A single inhibitory upstream open reading frame (uORF) is sufficient to regulate *Candida albicans GCN4* translation in response to amino acid starvation conditions

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ABSTRACT

***Candida albicans* is a major fungal pathogen that responds to various environmental cues as part of its infection mechanism. We show here that the expression of *C. albicans GCN4*, which encodes a transcription factor that regulates morphogenetic and metabolic responses, is translationally regulated in response to amino acid starvation induced by exposure to the histidine analog 3-aminotriazole (3AT). However, in contrast to the well-known translational control mechanisms that regulate yeast *GCN4* and mammalian *ATF4* expression via multiple upstream open reading frames (uORFs) in their 5′-leader sequences, a single inhibitory uORF is necessary and sufficient for *C. albicans GCN4* translational control. The 5′-leader sequence of *GCN4* contains three uORFs, but uORF3 alone is sufficient for translational regulation. Under nonstress conditions, uORF3 inhibits *GCN4* translation. Amino acid starvation conditions promote Gcn2-mediated phosphorylation of eIF2α and leaky ribosomal scanning to bypass uORF3, inducing *GCN4* translation. *GCN4* expression is also transcriptionally regulated, although maximal induction is observed at higher concentrations of 3AT compared with translational regulation. *C. albicans GCN4* expression is therefore highly regulated by both transcriptional and translational control mechanisms. We suggest that it is particularly important that Gcn4 levels are tightly controlled since Gcn4 regulates morphogenetic changes during amino acid starvation conditions, which are important determinants of virulence in this fungus.**

Keywords: translational regulation; upstream open reading frame; Gcn4; *Candida albicans*

INTRODUCTION

All cells must be able to maintain their intracellular homeostasis during exposure to diverse physiological and environmental stress conditions. This requires that they regulate their gene expression patterns to adapt to the altered conditions. A common regulatory mechanism is instigated by global inhibition of translation initiation (Proud 2005). Reducing the rate of protein synthesis not only prevents continued gene expression during potentially error-prone conditions but also allows for the turnover of existing mRNAs and proteins while gene expression is reprogrammed to deal with the stress. Inhibiting translation initiation via phosphorylation of the α subunit of translation initiation factor 2 (eIF2) is a common response to diverse stress conditions in most eukaryotic cells (Proud 2005; Shenton et al. 2006). eIF2 is an essential GTP-binding protein that interacts with the initiator methionyl-tRNA (Met-tRNA$_i^{Met}$) to form a ternary complex (TC) that is competent for translation initiation. Phosphorylation of eIF2α at a conserved serine residue (Ser51) blocks GDP-GTP exchange, resulting in reduced TC levels, which inhibit translation initiation (Pavitt et al. 1998; Harding et al. 2000).

Phosphorylation of eIF2α inhibits global translation initiation but also induces translation of specific mRNAs, such as that encoding the metazoan activating transcription factor 4 (*ATF4*) (Harding et al. 2000; Vattem and Wek 2004). Translational regulation of ATF4 requires two uORFs in the 5′-leader of the ATF4 mRNA. Translation of uORF1 promotes ribosomal reinitiation, which under normal growth conditions occurs at inhibitory uORF2. Reduced TC levels increase the time required for ribosomes to reinitiate following uORF1 translation, and more ribosomes bypass uORF2 to reinitiate translation at the ATF4 start codon. Activation of ATF4 is considered part of a stress response signaling pathway termed the integrated stress response (ISR) which is initiated by eIF2α phosphorylation (Harding et al. 2003).

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ATF4 ultimately regulates the expression of genes that protect against the inducing stress conditions. In the yeast *Saccharomyces cerevisiae*, Gcn2 phosphorylates eIF2α in response to amino acid starvation conditions. This not only reduces global protein synthesis but also enhances translation of the *GCN4* mRNA (Hinnebusch 2005). Gcn4 is a transcription factor, which, analogous to the mammalian ISR, activates amino acid biosynthetic genes to overcome the imposed starvation that initially led to its translational control. Similar to ATF4, translation of the *GCN4* mRNA is activated in response to low TC levels in a mechanism involving four short uORFs (Hinnebusch 2005). This translational control mechanism depends on the sequence context immediately surrounding the stop codons of the uORFs, which modulate the ability of ribosomes to reinitiate translation. During normal unstressed conditions, ribosomes translate uORF1 and can reinitiate at inhibitory uORFs2–4. Decreased TC levels mean that ribosomes take longer to bind the TC and are more likely to scan past uORF2–4 and reinitiate at *GCN4* (Hinnebusch 2005).

Candida albicans is a major human fungal pathogen that causes opportunistic infections (Pfaller and Diekema 2007). Systemic *C. albicans* infections are life-threatening, particularly in immunocompromised patients undergoing organ transplantation and chemotherapy and in those with HIV/AIDS. This means that *Candida* infections are a leading cause of mortality arising from hospital-acquired bloodstream infections (Viudes et al. 2002; Wisplinghoff et al. 2004). Amino acid starvation conditions can promote morphogenetic changes in *C. albicans,* including hyphal differentiation and biofilm formation, which are important determinants of virulence (Tripathi et al. 2002; Rubin-Bejerano et al. 2003). *C. albicans* can mount a GCN (general amino acid control) response, where it induces the expression of most amino acid biosynthetic pathways in response to amino acid starvation, dependent on the Gcn4 transcription factor (Tripathi et al. 2002). Surprisingly, however, *GCN4* expression was thought to mainly be regulated at the transcriptional level in *C. albicans*, and Gcn2 has been proposed to play only a minor role in the activation of general amino acid control (Tournu et al. 2005). We show here that *C. albicans* Gcn4 expression is subject to both transcriptional and translational controls, but the induction of *GCN4* expression is predominantly regulated at the translational level in response to amino acid starvation conditions. Activation of *GCN4* expression requires Gcn2-dependent phosphorylation of eIF2α, but in contrast to yeast GCN4 and mammalian ATF4, regulation is predominantly mediated by a single inhibitory uORF, which is necessary and sufficient to control *GCN4* translation.

