies which demonstrate a virulence defect in a *pka1* mutant and hypervirulence in a *pkr1* strain (constitutive activation of PKA) (D'Souza *et al.*, 2001, Hicks *et al.*, 2004). In our study we show that in addition with induction, PKA also exerts a stabilizing effect on newly synthesized RP mRNAs that enable the transcripts to be readily available to resume protein synthesis and ribosome biogenesis. Moreover, stabilization of RP transcripts by exogenous cAMP is witnessed even in the absence of glucose, thereby suggesting a novel role for PKA in mRNA metabolism. Activation of PKA that occurs in the absence of glucose did not relieve translational repression, suggesting that the starvation signal via Hog1 is sufficient for translation repression. Hog1 is known to be activated by ROS, and the response to starvation included the induction of ROS-sensitve genes including *SOD2* and *CAT3* that was Hog1-depedent, suggesting that ROS production is a component of the starvation response. It is possible, however, that Hog1 is activated by another, novel signal during starvation that leads to the ROS-responsive program downstream of Hog1 activation.

The mechanism by which PKA and Hog1 communicate with the mRNA decay machinery remains unknown. Recent quantitative proteomic analysis of *C. neoformans* strains overexpressing Pka1 demonstrated regulation of the abundance of ribosomal components, consistent with a role for Pka1 in direct modulation of translation (Geddes et al., 2016). The experimental conditions used in this study, namely galactose induction, may not directly correlate with our *in vitro* conditions or *in vivo* conditions encountered in the human host.

Nevertheless, findings from both the studies pave the way for further research to determine the targets and mechanisms involved in Pka1 mediated regulation of ribosome biogenesis and translation with respect to glucose availability. No direct targets of Hog1 phosphorylation have been described, and so further investigation is needed to determine the direct targets of Hog1 that regulate translation and mRNA decay.

Our study describes an elegant regulatory control between two signaling pathways to modulate gene expression in *C. neoformans* in response to glucose availability. This mechanism of translation repression and resumption of protein synthesis by modulating RP mRNA stability via an antagonistic action of Hog1 and PKA signaling is both interesting and novel. Deciphering the biology of these processes will help in the better understanding of pathogenesis and stress response in this important human pathogen.

Experimental Procedures

Strains, media and chemicals—*C. neoformans* strain H99 (serotype A), *ccr4* mutant (Bloom *et al.*, 2013, Panepinto *et al.*, 2007), *pka1* mutant (D'Souza *et al.*, 2001) *gpa1* mutant and (Alspaugh *et al.*, 1997) *hog1* mutant (Bahn *et al.*, 2005) were gifts of Joe Heitman (Duke University), *pkh2-02* mutant and H99 against which the mutant was created are a part of the Madhani collection (Liu *et al.*, 2008) and obtained from the Fungal Genetics Stock Center at Kansas State University.

Production of *snf1* deletion strain: A *C. neoformans snf1* strain was constructed as described previously (Panepinto *et al.*, 2005). The knockout construct was verified by sequencing and the construct was introduced into wild type H99 strain using biolistic gene transfer method (Toffaletti *et al.*, 1993). NAT-resistant colonies were screened by PCR with 5′ SNF1 and 3′SNF1 screening primers (S1.Table 1). PCR-positive clones were further verified by Northern and Southern blot.

All strains were maintained in YPD broth (BD Difco) with 80% glycerol (Fisher Chemicals) and streaked fresh on YPD agar plates before conducting experiments. Nutrient rich, mid-log cells were grown in YPD broth whereas glucose starvation experiments were performed either in YNB medium alone (YNB without amino acids, BD Difco) or YNB supplemented with 2% glycerol, 2% 2-Deoxy-D-glucose (Sigma Aldrich), 10 mM cyclic AMP (Sigma Aldrich), or 1 μg ml^{-1} Rapamycin (Sigma Aldrich). For generating oxidative stress, YPD medium was supplemented with 1 mM Hydrogen Peroxide solution (J.T Baker) and 1 μM Antimycin (Sigma Aldrich). All the anti-oxidants used in this study, α-Tocopherol, EUK-134 and Mito-Tempo was obtained from Sigma Aldrich. 1,10-phenanthroline (J.T Baker) was used as a transcriptional inhibitor and cycloheximide (Sigma Aldrich) was used as a translational inhibitor in our studies.

