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The eIF2 kinase Gcn2 modulates *Candida albicans* virulence to *Caenorhabditis elegans*

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Abstract

Morphogenesis is related to *Candida albicans* pathogenesis and nutritional/ environmental factors linked to eIF2-mediated translational regulation, modulated by Gcn2 and Gcn4, are common inducers of morphological changes. Here we show that *C. albicans GCN2* knockout is less virulent to *Caenorhabditis elegans* and worms survive longer ($p < 0.05$) than control worms infected with wild-type yeast. Besides, *C. albicans GCN4* knockout showed slightly reduced virulence to *C. elegans* when compared to controls ($p < 0.05$). Interestingly, *C. elegans* decreased susceptibility to *C. albicans GCN2* knockout correlates with its decreased filamentous growth and biofilm formation ability in amino acid rich media used to survival assays ($p < 0.001$). We conclude that Gcn2 kinase, regulator of General Amino Acid control pathway (GAAC) and its target Gcn4 are important to *C. albicans* virulence in *C. elegans* model of infection.

Introduction

Morphological changes related to microbial pathogenesis are commonly referred as virulence factors and are observed in some pathogenic fungi including the genera *Candida* that cause a frequent diagnosed human fungal infection [1,2]. Similar to other dimorphic pathogenic fungi, *Candida albicans* can differentiate from yeast to micelial form and vice versa and this dimorphism is central to biofilm formation, infection and disease [3-6].

Ambient temperatures >35°C, serum, neutral pH, nutrient starvation, N-acetylglucosamine and CO_2 promote yeast to hypha morphogenesis in *C. albicans* [7,8]. Morphogenesis in *C. albicans* is also regulated by quorum sensing [9] and MAPK/ Ras-cAMP signaling pathways in response to starvation and/or serum signals [10-13]. In addition, previous work reported that besides their role as nutritional stress sensors, eIF2 kinase Gcn2 and its indirect target Gcn4 participate in *C. albicans* morphogenesis during amino acid starvation [14,15]. Here we report that Gcn2 coordinates the virulence of *C. albicans* in the well-established *C. elegans* model of infection [16-18] and is related to morphological switch between yeast and micelial forms, as well as involved in biofilm formation during amino acid rich growth conditions.

Materials and methods

Strains and media

The *C. albicans* strain CAI-4 (*ura3::λ imm434/ura3::λ imm434*), *C. albicans* Δ*gcn2 (*like CAI-4 except, *gcn2::hisG/gcn2::hisG)* [14], *C. albicans* Δ*gcn4* (like CAI-4 except, *gcn4::hisG/gcn4::hisG*) [14] were maintained on yeast extract peptone dextrose agar (YPD - Difco). The standard *C. elegans* strain N2 Bristol was maintained at 15°C and propagated on *E. coli* strain OP50 using established procedures [19,20].

C. elegans survival assays

Yeast lawns were grown for survival assays as follows: yeast strains were inoculated into 2 ml of YPD and grown at 30°C for 18 h; 30 µl of the culture was spread on 35-mm tissue-culture plates (Falcon) containing nematode growth media (NGM) [19]. The plates were incubated at 30°C overnight and then for two days at 25°C. At the experiment day, a Pasteur pipette molded into the shape of hockey stick was used to gently scrape excess of yeast of the top of the thick yeast lawn. This step facilitated scoring the animals as live or dead on subsequent days. For all experiments Ampicillin (100 µg/ml - Sigma) was added to the medium to selectively prevent growth of *E. coli* OP50 carried over on transfer of worms to the yeast-containing plates. Between 30 and 40 *C. elegans* animals at the young adult developmental stage (L4) were transferred from a lawn of *E. coli* OP50 on NGM to a lawn of the yeast to be tested on NGM media, incubated at 25°C, and examined for viability at 24h intervals with a dissecting microscope. Worms were considered dead when they did not respond to touch with a platinum wire pick. Each experimental condition was tested in triplicate. Plotting of killing curves, calculation of LT_{50} (time for half of the worms to die) and estimation of differences in survival (log-rank and Wilcoxon tests) with the Kaplan–Meier method were performed by using STATA 6 statistical software (Stata, College Station, TX). P values < 0.05 were considered significant.