RESULTS

C. albicans Gcn2 is required for the response to amino acid starvation conditions

Amino acid starvation caused by exposure to the histidine analog 3-aminotriazole (3AT) has been extensively used to induce Gcn2-mediated phosphorylation of eIF2α in *S. cerevisiae* (Hinnebusch 2005). We first established amino acid starvation conditions using 3AT in *C. albicans* and examined eIF2α phosphorylation by immunoblot analysis. Exposure of *C. albicans* to 3AT at concentrations between 1 mM and 40 mM for 1 h caused a dose-dependent increase in eIF2α phosphorylation compared with untreated control cultures (Fig. 1A). No phosphorylation of eIF2α was detected in a *gcn2* mutant, indicating that phosphorylation is entirely dependent on the Gcn2 protein kinase. Translational activity was analyzed by examining the distribution of polysomes under the same growth conditions. Polysomes are ribosomes that are actively translating mRNAs and can be separated and detected on sucrose density gradients. There was a shift of ribosomes from the polysomal region into the monosome peak following treatment with 3AT, which is indicative of decreased translation initiation (Fig. 1B). No inhibition of

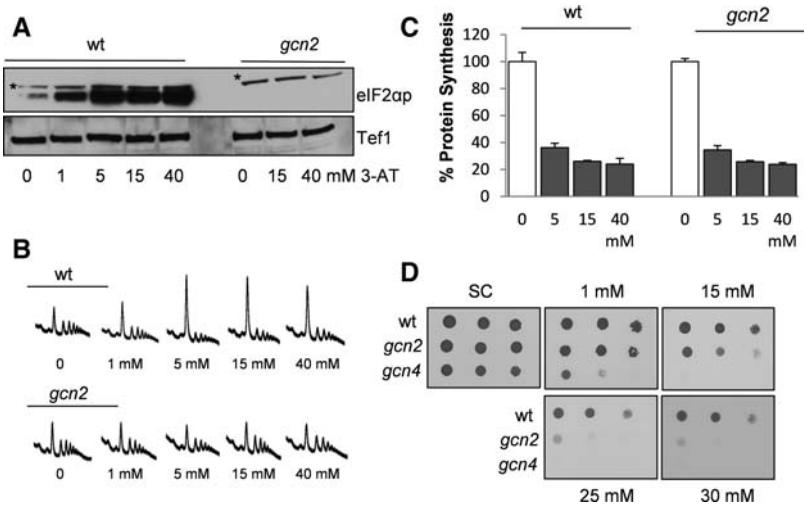

FIGURE 1. Regulation of *C. albicans* translation initiation by Gcn2. (*A*) The wild-type and *gcn2* mutant strains were treated with the indicated concentrations of 3AT for 1 h. Immunoblots are shown probed with antibodies specific for phosphorylated eIF2α and translation elongation factor 1 (Tef1) as a loading control. (*) A nonspecific band recognized by anti-eIF2αP. (*B*) Polyribosome traces are shown for strains treated with the indicated concentrations of 3AT for 1 h. Peaks correspond to small ribosomal subunits (40S), large ribosomal subunits (60S), monosomes (80S), and increasing numbers of ribosomes bound to mRNAs (polysomes). (*C*) Protein synthesis was measured by pulse labeling with [^{35}S]-cysteine/methionine for 5 min. Data are shown for untreated cultures (100%) and following treatments with different concentrations of 3AT. (*D*) Strains were grown to stationary phase and diluted cultures (A_{600} = 1.0, 0.1, and 0.01) spotted onto SCD agar plates containing the indicated concentrations of 3AT.

translation initiation was observed in a *gcn2* mutant following exposure to 3AT. These data confirm that similar to other eukaryotes, *C. albicans* inhibits translation initiation in response to amino acid starvation conditions via Gcn2-mediated phosphorylation of eIF2α. The rate of protein synthesis was measured during the final 5 min of 3AT treatment by the incorporation of [^{35}S]cysteine/methionine. Exposure of the wild-type strain to 5 mM 3AT inhibited translation by ~70%, whereas 15 mM and 40 mM 3AT treatments caused ~80% inhibition (Fig. 1C). The rate of protein synthesis was still inhibited to a similar extent in a *gcn2* mutant that could not inhibit translation initiation. This is consistent with histidine starvation causing an additional Gcn2-independent inhibition of protein synthesis, presumably at the level of translation elongation.

Loss of *GCN2* was reported to cause only a minor decrease in the resistance of *C. albicans* to 3AT, suggesting that the *C. albicans* GCN response is not Gcn2 dependent (Tournu et al. 2005). To further examine this finding, we compared the sensitivity of *gcn2* and *gcn4* mutants to a range of 3AT concentrations. In agreement with previous observations (Tripathi et al. 2002; Tournu et al. 2005), *gcn4* mutants are hypersensitive to amino acid starvation conditions and were unable to grow on plates containing 1 mM 3AT (Fig. 1D). In contrast, *gcn2* mutants were less sensitive and could grow on plates containing 15 mM 3AT. However, *gcn2* mutants are sensitive to 3AT since they could not grow at 3AT concentrations >25 mM, a concentration of 3AT that did not affect the growth of the wild-type strain. These data indicate that *C. albicans* Gcn2 is required for tolerance to amino acid starvation conditions.

Transcriptional and translational regulation of *C. albicans GCN4* expression

The transcription start site of *GCN4* was mapped by 5′-RACE and DNA sequencing. This analysis identified a similar 577-nt 5′-leader sequence in the *GCN4* mRNA under both nonstressed and 3AT stress conditions (Fig. 2A,B). To examine the translational activity of the *GCN4* mRNA, its 5′-leader sequence was cloned upstream of a luciferase reporter gene. The expression of *GCN4::Luc* was induced following exposure to 3AT with a maximal increase of about 10-fold following treatment with 15 mM 3AT for 3 h. (Fig. 3A). In contrast, the induction of *GCN4::Luc* expression was largely abrogated in a *gcn2* mutant, supporting a translational control mechanism for increasing *GCN4* expression. A much reduced two- to threefold increase in *GCN4::Luc* expression was still observed in the *gcn2* mutant, which presumably arises as a consequence of increased transcriptional activity. We used quantitative RT-PCR analysis to confirm that *GCN4* mRNA levels are elevated in response to 3AT exposure (Fig. 3B). *GCN4-Luc* mRNA levels were increased in response to 3AT, with maximal induction observed at 40 mM 3AT exposure in both the wild-type and *gcn2* mutant strains, consistent with transcriptional regulation of *GCN4* expression.

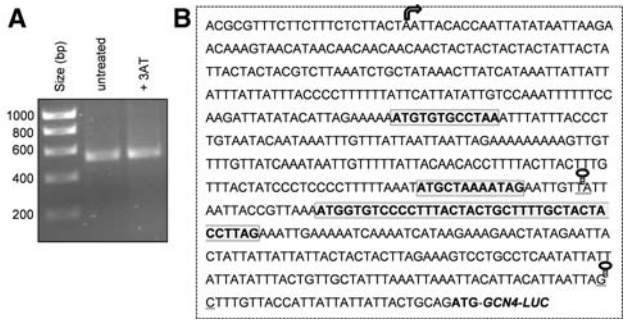

FIGURE 2. Mapping the transcription start site of *GCN4* by 5′-RACE. (*A*) The transcription start site of *GCN4* was mapped by 5′-RACE under both nonstressed and following 15 mM 3AT stress for 3 h. (*B*) The transcription start site is indicated with an arrow on the leader sequence of *GCN4*. uORF1, uORF2, and uORF3 are indicated with boxes. The positions of the stem–loop structures introduced upstream of and downstream from uORF3 are indicated.