Steady-state and stability time-courses

Overnight cultures of respective strains were inoculated in YPD at an OD_{600}=0.2 and grown to mid-log phase (OD_{600}=0.6) at 30°C. Cells were harvested by centrifugation at 4000 X g for 5 minutes. For steady state analyses of transcripts, cells were resuspended in 30 ml YNB media and incubated for 30 minutes or one hour as required for each experiment. 5 ml

aliquots were pelleted by centrifugation at specific time intervals depending upon the experiments and further processed for RNA extraction. For transcript stability analyses, mid-log cells were re-suspended in 30 ml YNB alone or supplemented with reagents as mentioned for each experiment. A transcriptional inhibitor, 1,10-phenanthroline (250 μg ml^{-1}) was added to the medium and cells were incubated for an hour. Aliquots of 5 ml were harvested every 15 minutes over a one hour time period and processed for RNA extraction.

RNA extraction and Northern blot analyses

RNA was extracted using Qiagen RNAeasy Kit following manufacturer's instructions. RNA was quantified and 2 μg RNA was loaded on formaldehyde-agarose gel. Upon electrophoresis, RNA was transferred to nitrocellulose membrane, cross-linked, and probed with P^{32}-labelled DNA probes that hybridized to the transcripts being tested, as described previously (Bloom *et al.*, 2013). After washing, blots were exposed to phosphor screen, and imaged on a Typhoon 9400 Variable Mode Imager. Hybridization signal was quantified using Quantity One software and normalized to the signal of the ribosomal bands. Statistical analysis for steady state experiments were performed by Two-way ANOVA grouped analyses method to detect standard errors (SEM) between replicate data sets using Graphpad Prism software. Statistical analyses to calculate stability data were performed by determining the least squares fit of one-phase exponential decay non-linear regression with Graphpad Prism software. Significance between curves was detected by sum-of-squares *F*-test, with P<0.05 as significant P-value, which determined that the data from two comparable experiments fall on different regression lines thereby confirming different rates of decay and ultimately different half-lives of the transcripts.

Polysome profile analyses

Sample was prepared by harvesting mid-log cells growing in YPD or cells shifted to YNB medium for 15 or 30 minutes. Following the induction of starvation stress, translating polyribosomes were halted on the mRNAs by the addition of cycloheximide to 0.1 mg/ml and chilled on ice for 5 minutes prior to centrifugation. Cells were washed twice with 10 ml water followed by a final wash with 15 ml of lysis buffer (20 mM Tris-HCl [pH 8], 140 mM KCl, 5 mM MgCl$_2$, 0.5mM DTT, 0.1 mg ml^{-1} cycloheximide, and 0.5 mg ml^{-1} heparin, 1% Triton X-100), flash frozen in liquid nitrogen and stored at -80°C. Cells were thawed, 200 μl of lysis buffer was added to resuspend the cells, and an equal volume of pre-chilled glass beads were added. Cells were mechanically crushed by vortexing every 20 seconds followed by incubation on ice for 40 seconds for a total of 5 minutes. The lysate was cleared by two subsequent microcentrifugation steps (15,000 x g, for 5 min at 4°C) and volume was brought to an equal level by adding buffer. Equal volumes of the samples were loaded onto a 10 ml linear sucrose gradients (10%-50%) prepared in gradient buffer (20mM Tris-HCl, 140 mM KCl, 5 mM MgCl$_2$, 0.5 mM DTT, 0.5 mg ml^{-1} heparin, and 0.1 mg ml^{-1} cycloheximide). The gradients were centrifuged at 39,000 × rpm for 2 hours at 4°C, using an SW41 Ti rotor. Following centrifugation, sucrose gradients were pushed through a flow cell and RNA was recorded using UV-Vis detector at 254 nm.

All data represented in the work are representatives of 3 biological replicates. Supplementary material includes list of primers used in the study (S1.Table 1)