Phenotypic analysis of *C. albicans* knockouts

To evaluate potential morphologic changes of *C. albicans* during *C. elegans* survival assays, one isolated colony of wild type and knockouts (Δ*gcn2* or Δ*gcn4*), growth in YPD plates at 30°C, were inoculated in fresh liquid YPD and incubated 18 hours at 30°C. The cultures were

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diluted (1:10) in YPD, incubated until reach $O.D._{600}$ = 0.3 and then spotted on NGM plates and YPD plates. Morphological changes were evaluated by macroscopic and microscopic observation of spots after incubation at 25°C and 30°C for 72 hours.

Biofilm formation assay

To prepare a standard cell suspension, a single colony of wild type and knockout *C. albicans* strains was cultured in Synthetic Complete medium (SC) and incubated for 18 h at 30°C with agitation. The fungal cells were harvested by centrifugation, washed twice in PBS (pH 7.2), and resuspended at 1×10^7 cells/ml. RPMI-1640 media (Invitrogen) and liquid NGM media were used for hyphal induction at 37°C. Fungal biofilms were prepared and analyzed as previously described [21] on commercially available, pre-sterilized, flat-bottomed 96-well polystyrene microtitre plates (Corning). Standard yeast cell suspension (100 µl) was transferred into the plate wells and incubated for 24h at 37°C with agitation. After adhesion phase, the liquid was aspirated, and each well was washed twice with PBS to remove loosely attached cells. The metabolic activity of the *C. albicans* biofilms was determined quantitatively using a standard 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction assay. The data were analyzed employing a two-way ANOVA and Bonferroni post-test.

Results

Survival assays of *C. elegans* infected with *C. albicans*

To verify if *GCN2* and *GCN4* are important to yeast virulence, we performed *C. elegans* survival assays with *C. albicans* WT (CAI-4), Δ*gcn2* or Δ*gcn4*. As shown in figure 1A, *C. elegans* infected with *C. albicans* Δ*gcn2* is significantly more resistant to fungal damages (LT_{50} = 5 days, time required to kill 50% of worms; p <0.05) than worms infected with wild type *C. albicans* (LT_{50}=3 days). Deletion of *GCN4*, the indirect target of Gcn2, in *C. albicans* showed decrease in nematode survival after infection at the first days of our assays (LT_{50}= 2 days; p <0.05) when compared to WT infection (LT_{50}= 3 days). Despite the survival assay results, *C. elegans* showed decreased susceptibility to *C. albicans* Δ*gcn4* at the end of the experiment (Figure 1B), since worms stop to die 3 days post-infection.

Phenotypic analysis of *C. albicans* knockout strains in NGM culture medium

We performed macroscopic and microscopic analysis of *C. albicans* Δ*gcn2* and Δ*gcn4* grown on YPD and NGM agar to verify potential changes in growth patterns. We did not observe differences in the growth rate of *C. albicans* WT and knockouts spotted on YPD agar (data not shown). As observed in figure 2, we demonstrate that NGM media is permissive to *C. albicans* morphologic transition at 30°C and at the temperature used in *C. elegans* survival assays (25°C). At analyzed temperatures *C. albicans* WT showed differentiation from yeast to filamentous form (Figure 2A, B and C). Interestingly, *C. albicans* Δ*gcn4* showed the same morphologic changes observed in wild type strain but with fewer filaments (Figure 2D, E and F). On the other side, *C. albicans* Δ*gcn2* showed decreased growth capacity and complete absence of filamentous growth in NGM (Figure 2G, H and I).

C. albicans GCN2 knockout biofilm formation assay

In order to evaluate if absence of filamentous growth in *C. albicans GCN2* knockout observed in NGM media correlates with biofilm formation ability, we performed a biofilm formation assay using MTT reduction assay as maker of yeast proliferative activity. We found