To further examine the importance of Gcn2-mediated translational regulation of *GCN4* expression, we used a previously described Gcn4-response element (GCRE) reporter construct that can be used to measure Gcn4-mediated transcriptional activation (Tripathi et al. 2002; Tournu et al. 2005). This reporter construct contains five copies of the GCRE cloned in a basal promoter upstream of a luciferase reporter gene. The expression of *GCRE-Luc* was increased in response to 3AT treatment with maximal increases observed at lower concentrations of 3AT (Fig. 3C). This increased activation of the GCRE reporter was largely abrogated in a *gcn2* mutant, confirming that *GCN4* activity is predominantly regulated at the translational level in response to amino acid starvation conditions.

A single uORF is required for translational regulation of *GCN4* expression

The 5′-leader sequence of *GCN4* is predicted to contain three uORFs preceding the *GCN4* coding region (Fig. 2B). uORF1 and uORF2 encode small three amino acid polypeptides, whereas uORF3 encodes a larger 12-amino-acid polypeptide. By analogy with yeast *GCN4* and mammalian *ATF4*, these uORFs would be expected to play a role in regulating the translational activation of *GCN4* expression. We therefore mutated the initiation codon of each uORF to prevent its translation and to determine the role of each uORF in regulating *GCN4* expression. *GCN4::Luc* expression was measured following treatment of cells with 15 mM 3AT, which increased the expression of the wild-type *GCN4* construct by about 10-fold in a largely Gcn2-dependent manner (Fig. 4A). Simultaneous mutation of uORF1, uORF2, and uORF3 resulted in a greater than 100-fold increase in *GCN4-Luc* expression, indicating that these uORFs normally act to inhibit *GCN4* translation as expected (Fig. 4B). Quantitative RT-PCR analysis indicated that *GCN4-Luc* mRNA levels are induced by approximately sevenfold in response to 3AT stress

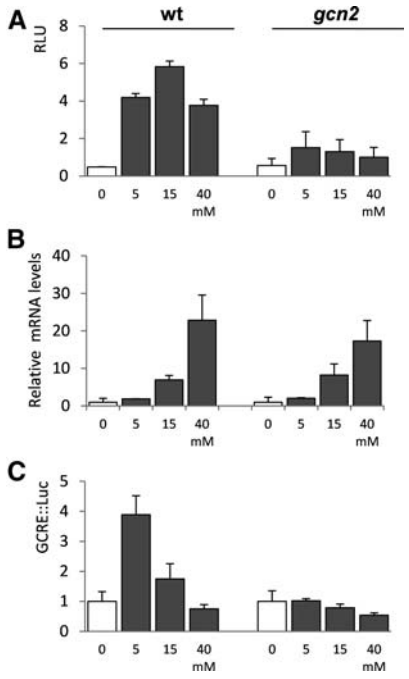

FIGURE 3. Transcriptional and translational regulation of *C. albicans GCN4* expression. (*A*) The wild-type and *gcn2* mutant strains were treated with the indicated concentrations of 3AT for 3 h, and *GCN4-Luc* activity was measured (RLU). (*B*) The wild-type and *gcn2* mutant were treated with 3AT as above, and *GCN4-Luc* mRNA levels are expressed relative to GAPDH. (*C*) *GCRE-Luc* activity is shown for the wild-type and *gcn2* mutant treated with 3AT as above.

conditions for both constructs in wild-type and *gcn2* mutant cells, confirming that translational alterations account for the differences in *GCN4-Luc* expression (Fig. 4C). Similar increases in *GCN4* mRNA levels were observed in response to 3AT compared with *GCN4-Luc* mRNA levels, confirming that the *GCN4::Luc* constructs are transcriptionally regulated similar to endogenous *GCN4*.

We next examined the requirement for each uORF in regulating *GCN4* expression. Surprisingly, mutation of uORF1 did not significantly affect *GCN4* translational regulation. Basal expression was moderately increased by 1.5-fold, but *GCN4* expression was still induced by about 12-fold in the uORF1 mutant in response to 3AT stress (Fig. 5A). This is unexpected since the 5′-proximal uORFs of yeast *GCN4* and mammalian *ATF4* play positive roles in promoting ribosomal scanning and reinitiation to promote *GCN4*/*ATF4* expression under amino acid starvation conditions (Vattem and Wek 2004; Hinnebusch 2005). Mutation of uORF2 increased the basal expression levels of *GCN4* by approximately twofold, but *GCN4-Luc* expression was still induced by sevenfold in response to 3AT stress. Mutation of uORF3 had the most dramatic effect on *GCN4* expression; basal *GCN4-Luc* expression was increased by 120-fold, and little or no further induction was observed in response to 3AT stress (Fig. 5A). Taken together, these data indicate that uORF3 appears to play the predominant role as an inhibitory uORF reducing *GCN4* translation.

To further define the role of uORFs in regulating *GCN4* expression, we mutated each uORF in pairs. Mutants lacking combinations of uORF1 and uORF3 or of uORF2 and uORF3 displayed significant increases in basal *GCN4* expression and were largely unable to induce *GCN4* expression further in response to 3AT stress (Fig. 5B). In contrast, regulation was still largely maintained in a construct lacking both uORF1 and uORF2, indicating that uORF3 is necessary and largely sufficient to control *GCN4* translation in response to amino acid starvation conditions (Fig. 5B). To confirm that the *GCN4-Luc* reporter is a faithful model of the authentic *GCN4* gene, a wild-type Gcn4 construct and a Gcn4 construct containing uORF3 alone were reintroduced into a *gcn4* deletion strain. Both constructs were able to complement the 3AT sensitivity of the *gcn4* mutant, confirming that uORFs1–2 are dispensable for regulation and uORF3 alone is sufficient to maintain *GCN4* function (Fig. 5C).