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Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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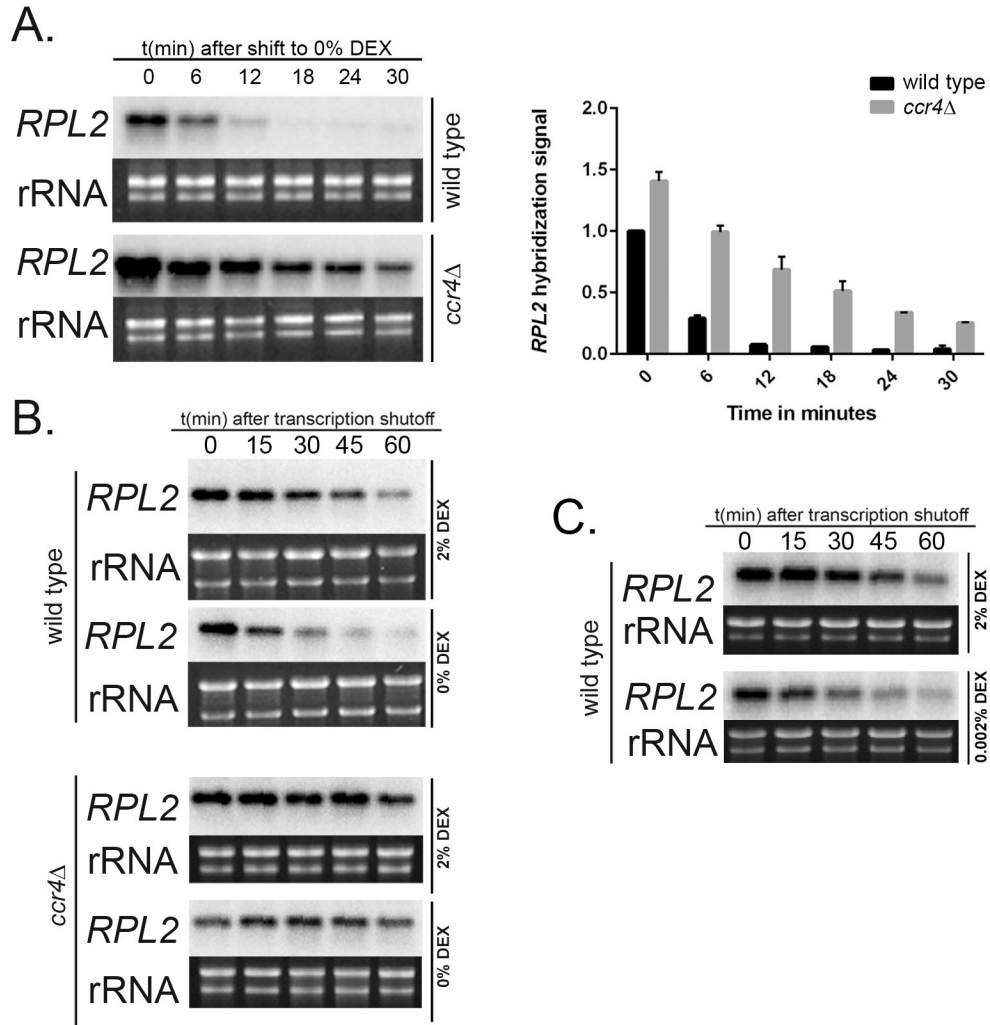
Figure 1. Carbon starvation in *C. neoformans* results in RP repression by Ccr4 mediated accelerated decay of RP mRNA