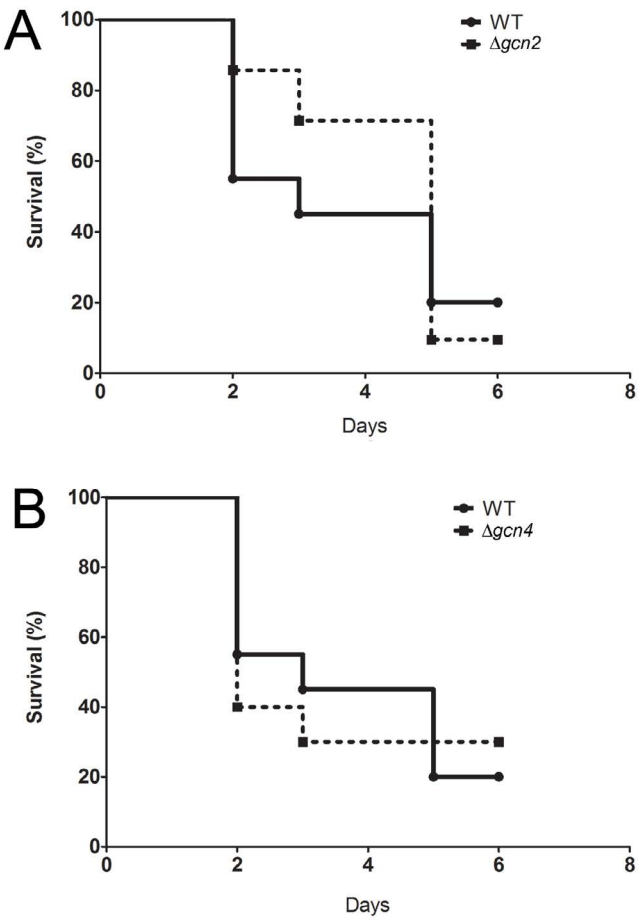

Figure 1. *GCN2* and *GCN4* are essential for *C. albicans* virulence in *C. elegans*.
A) *C. albicans* Δ*gcn2* showing significant reduction of virulence (p <0.05) to *C. elegans* (LT_{50} = 5 days) when compared to control yeast (wild-type – WT, CAI-4) (LT_{50} = 3 days). B) Survival of *C. elegans* after infection with wild type (LT_{50}=3 days) or *C. albicans* Δ*gcn4* (LT_{50} = 2 days) showing worms resistance 3 days after infection when compared to WT.

that the *C. albicans* Δ*gcn2* have significantly reduced capacity to form biofilm in RMPI-1640 and NGM media (p <0.001) when compared to wild-type yeast at 25°C (data not shown) and 30°C (Figure 3).

Discussion

Inhibition of global protein synthesis is a main pathway driving stress responses in eukaryotes resulting in remodeling of gene expression patterns, changing the proteome and maintaining homeostasis [22,23]. In yeast, translation can be regulated by eIF2-mediated mechanisms involving only one conserved eIF2 kinase (Gcn2) that phosphorylates the alpha subunit of eIF2, shutting-off global protein synthesis and promoting the translation of mRNA encoding the transcriptional regulator Gcn4 [22].

Gcn2 have important roles on the morphogenesis and virulence of protozoa regulating adaptation of proliferative forms to host and enabling parasite to overcome exposure to the host extracellular environment [24-27]. There is limited information on the role of Gcn2 and Gcn4 in adaptation and virulence of fungal pathogens to their hosts. *In vitro* analysis showed that Gcn2 and Gcn4 are involved in *C. albicans* morphogenesis induced by N-acetylglucosamine (GlcNAc), suggesting relevant role of Gcn2 during *C. albicans* pathogenesis [28].

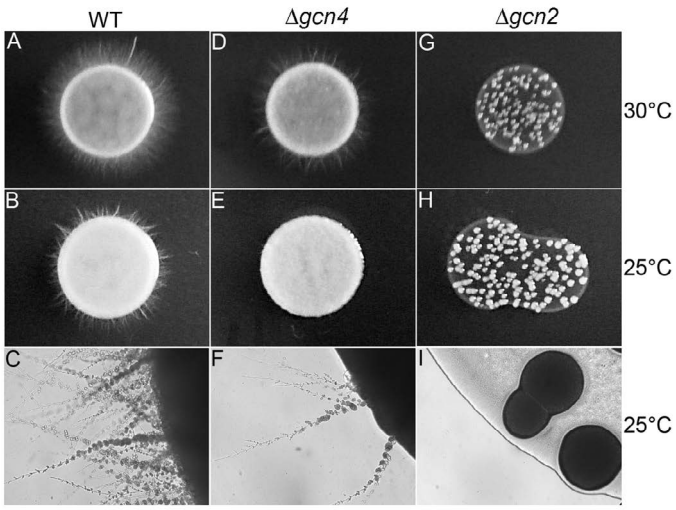

Figure 2. Virulence of *C. albicans* Δ*gcn2* and Δ*gcn4* to *C. elegans* is related to hyphae production in Nematode Growth Media. *C. albicans* WT, Δ*gcn2* and Δ*gcn4* were grown in NGM agar plates at 30°C and 25°C, as indicated. Wild-type *C. albicans* changed to its filamentous form at both tested temperatures (A, B and C). *GCN4* (Δ*gcn4*) knockout showed reduced morphological transition at 30°C and 25°C when compared to WT strain (D, E and F); *GCN2* (Δ*gcn2*) knockout showed decreased growth rate and absence of morphological transition to hyphae on NGM (G,H and I). A, B, D, E, G and H, yeast spot photo. C, F and I, 10X magnification.