Regulation of *GCN4* expression depends on a leaky scanning mechanism at uORF3

DNA segments encoding stem–loop structures were inserted into the *GCN4* mRNA leader to confirm that regulation of *GCN4* translation requires a scanning mechanism rather than internal initiation. In one set of constructs, the sequence

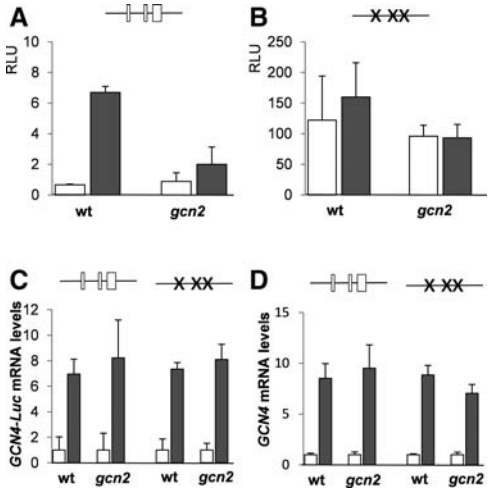

FIGURE 4. Regulation of *GCN4* translation by uORFs. (*A*) The wild-type and *gcn2* mutant strains containing a wild-type *GCN4-Luc* construct were treated with 15 mM 3AT for 3 h. The uORFs in *GCN4* are indicated as boxes in the schematic. (*B*) The wild-type and *gcn2* mutant strains containing a *GCN4-Luc* construct where uORF1–3 were mutated were treated with 15 mM 3AT for 1 h. Mutations in the uORFs of *GCN4* are indicated as crosses in the schematic. (*C*) Quantitative RT-PCR analysis of *GCN4-Luc* mRNA levels relative to GAPDH mRNA levels. (*D*) Quantitative RT-PCR analysis of GCN4 mRNA levels relative to GAPDH mRNA levels. White-colored bars denote values obtained from nonstressed cells; black-colored bars, values obtained from cells treated with 15 mM 3AT for 3 h.

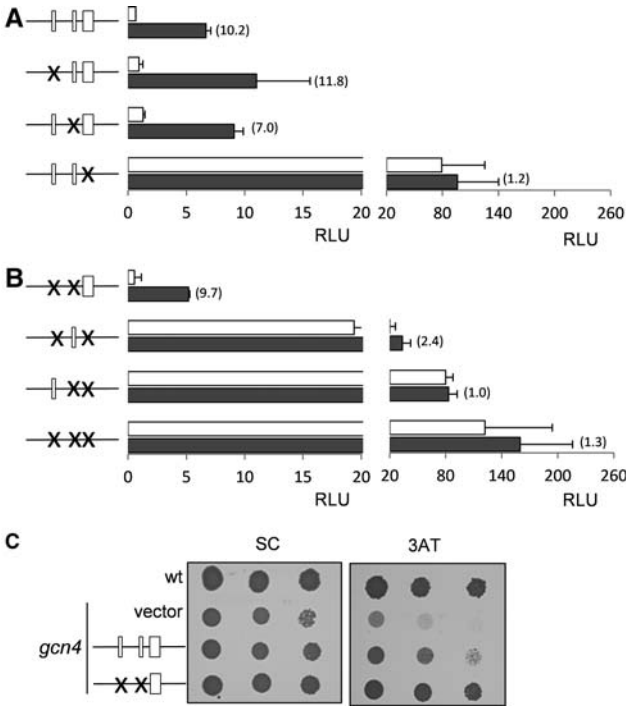

FIGURE 5. uORF3 is required for translational regulation of *GCN4* expression. (*A*) The start codons of each individual uORF were mutated in *GCN4-Luc* and are indicated as crosses in the schematic. Fold-induction is indicated in brackets. (*B*) Pairs of uORFs were mutated as indicated. White-colored bars denote values obtained from nonstressed cells; black-colored bars, values obtained from cells treated with 15 mM 3AT for 3 h. (*C*) The 3AT sensitivity of a *gcn4* mutant is complemented by reintroduction of wild-type *GCN4* and a *GCN4* construct containing uORF3 alone. The wild-type strain and *gcn4* mutant are shown containing the empty vector. Strains were grown to stationary phase and diluted cultures (A_{600} = 1.0, 0.1, and 0.01) spotted onto SCD agar plates containing 1 mM 3AT.

5′-GAATTCCCATCTTGGGAATTC-3′ (ΔG = −8.7 kcal) was introduced 16 nt upstream of uORF3 or 57 nt downstream from uORF3 (Fig. 2B). The secondary structure provided by this sequence has previously been shown to inhibit yeast *GCN4* expression (Abastado et al. 1991). In a second set of constructs, the sequence 5′-CTGCAGCCACCACG GCCCCCAAGCTTGGGCCGTGGTGGCTGCAG-3′ (ΔG = −41 kcal), which has previously been shown to inhibit mammalian *ATF4* expression (Vattem and Wek 2004), was introduced at the same locations. Both stem–loop structures were initially introduced into a construct lacking uORF1–3, where they were found to significantly decrease *GCN4-Luc* expression by about 50-fold and 200-fold, respectively (Fig. 6A). Interestingly, *GCN4-Luc* expression was induced by two- to fourfold in these constructs, suggesting that scanning ribosomes may be better able to scan past secondary structures during conditions of limiting TC availability. Alternatively, increased transcriptional induction may account for this increase in *GCN4-Luc* expression under 3AT starvation conditions. Both stem–loop structures also decreased *GCN4-Luc* expression in a wild-type construct, supporting a mechanism where ribosomes scan the leader sequence past uORF3 under starvation conditions to initiate *GCN4* translation (Fig. 6B). These stem–loop structures were also less inhibitory under derepressing conditions in a construct containing uORF1–3 (Fig. 6B). The reasons for this difference are unknown at present but may, again, suggest that *C. albicans* ribosomes are better able to scan past secondary structures during conditions of limiting TC availability.