A) Northern Blot analysis of steady-state RNA extracted from wild type and *ccr4* mutant cells shifted from YPD (2% Dextrose) to YNB (0% Dextrose) showed reduced levels of *RPL2* in wild type in contrast to comparatively higher abundance of the same in the mutant. Graph represents relative abundance of *RPL2* in wild type and *ccr4* mutant cells with SEM values. Northern Blot analyses showed *RPL2* decay kinetics when (B, top panel) wild type and (B, bottom panel) *ccr4* mutant were shifted to minimal media. *RPL2* was rapidly destabilized with a shorter high life in wild type when starved whereas a significant stabilization of *RPL2* was observed in the *ccr4* mutant following starvation. Hybridization signal in bands corresponding to indicated transcripts were normalized to rRNA. C) Northern blot analysis showed rapid destabilization of *RPL2* transcript with shorter half-life in wild type following a shift from YPD to YNB supplemented with 0.002% dextrose.
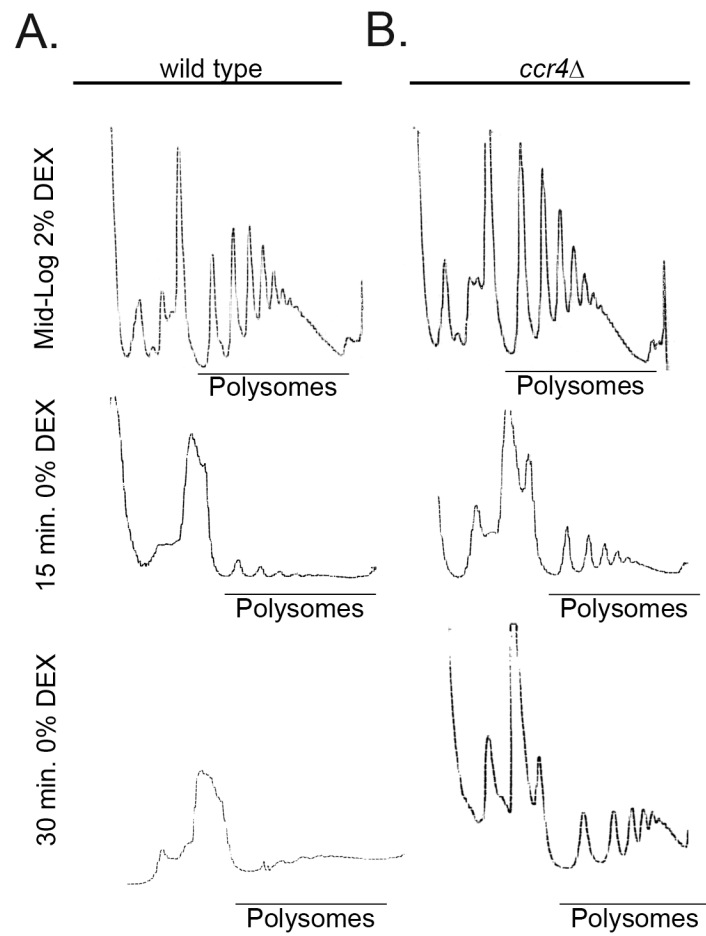
Figure 2. Glucose withdrawal leads to loss of polysomes following RP decay
Polysome profile analysis of wild type (A) showed reduced abundance of polysomes following a 15 minutes and 30 minutes shift to glucose deficient YNB medium whereas (B) a significant retention of polysomes was observed in the *ccr4* mutant at both time points following starvation.
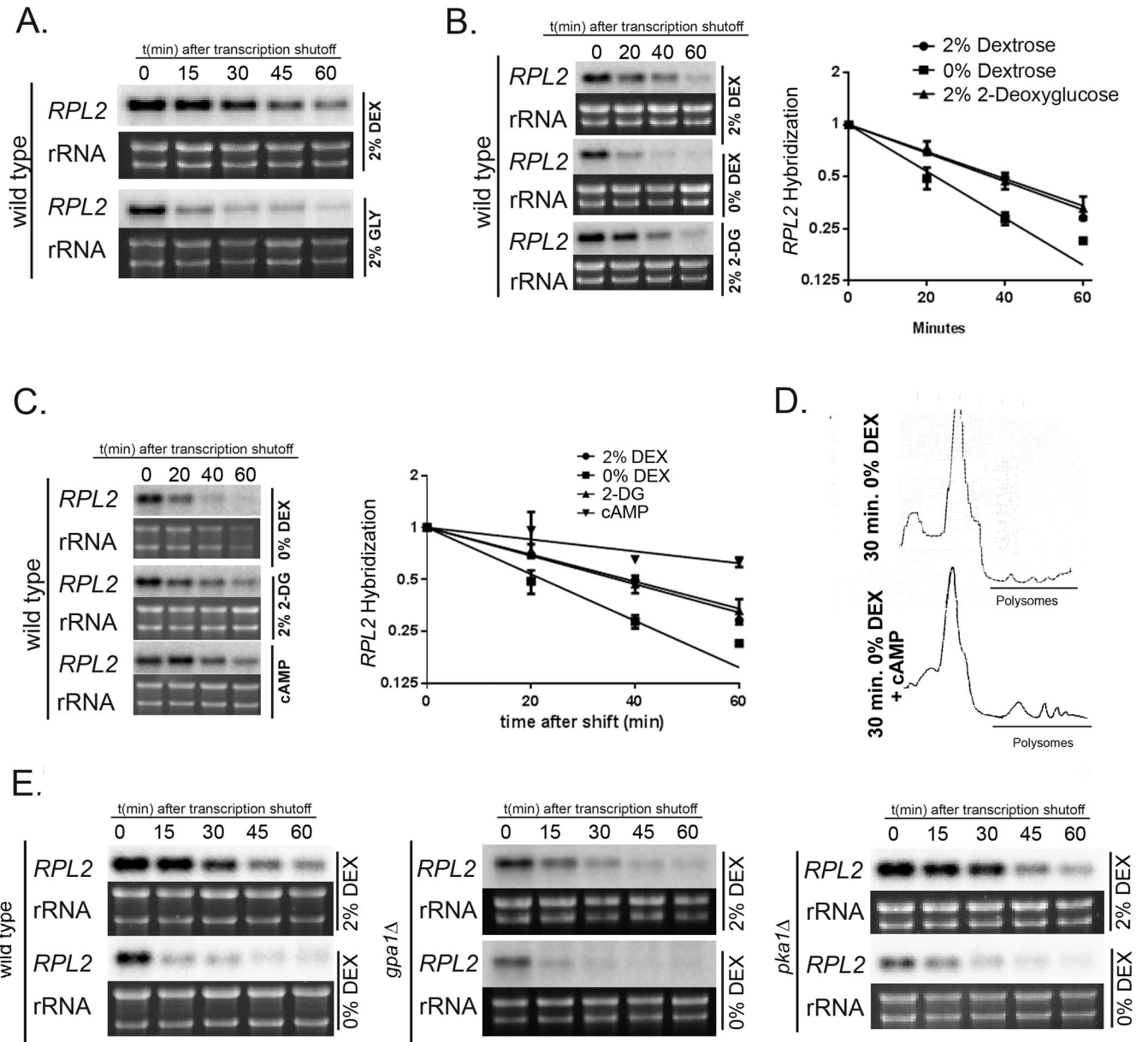
Figure 3. Activation of cAMP/PKA signaling stabilizes RP transcripts