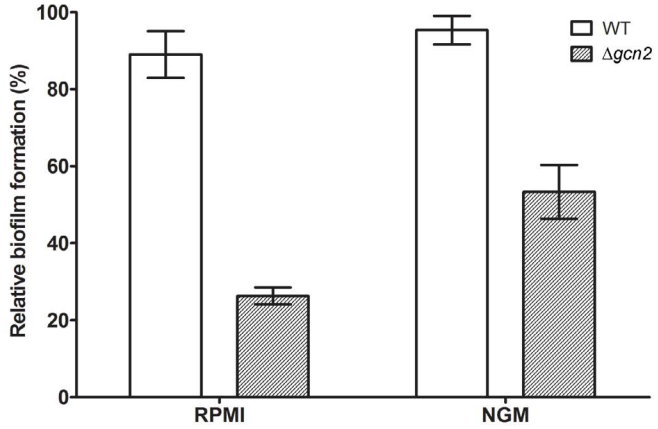

Figure 3. *C. albicans* Δ*gcn2* is defective in biofilm formation. Biofilm formation of *C. albicans* Δ*gcn2* was examined in RPMI-1640 and NGM medium (30°C). The biofilm metabolic activity was assessed quantitatively using MTT reduction assay. The activity of wild type *C. albicans* was taken as 100%. Results shown were the average of three independent experiments ± SD. p <0.0001 when compared with WT yeast.

Here we show that eIF2-mediated translation regulation, besides its function during adaptation to nutritional and environmental stress [14,29,30], is also essential to yeast virulence *in vivo*. Absence of *GCN2* in *C. albicans* renders *C. elegans* resistant to infection-induced lethality (Figure 1A) and absence of *GCN4* partially abolish the virulence of *C. albicans* (Figure 1B).

Interestingly, growth conditions used in our assays induces the filamentation of wild type *C. albicans* (Figure 2) corroborating the decreased survival rates observed in *C. elegans* after infection, since *C. albicans* is converted in a more virulent form in our tested conditions. In addition, resistance of N2 worms to infection with *C. albicans GCN2* or *GCN4* knockouts is linked to strain filamentation ability. We show that *C. albicans GCN4* knockout has decreased filamentous growth (Figure 2E, 2F) and slightly reduced virulence to *C. elegans*, while *GCN2* knockout show slow-growth phenotype and hyphae absence (Figure 2H, 2I) that correlates with marked *C. elegans* resistance to infection when compared to worms infected with wild type *C. albicans* (Figure 1A).

Here, we confirmed that Gcn2 loss of function in *C. albicans* change yeast morphogenesis reducing the biofilm formation capacity, as reported previously [14,29] and renders yeasts less virulent to the invertebrate model of infection *C. elegans*. Tripathi and co-workers showed that Gcn2 and Gcn4 control *C. albicans* morphogenesis during amino acid starvation [29] and in this work we show that Gcn2 and Gcn4 loss of function are important to yeast virulence and morphogenesis in the presence of amino acids. In NGM medium there is no glucose supplementation and amino acids provided by bacto-peptone are the only carbon source. Vylkova and co-workers have shown that *C. albicans* growing in conditions in which amino acids are the primary carbon source result in generation of large quantities of ammonia, raising the extracellular pH and inducing the hyphal switch *in vitro* [31]. Also, amino acids induce *C. albicans* phagosomal pH neutralization, hyphal morphogenesis, and escape from macrophages [32]. Considering these data along with our findings, we demonstrate that *C. elegans* infection with *C. albicans* in solid NGM could be a valuable tool to study pathogen virulence traits and host responses.

Taken together, we show eIF2-mediated translation regulation modulates *C. albicans* virulence to *C. elegans* through Gcn2 function in morphogenesis during amino acid rich conditions. More studies are necessary to address how Gcn2 and Gcn4 regulate and/or integrate with previously described morphogenesis pathways in *C. albicans*.

Accession numbers

Accession number for *C. albicans* genes *GCN2* (C7_01330C) and *GCN4* (C2_09940W) accessed at http://www.candidagenome.org.

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Author contributions

VSA and EM conceived and designed the experiments. VSA performed the experiments. VSA and EM analyzed the data and wrote the paper.

Competing interests

The authors have declared that no competing interests exist.

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