Extensive overlap between uORF3 and *GCN4* coding sequences and increasing the spacing between uORF2 and uORF3 has little effect on *GCN4* expression

To rigorously test the model that ribosomes leaky scan past uORF3 under starvation conditions, we extended uORF3 such that it terminates downstream from the *GCN4* ATG codon. This was done by mutating the uORF3 stop codon and removing a subsequent in-frame stop codon. This creates an extended 67-codon version of uORF3 that terminates 30 nt downstream from the start codon of *GCN4*. This alteration had little or no effect on the expression of the *GCN4-LUC* reporter consistent with a model where ribosomes leaky scan past uORF3 under starvation conditions to reach *GCN4* (Fig. 7A). As a further test to rule out any requirement for a positively acting uORF that promotes scanning and reinitiation at *GCN4*, we inserted a 150-nt sequence upstream of uORF3. When a similar insertion was made in *S. cerevisiae GCN4* increasing the separation between uORF1 and uORF4, it did not affect expression under nonstarvation conditions but significantly reduced expression under derepressing conditions (Abastado et al. 1991). This is consistent with the idea that *S. cerevisiae* uORF1 is required to promote reinitiation at *GCN4* under starvation conditions. In contrast, increasing the separation between uORF1–2 and uORF3 in *C. albicans GCN4* did not reduce *GCN4* expression under starvation conditions (Fig. 7A). Basal expression under nonstarvation conditions was somewhat increased, suggesting that increasing the separation between uORF2 and uORF3, or the sequence introduced, somehow decreased the ability of ribosomes to recognize and initiate translation at the uORF3 start codon. Nevertheless, these data are inconsistent with the *S. cerevisiae GCN4* model where ribosomes initiate translation at an uORF and control depends on their ability to scan past an inhibitory uORF in order to reinitiate at *GCN4*.

The sequence of uORF3 is not important for regulating *GCN4* expression

Codon context plays an important role in the recognition of AUG start codons, and one possibility is that ribosomes scan past uORF3 to translate *GCN4* under amino acid starvation conditions. However, the start codon context of uORF3 (GTTAAA**ATG**G) is similar to the Kozak consensus sequence 5′-gcc(A/G)cc**ATG**G-3′, where the A/G at −3 and

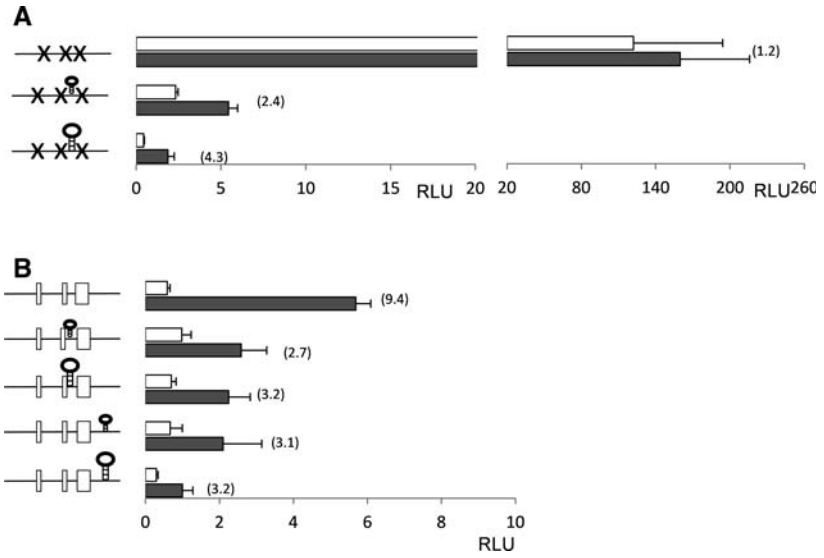

FIGURE 6. Regulation of *GCN4* expression depends on a leaky scanning mechanism at uORF3. Two different stem–loop structures (ΔG = −8.7 kcal and −41 kcal) were introduced into *GCN4-Luc* lacking uORFs (*A*) or containing uORFs (*B*) as indicated. Fold-induction is indicated in brackets. White-colored bars denote values obtained from nonstressed cells; black-colored bars, values obtained from cells treated with 15 mM 3AT for 3 h.

thought to provide flexibility in controlling *ATF4* expression, which is important in the response to differing stress conditions as part of the ISR (Dey et al. 2010). Amino acid starvation conditions can promote morphogenetic changes in *C. albicans,* including hyphal differentiation and biofilm formation, which are important determinants of virulence (Tripathi et al. 2002; Rubin-Bejerano et al. 2003; Garcia-Sanchez et al. 2004). *GCN4* is implicated in these morphogenetic changes, and it is therefore important that *C. albicans GCN4* expression is carefully controlled.

Maximal transcriptional induction of *GCN4* was observed at higher concentrations of 3AT compared with translational induction. Translational regulation of *GCN4* expression therefore appears to be the first response to low levels of amino acid starvation. Increasing 3AT

the G at +4 are thought to be the most critical residues (Kozak 1986). Nevertheless, we changed the sequence around the AUG codon of uORF3 to match the Kozak consensus sequence. However, the induction of *GCN4* expression in response to amino acid starvation conditions was largely maintained, although there was a moderate increase in expression under both normal and stressed conditions (Fig. 7B). The peptide sequence encoded by uORF3 (MVSP LLLLLLLP) is unusual since it contains a run of seven Leu residues. We therefore tested the requirement for the coding sequence of uORF3 by inserting a C residue at +5 and deleting a C residue at +35 to generate an unrelated uORF3 peptide sequence (MAVPFTTAFATT). Replacing uORF3 with an uORF encoding an unrelated peptide sequence did not significantly alter *GCN4* translational regulation, indicating that the peptide sequence of uORF3 is not important for control (Fig. 7B).

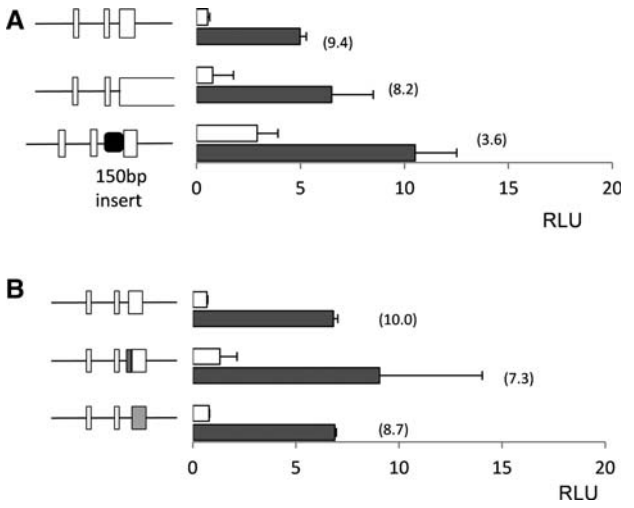

FIGURE 7. Regulation of GCN4 expression. (*A*) Extensive overlap between uORF3 and *GCN4* coding sequences has little effect on *GCN4* expression. The uORF3 stop codon and a subsequent in-frame stop codon were removed. This creates an extended 67-codon version of uORF3 that terminates 30 nt downstream from the start codon of *GCN4.* Increasing the spacing between uORF2 and uORF3 does not reduce *GCN4* expression under starvation conditions. A 150-nt sequence, normally found between uORF3 and *GCN4,* was inserted between uORF2 and uORF3 as indicated. (*B*) The start codon context of uORF3 is not important for *GCN4* translational control. The start codon context of uORF3 (GTTAAAATGG) was changed to more closely match the Kozak consensus sequence (GCCACCATGG), indicated as a shaded box in the schematic. The coding sequence of uORF3 is not important for *GCN4* translational control. The peptide sequence encoded by uORF3 (MVSPLLLLLLLP) was changed (MAVPFTTAFATT), indicated as a hatched box in the schematic. White-colored bars denote values obtained from nonstressed cells; black-colored bars, values obtained from cells treated with 15 mM 3AT for 3 h.