A) Northern Blot analysis of wild type cells when shifted from YNB+2% dextrose to YNB supplemented with 2% glycerol as alternate carbon source showed a rapid decay of *RPL2* mRNA with a shorter half-life. Activation of glucose signaling by incubation of wild type cells in the presence of (B) exogenous 2-Deoxy-D-glucose (C) and cyclic AMP led to a stabilization of *RPL2* even in the absence of glucose. Decay kinetics was plotted and nonlinear regression analysis was used to determine half-lives. D) Polysome profile analysis of mid-log wild type cells incubated in YNB supplemented with cAMP showed reduced levels of polysomes similar to the profile obtained during glucose starvation. E) Inactivation of cAMP/PKA pathway did not influence *RPL2* decay as exhibited in Northern Blot

analyses in *gpa1* and *pka1* mutants, both in YPD (2% Dextrose) and YNB media (0% Dextrose).
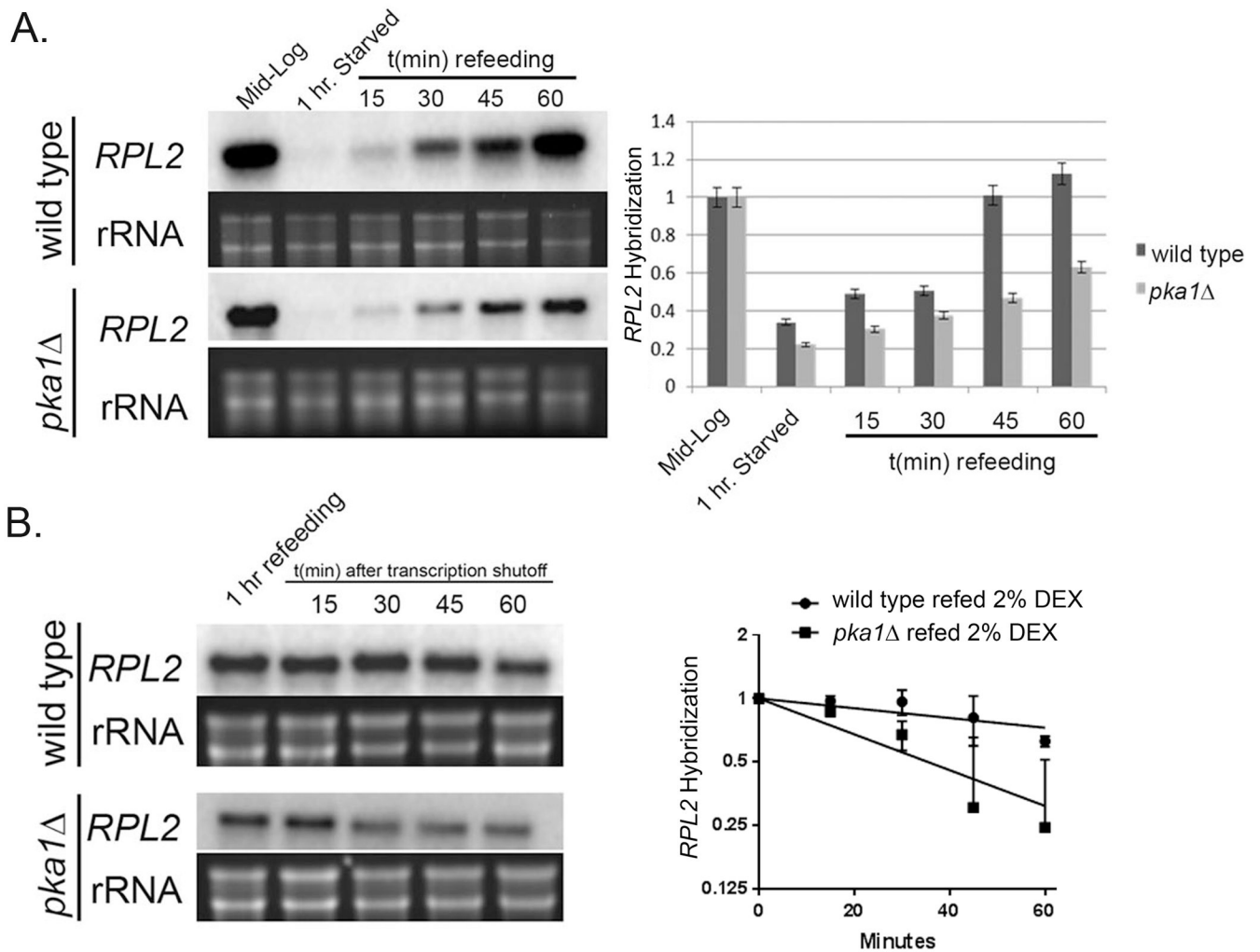
Figure 4. Stabilization of RP transcripts by PKA is important during refeeding of glucose