DISCUSSION

Our data indicate that *C. albicans GCN4,* which encodes a central regulator of amino acid biosynthetic genes, is regulated at both the transcriptional and post-transcriptional levels in response to amino acid starvation. Yeast *GCN4* is minimally regulated at the transcriptional level and has long served as a model of translational gene regulation (Hinnebusch 2005), whereas mammalian *ATF4* gene expression is subject to both transcriptional and translational regulation (Dey et al. 2010). Regulation of *C. albicans GCN4* is therefore more similar to mammalian *ATF4* than to yeast *GCN4.* Combining transcriptional and translational regulation is

concentrations induced *GCN4* transcription, which may reflect the need to increase *GCN4* mRNA levels under conditions where global translation is strongly inhibited. Indeed, protein synthesis measured by radiolabelling was strongly inhibited in wild-type and *gcn2* mutant cells at higher concentrations of 3AT. This inhibition of protein synthesis at the post-initiation phase of translation may explain why no increase in *GCN4-Luc* expression is observed in a *gcn2* mutant despite the strong transcriptional induction that is observed following exposure to 40 mM 3AT. It is interesting that induction of the *GCRE-Luc* reporter, which provides a measure of gene activation by Gcn4 (Tripathi et al. 2002), was mainly induced at low concentrations of 3AT compared with both the transcriptional and translational induction observed in response to 3AT. Additional regulatory inputs are known to regulate yeast Gcn4-target gene expression, including transcription factors and coactivators, which may account for this difference (Takemaru et al. 1998; Patil et al. 2004).

In contrast to both *ATF4* and yeast *GCN4,* which are translationally regulated via mechanisms that require multiple uORFs in their 5′-leader sequences, a single inhibitory uORF is sufficient for *C. albicans GCN4* translational control. uORF3 appears to play the predominant role as an inhibitory uORF reducing *GCN4* translation. This is similar to yeast *GCN4*, where the 3′-proximal uORF4 plays the predominant role as an inhibitory uORF (Abastado et al. 1991). *GCN4* translational regulation is maintained in a construct containing a single uORF3, which raises the question as to the requirement for uORF1 and uORF2. The presence of any positively acting short uORFs that might be initiated by a near-cognate start codon is unlikely since inserting a large 150-nt sequence upstream of uORF3 did not alter *GCN4* translational control. Inserting a similar-sized sequence between uORF1 and uORF4 in *S. cerevisiae GCN4* significantly reduced *GCN4* expression under derepressing conditions, consistent with the idea that the increased scanning time enabled ribosomes to reacquire TC and to reinitiate at inhibitory uORF4 (Abastado et al. 1991).

Comparison of the construct lacking any uORFs with constructs containing uORF1 and uORF2 alone indicates that they inhibit *GCN4* translation by ~35% and 85%, respectively. One possibility is that uORF1 and uORF2 act to moderate *GCN4* expression by regulating ribosomal scanning on the *GCN4* mRNA. This may be important in order to respond to different levels of amino acid starvation and eIF2α phosphorylation. It is not clear why uORF3 is more inhibitory than uORF1 and uORF2. Codon context plays an important role in the recognition of AUG start codons and, for example, is important in the regulation of translation at mammalian uORFs (Kolitz and Lorsch 2010; Palam et al. 2011). The start codon contexts of uORF1 (GAAAAA**ATG**T) and uORF2 (TTAAAT**ATG**C) may be less optimal than uORF3 since they do not contain a G at the +4 position. Additionally, uORF3 may be more inhibitory since it is longer, encoding a 12-amino-acid polypeptide, compared with uORF1 and uORF2, which encode short three-amino-acid polypeptides. Interestingly, uORF1 does appear to somewhat mitigate the inhibitory effect of uORF2 under nonstarvation conditions based on the comparison of constructs containing both uORF1 and uORF2 with constructs containing uORF1 or uORF2 alone (Fig. 5). These data suggest that uORF1 and uORF2 may be able to play regulatory roles under certain growth conditions.

Changing the start codon at uORF3 to match the Kozak consensus sequence did not abrogate *GCN4* translational control. It should be stressed, however, that little is known regarding the importance of start codon context in *C. albicans*, and it is possible that the sequence around the uORF3 start codon is important for regulating *C. albicans GCN4* translation. Many fungal systems contain frequent uORFs in the 5′-leader regions of diverse genes (Hood et al. 2009). Although it is thought that a number of these may play a regulatory role by controlling ribosomal scanning to their downstream coding regions, few examples have been examined experimentally. Regulation of *C. albicans GCN4* translation involves a scanning mechanism to reach the *GCN4* coding region since the introduction of stem–loop structures around uORF3 decreased the induction of *GCN4* expression normally seen in response to amino acid starvation conditions. A scanning mechanism is also consistent with the finding that the uORF3 reading frame could be extended such that it overlapped the start of *GCN4* without affecting translational regulation in response to amino acid starvation conditions.

Our model for translational regulation of *C. albicans GCN4* is that under normal growth conditions, most ribosomes initiate translation at uORF3 and, to a lesser extent, at uORF1 and uORF2, preventing scanning to the *GCN4* ORF. Hence, inserting stem–loop structures into a wild-type construct did not have the same large impact on scanning to *GCN4* compared with a construct lacking uORFs since most scanning to *GCN4* is already blocked by uORF3. It is possible that the low-basal level of translation seen in the presence of stem–loop structures (or absence of Gcn2) does not require scanning from the predominant 5′ end of the mRNA. This may be explained by proposing the existence of a small percentage of transcripts with 5′ ends located between the stem–loop insertion points and the *GCN4* start codon, which would be below the detection of the 5′-RACE analysis. Under amino acid starvation conditions, phosphorylation of eIF2α increases leaky scanning past uORF3 and promotes *GCN4* translation. This is similar to the bypass of uORFs with poor initiation codon context, which has been observed in response to phosphorylation of eIF2α (Palam et al. 2011). One possibility is that under conditions of limiting TC levels, ribosomes are able to leaky scan past small uORFs such as uORF3, but large structured coding regions like the *GCN4* ORF are able to "catch" these ribosomes, initiating *GCN4* translation. It should be emphasized that the majority of scanning ribosomes (>80%) still initiate at uORF3, and only a modest decrease in translation at

uORF3 accounts for the 10-fold increase in GCN4 translation. Hence, uORF3 appears to act as a moderator of *C. albicans GCN4* expression, regulating the number of scanning ribosomes that initiate *GCN4* translation in response to amino acid starvation conditions.

MATERIALS AND METHODS

Strains and growth conditions

The *C. albicans gcn2* and *gcn4* mutants were isogenic derivatives of CAI-4 (*ura3*::λ *imm434/ura3*::λ *imm434*) as described previously (Tournu et al. 2005). Strains were grown in complex YEPD (2% w/v glucose, 2% w/v bactopeptone, 1% w/v yeast extract) or synthetic complete (SC) medium (2% w/v glucose, 0.67% w/v yeast nitrogen base, 0.185% w/v complete amino acid supplement [Formedium] mixtures). Uridine was supplemented to a final concentration of 25 μg/mL. Strains were grown at 30°C and 180 rpm. Media were solidified by the addition of 2% (w/v) agar. Stress sensitivity was determined by growing cells to stationary phase and spotting diluted cultures (A_{600} = 1.0, 0.1 and 0.01) onto agar plates containing various concentrations of 3AT. Liquid cultures were treated with 3-AT by growing cells to exponential phase and treating with different concentrations of 3-AT as indicated.

Plasmids and strain construction

The 600 bp immediately upstream of the *C. albicans GCN4* coding sequence were synthesized by Life Technologies. Sequences were also synthesized containing single point mutations (ATG to TTG) at the start codons of uORF1, uORF2, and uORF3. An extended uORF3 construct was synthesized by changing the normal uORF3 stop codon (TAG) to TAC and a subsequent in-frame TAG codon (33 nt downstream from uORF3) to TAC. The separation between uORF2 and uORF3 was increased by inserting a 150-nt sequence normally found between uORF3 and *GCN4*, 16 nt upstream of uORF3. Constructs containing multiple uORF start codon mutations were made using the QuikChange method (Agilent). Similarly, the 6 nt immediately upstream of uORF3 was changed to match the mammalian kozak consensus sequence (GCCACC ATGG), and the uORF3 coding sequence was changed by inserting a C residue at +5 and deleting a C residue at +35 using the QuikChange method. Two sequences with the potential to form stem–loop structures (5′-GAATTCCCATCTTGGGAATTC-3′) and (5′-CTGCAGCCACCACGGCCCCCAAGCTTGGGCCGTGG TGGCTGCAG-3′) were introduced 16 nt upstream of uORF3 and 57 nt downstream from uORF3 using the Q5 site-directed mutagenesis kit (New England Biolabs). Constructs were ligated immediately upstream of *RLUC* in the pCRW3N basal vector using MluI and PstI restriction sites as described previously (Tripathi et al. 2002). Plasmids were integrated at the *ADE2* locus of wild-type and *gcn2* mutant strains, and three independent correct colonies were used for each experiment. The reporter construct containing five copies of the GCRE cloned in a basal promoter upstream of a luciferase reporter gene has been described previously (Tripathi et al. 2002; Tournu et al. 2005). To check for complementation of the *gcn4* mutant, wild-type *GCN4* and *GCN4* with mutated uORF1 and uORF2 was PCR amplified and cloned in the pCRW3N vector using PstI and Xho1 restriction sites. Plasmids were integrated at the *ADE2* locus.

Transcription start site mapping of GCN4

Total RNA was extracted using the RNEasy mini kit (Qiagen). Five micrograms of total RNA was used to identify the transcriptional start site of *GCN4* using the FirstChoice RLM-RACE Kit (Ambion) according to the manufacturer's instructions. Two rounds of nested PCR were performed using 5′-RACE outer primer (forward 5′-GCTGATGGCGATGAATGAACACTG-3′), GCN4 outer primer (reverse 5′-TGCTGAAACCCGACTCCAAT-3′), 5-RACE inner primer (forward 5′-CGCGGATCCGAACACTGCGTTTGCTGGC TTTGATG-3′), and GCN4 inner primer (reverse 5′-TTGAGGC AGGACTTTCTAAGTAGT-3′). PCR products were run in 1.5% agarose gels and purified using a PCR purification kit (Macherey-Nagel). Purified PCR products were cloned using a TA cloning kit (Invitrogen), and transcription start sites were identified by DNA sequencing.

Quantitative RT-PCR analysis

RNA was extracted using the RNeasy mini kit (Qiagen) and treated with recombinant DNase I (Ambion). Two hundred nanograms of total RNA was reverse transcribed into cDNA using Oligo(dT)20 primer and iScript reverse transcriptase (Biorad). RT-PCR was performed using iTaq Universal SYBR green supermix (Biorad) in a CFX connect real-time PCR detection system (Biorad). Primers used for detection of *RLUC* mRNA forward and reverse primers were 5′-GGAATTATAATGCTTATCTACGTGC-3′ and 5′-CTTGC GAAAAATGAAGACCTTTTAC-3′; *GAPDH* mRNA forward and reverse primers were 5′-CGAAGGTGCTCAAAAACACA-3′ and 5′-TGTACCACCAACTGTTTGGC-3′; and *GCN4* mRNA forward and reverse primers were 5′-CCAGAAATGCAAAAGGCTTC-3′ and 5′-GACTTTGGCTCCGTCCATAA-3′.

Analysis of protein synthesis

For the analysis of ribosome distribution on sucrose density gradients, cultures were grown to exponential phase and treated with 3AT as indicated. Extracts were prepared in the presence of 1 mg/mL cycloheximide/mL and layered onto 15%–50% sucrose gradients. The gradients were sedimented via centrifugation at 40,000 rpm in a Beckman ultracentrifuge for 2.5 h, and the A_{254} was measured continuously to give the traces shown, as described previously (Shenton et al. 2006). Monosome and polysome peaks were quantified using the National Institutes of Health Image J software (http://rsb.info.nih.gov/ij/). The rate of protein synthesis was measured in cells treated with various concentrations of 3AT. Cells were treated with 3AT for 1 h and pulse-labeled for the last 5 min of the treatment with 85 μM L-[^{35}S] cysteine/methionine as described previously (Shenton and Grant 2003).

Western blot analysis

Protein extracts were electrophoresed under reducing conditions on SDS-PAGE minigels and electroblotted onto PVDF membrane (Amersham Pharmacia Biotech). Blots were probed using

phosphospecific eIF2α antibodies as described previously (Holmes et al. 2004). Antibodies raised against yeast elongation factor 1 (Tef1) were used as a loading control.