A) Northern Blot steady state analysis of starved wild type cells showed a progressive induction of *RPL2* following refeeding with glucose over a one hour period. This increase in *RPL2* abundance was defective in a *pka1* mutant. Mean expression of *RPL2* from 3 biological replicates are demonstrated in the histogram with error bars depicting SEM. B) Northern Blot analysis in starved wild type cells showed a greater stabilization of *RPL2* following refeeding with 2% dextrose. This stabilization was comparatively deficient in a *pka1* mutant with a shorter half life as depicted in the regression plot.
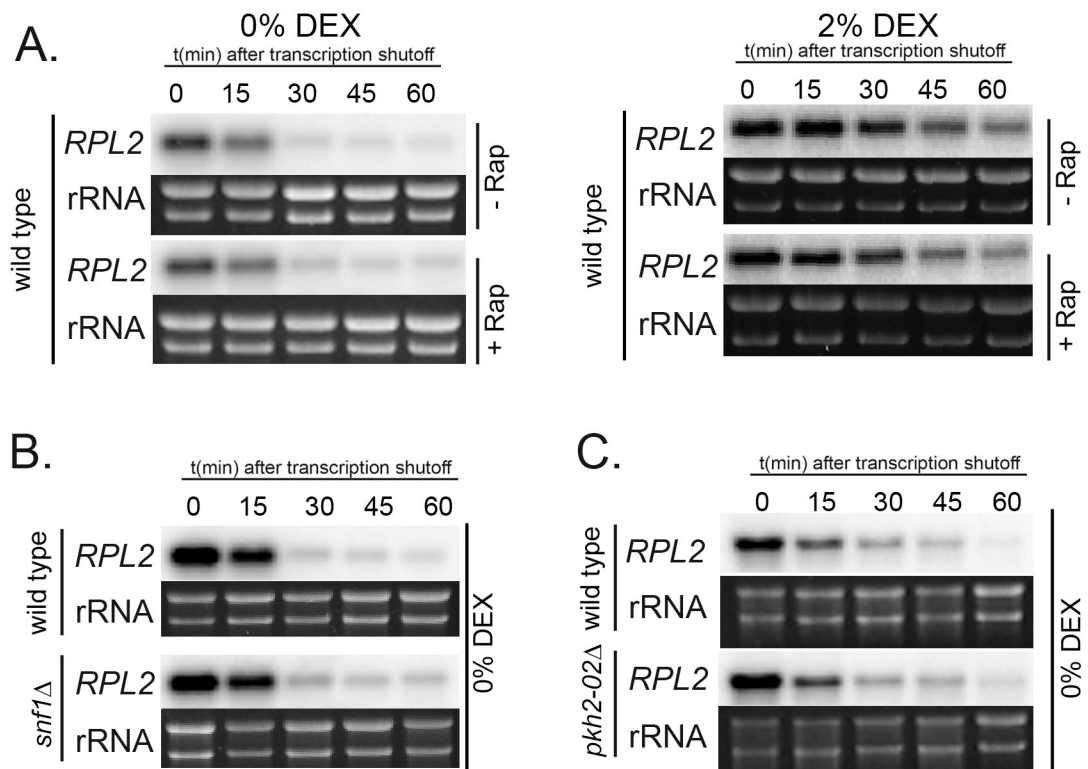
Figure 5. Common stress signaling pathways do not mediate starvation induced RP decay
Northern Blot analyses of (A, left panel) rapamycin treated wild type cells in YNB (A, right panel) rapamycin treated wild type cells in YPD (B) *snf1* mutant and (C) *pkh2 -02* mutant did not exhibit any stabilization of *RPL2* mRNA during glucose unavailability, excluding their role as a signaling regulator for RP decay.
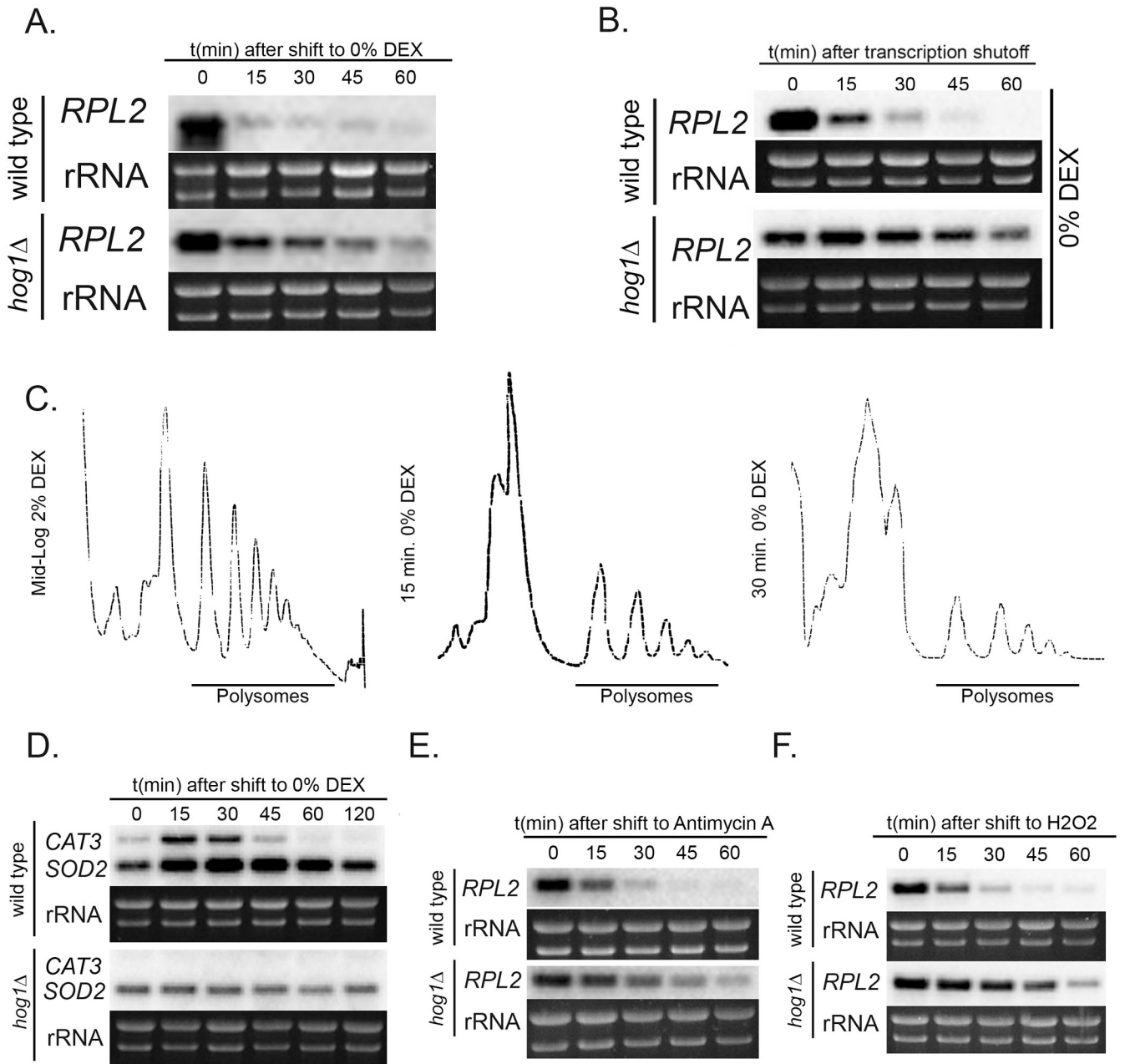
Figure 6. Hog1 signals accelerated RP transcript degradation during starvation stress