Reporter assays

Luciferase analysis was performed essentially as explained previously by Srikantha et al. (1996). Briefly, cells were harvested and extracted using RLUC buffer (0.5 M NaCl, 0.1 M K_2HPO_4 at pH 6.7, 1 mM Na_2 EDTA, 0.6 mM sodium azide, 1 mM phenylmethylsulfonyl fluoride, 0.02% bovine serum albumin). Luciferase assays were started by adding 1.25 μM coelenterazine h (Promega) to cell extracts, and activity was measured using a GloMax 20/20 luminometer (Promega). Luciferase activity (RLU) is expressed as relative luminescence per 10 sec/mg protein.

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REFERENCES

Abastado JP, Miller PF, Jackson BM, Hinnebusch AG. 1991. Suppression of ribosomal reinitiation at upstream open reading frames in amino acid-starved cells forms the basis for *GCN4* translational control. *Mol Cell Biol* **11:** 486–496.

Dey S, Baird TD, Zhou D, Palam LR, Spandau DF, Wek RC. 2010. Both transcriptional regulation and translational control of ATF4 are central to the integrated stress response. *J Biol Chem* **285:** 33165–33174.

Garcia-Sanchez S, Aubert S, Iraqui I, Janbon G, Ghigo JM, d'Enfert C. 2004. *Candida albicans* biofilms: A developmental state associated with specific and stable gene expression patterns. *Eukaryot Cell* **3:** 536–545.

Harding HP, Novoa I, Zhang Y, Zeng H, Wek R, Schapira M, Ron D. 2000. Regulated translation initiation controls stress-induced gene expression in mammalian cells. *Mol Cell* **6:** 1099–1108.

Harding HP, Zhang Y, Zeng H, Novoa I, Lu PD, Calfon M, Sadri N, Yun C, Popko B, Paules R, et al. 2003. An integrated stress response regulates amino acid metabolism and resistance to oxidative stress. *Mol Cell* **11:** 619–633.

Hinnebusch AG. 2005. Translational regulation of *GCN4* and the general amino acid control of yeast. *Annu Rev Microbiol* **59:** 407–450.

Holmes LE, Campbell SG, De Long SK, Sachs AB, Ashe MP. 2004. Loss of translational control in yeast compromised for the major mRNA decay pathway. *Mol Cell Biol* **24:** 2998–3010.

Hood HM, Neafsey DE, Galagan J, Sachs MS. 2009. Evolutionary roles of upstream open reading frames in mediating gene regulation in fungi. *Annu Rev Microbiol* **63:** 385–409.

Kolitz SE, Lorsch JR. 2010. Eukaryotic initiator tRNA: Finely tuned and ready for action. *FEBS Lett* **584:** 396–404.

Kozak M. 1986. Point mutations define a sequence flanking the AUG initiator codon that modulates translation by eukaryotic ribosomes. *Cell* **44:** 283–292.

Palam LR, Baird TD, Wek RC. 2011. Phosphorylation of eIF2 facilitates ribosomal bypass of an inhibitory upstream ORF to enhance *CHOP* translation. *J Biol Chem* **286:** 10939–10949.

Patil CK, Li H, Walter P. 2004. Gcn4p and novel upstream activating sequences regulate targets of the unfolded protein response. *PLoS Biol* **2:** E246.

Pavitt GD, Ramaiah KV, Kimball SR, Hinnebusch AG. 1998. eIF2 independently binds two distinct eIF2B subcomplexes that catalyze and regulate guanine–nucleotide exchange. *Genes Dev* **12:** 514–526.

Pfaller MA, Diekema DJ. 2007. Epidemiology of invasive candidiasis: A persistent public health problem. *Clin Microbiol Rev* **20:** 133–163.

Proud CG. 2005. eIF2 and the control of cell physiology. *Semin Cell Dev Biol* **16:** 3–12.

Rubin-Bejerano I, Fraser I, Grisafi P, Fink GR. 2003. Phagocytosis by neutrophils induces an amino acid deprivation response in *Saccharomyces cerevisiae* and *Candida albicans*. *Proc Natl Acad Sci* **100:** 11007–11012.

Shenton D, Grant CM. 2003. Protein S-thiolation targets glycolysis and protein synthesis in response to oxidative stress in the yeast *Saccharomyces cerevisiae*. *Biochem J* **374:** 513–519.

Shenton D, Smirnova JB, Selley JN, Carroll K, Hubbard SJ, Pavitt GD, Ashe MP, Grant CM. 2006. Global translational responses to oxidative stress impact upon multiple levels of protein synthesis. *J Biol Chem* **281:** 29011–29021.

Srikantha T, Klapach A, Lorenz WW, Tsai LK, Laughlin LA, Gorman JA, Soll DR. 1996. The sea pansy *Renilla reniformis* luciferase serves as a sensitive bioluminescent reporter for differential gene expression in *Candida albicans*. *J Bacteriol* **178:** 121–129.

Takemaru K, Harashima S, Ueda H, Hirose S. 1998. Yeast coactivator MBF1 mediates GCN4-dependent transcriptional activation. *Mol Cell Biol* **18:** 4971–4976.

Tournu H, Tripathi G, Bertram G, Macaskill S, Mavor A, Walker L, Odds FC, Gow NA, Brown AJ. 2005. Global role of the protein kinase Gcn2 in the human pathogen *Candida albicans*. *Eukaryot Cell* **4:** 1687–1696.

Tripathi G, Wiltshire C, Macaskill S, Tournu H, Budge S, Brown AJ. 2002. Gcn4 co-ordinates morphogenetic and metabolic responses to amino acid starvation in *Candida albicans*. *EMBO J* **21:** 5448–5456.

Vattem KM, Wek RC. 2004. Reinitiation involving upstream ORFs regulates *ATF4* mRNA translation in mammalian cells. *Proc Natl Acad Sci* **101:** 11269–11274.

Viudes A, Peman J, Canton E, Ubeda P, Lopez-Ribot JL, Gobernado M. 2002. Candidemia at a tertiary-care hospital: Epidemiology, treatment, clinical outcome and risk factors for death. *Eur J Clin Microbiol Infect Dis* **21:** 767–774.

Wisplinghoff H, Bischoff T, Tallent SM, Seifert H, Wenzel RP, Edmond MB. 2004. Nosocomial bloodstream infections in US hospitals: Analysis of 24,179 cases from a prospective nationwide surveillance study. *Clin Infect Dis* **39:** 309–317.