(A) Northern blot steady state analysis in *hog1* mutant cells showed higher abundance of *RPL2* following a shift from YPD (2% Dextrose) to YNB (0% Dextrose) compared to wild type. (B) This increased steady state *RPL2* levels in the *hog1* mutant was due to a greater stabilization of the transcript compared to wild type as depicted by stability time course data. (C) Polysome profile analysis of *hog1* mutant showed retention of polysomes when cells were shifted to minimal media for 15 or 30 minutes. (D) Induction of oxidative stress during glucose starvation was demonstrated in wild type by a transient increase in steady state levels of *CAT3* and *SOD2* during growth in YNB. This induction was absent in *hog1* mutant. Treatment of wild type cells with ROS generating compounds (E) Hydrogen

peroxide and (F) antimycin exhibited a rapid and drastic repression in *RPL2* that was absent in *hog1* mutant.
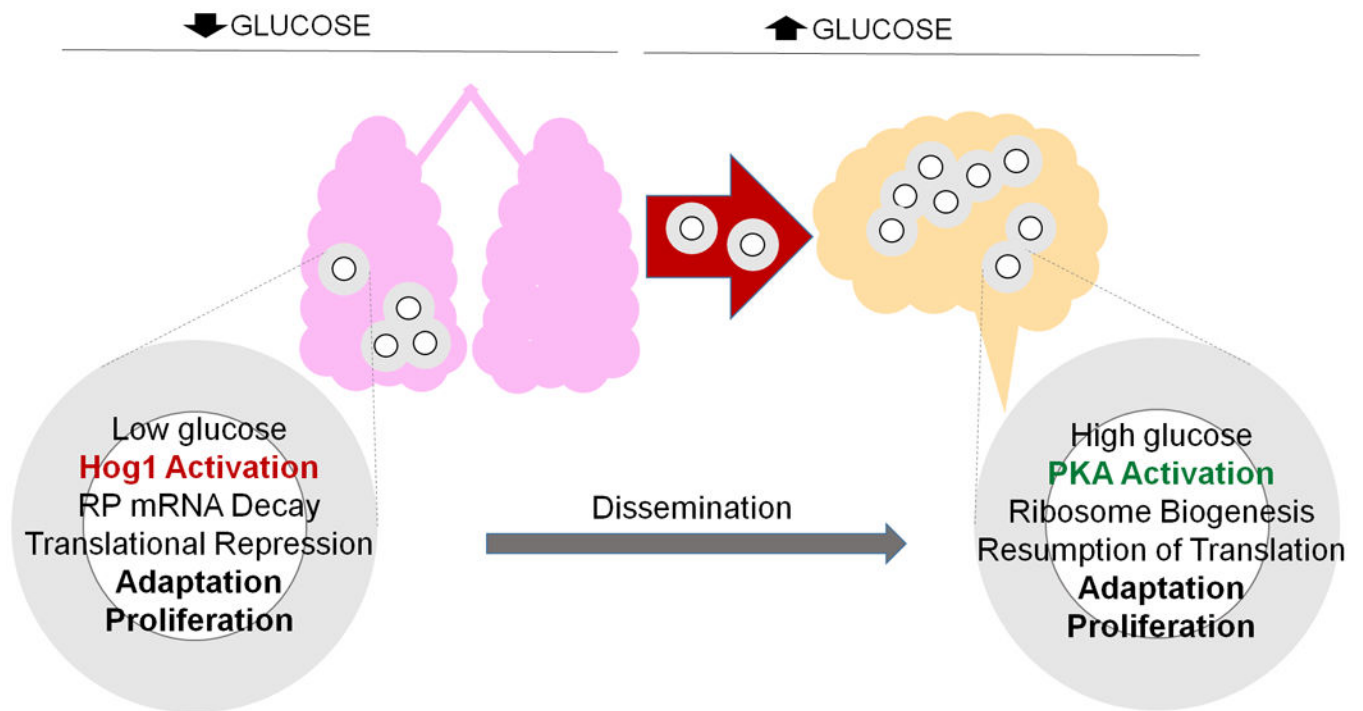
Figure 7. Hypothetical model for how the antagonism between Hog1 and PKA may promote host adaptation during *C. neoformans* infection

Glucose deprivation in the lungs results in Hog1 activation in *C. neoformans.* Hog1 signal mediates *RPL2* decay and translation repression that promotes adaptation to stress. The glucose-rich environment of the brain leads to PKA activation in *C. neoformans* that result in induction and stabilization of RP genes, and resumption of translation to promote adaptation and proliferation.