FEMS Yeast Research, 15, 2015, fov093

doi: 10.1093/femsyr/fov093
Advance Access Publication Date: 15 October 2015
Research Article

RESEARCH ARTICLE

The *GRF10* homeobox gene regulates filamentous growth in the human fungal pathogen *Candida albicans*

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One sentence summary: The homeodomain transcription factor Grf10 is important for formation of hyphae and virulence in the human pathogen *Candida albicans*.
Editor: Richard Calderone

ABSTRACT

Candida albicans is the most common human fungal pathogen and can cause life-threatening infections. Filamentous growth is critical in the pathogenicity of *C. albicans*, as the transition from yeast to hyphal forms is linked to virulence and is also a pivotal process in fungal biofilm development. Homeodomain-containing transcription factors have been linked to developmental processes in fungi and other eukaryotes. We report here on *GRF10*, a homeobox transcription factor-encoding gene that plays a role in *C. albicans* filamentation. Deletion of the *GRF10* gene, in both *C. albicans* SN152 and BWP17 strain backgrounds, results in mutants with strongly decreased hyphal growth. The mutants are defective in chlamydospore and biofilm formation, as well as showing dramatically attenuated virulence in a mouse infection model. Expression of the *GRF10* gene is highly induced during stationary phase and filamentation. In summary, our study emphasizes a new role for the homeodomain-containing transcription factor in morphogenesis and pathogenicity of *C. albicans*.

Keywords: *GRF10;* filamentation; chlamydospore formation; biofilm formation; virulence; *Candida albicans*

INTRODUCTION

Candida albicans is an opportunistic fungal pathogen and the most common cause of human fungal infections (Odds 1987). It causes the superficial infections in healthy individuals and life-threatening infections in immunocompromised people. *C. albicans* has the ability to grow as three distinctive forms, yeast, pseudohyphae and true hyphae [reviewed in (Sudbery, Gow and Berman 2004)]. The morphological transition of *C. albicans* from yeast to a filamentous form is closely linked to virulence (Lo *et al.* 1997; Thompson, Carlisle and Kadosh 2011). The filamentous forms are invasive and cause tissue damage. In addition, filamentation is a pivotal aspect of fungal biofilm development as the hyphal-specific proteins are required for

Received: 14 August 2015; **Accepted:** 7 October 2015

adherence (reviewed in Finkel and Mitchell 2011). Biofilms are inherently resistant to both drugs and host immunity, leading to medically complicated infections (Mathe and Van Dijck 2013). Thus, the healthcare burden of *Candida* infections is largely associated with the *C. albicans* in hyphal forms.

C. albicans grows as filamentous forms in response to a wide range of environmental cues such as serum, pH, elevated temperature and loss of nutrients (Sudbery, Gow and Berman 2004; Biswas, Van Dijck and Datta 2007; Whiteway and Bachewich 2007; Sudbery 2011). The Ras/cyclic AMP/protein kinase A pathway is principally involved in transducing extracellular signals to the transcriptional regulators that modulate the cellular response (Inglis and Sherlock 2013). These key transcriptional regulators include activators such as Cph1 (Liu, Köhler and Fink 1994), Efg1 (Stoldt *et al.* 1997), Tec1 (Schweizer *et al.* 2000), Ndt80 (Sellam *et al.* 2010) and Rim101 (Ramon, Porta and Fonzi 1999; Davis *et al.* 2000). Repressors, such as Tup1 (Braun and Johnson 1997), Rfg1 (Kadosh and Johnson 2001; Khalaf and Zitomer 2001), Nrg1 (Braun, Kadosh and Johnson 2001; Murad, Leng and Straffon 2001) and Rbf1 (Ishii *et al.* 1997), are also important for negatively regulating hyphal development. The coordinate regulation by these factors is responsible for integrating environmental signals with gene expression to obtain different phenotypic outcomes. While much progress on understanding the signals and factors necessary for hyphal formation has been identified, our understanding of the genetic control of this developmental process is still incomplete.

The homeotic genes are master regulators of development in eukaryotes. The first homeotic genes were identified in *Drosophila melanogaster*, and numerous genes have been identified in diverse organisms, including animals, plants and fungi since that time (reviewed in Holland 2013). These genes share a common sequence of 180 bp, the *homeobox*, which encodes a highly conserved DNA binding domain of about 60 amino acids called the homeodomain (Burglin 2011). In animals, homeodomain proteins specify the body plan along the anteroposterior axis, whereas in plants, homeodomain proteins are involved in regulating the patterning and morphogenesis of flowers, vegetative shoots and leaves. The homeobox genes have been linked with developmental pathways such as mating and filamentation in fungi, although they have been less studied in these organisms. The best studied cases are the heterodimers formed between the homeodomain proteins Mtla1 and Mtlα2, which regulates white-opaque switching and mating in *C. albicans* (Miller and Johnson 2002), and the homologous proteins Mata1 and Matα2 in other fungal species (Herskowitz, Rine and Strathern 1992; Kruzel and Hull 2010).

In *C. albicans*, *GRF10* encodes a homeodomain containing transcription factor. It has been implicated in adenylate biosynthesis, biofilm formation and virulence (Homann *et al.* 2009; Nobile, Fox and Nett 2012; Romanowski, Zaborin and Valuckaite 2012). In this report, we investigated whether it plays a role in morphogenesis. We found that strains with mutations in the *grf10*Δ gene exhibit filamentous growth defects in liquid and on solid medium, are unable to form normal chlamydospores, and they exhibit a decrease in biofilm formation. The *grf10* null mutants also exhibited attenuated virulence in a mouse model of systemic infections. We also found that expression of *GRF10* is induced during stationary phase and under hyphal-inducing conditions. Together, these findings support a critical role for the *GRF10* homeobox gene in the regulation of *C. albicans* morphogenesis.

Table 1. Yeast strains.

Strain	Relevant genotype	Source
SC5314	*URA3/URA3*	Fonzi and Irwin (1993)
SN152	*arg4*Δ/*arg4*Δ *leu2*Δ/*leu2*Δ *his1*Δ/*his1*Δ *URA3/ura3*Δ *IRO1/iro1*Δ	Noble and Johnson (2005)
TF021	*arg4*Δ/*arg4*Δ *leu2*Δ/*leu2*Δ *his1*Δ/*his1*Δ *URA3/ura3*Δ *IRO1/iro1*Δ *grf10*Δ::*HIS1/grf10*Δ::*LEU2*	Homann *et al.* (2009)
OHWT	*arg4*Δ/*arg4*Δ::*ARG4* *leu2*Δ/*leu2*Δ::*LEU2* *his1*Δ/*his1*Δ::*HIS1* *URA3/ura3*Δ *IRO1/iro1*Δ	Homann *et al.* (2009)
BWP17	*ura3*::λ*imm434/ura3*::λ*imm434* *his1*::*hisG/his1*::*hisG* *arg4*::*hisG/arg4*::*hisG*	Wilson, Davis and Mitchell (1999)
DAY185	*ura3*::λ*imm434/ura3*::λ*imm434* *HIS1*::*his1*::*hisG/ his1*::*hisG* *ARG4*::*URA3*::*arg4*::*hisG/arg4*::*hisG*	Davis *et al.* (2000)
RAC114	*ura3*::λ*imm434/ura3*::λ*imm434* *his1*::*hisG/his1*::*hisG* *arg4*::*hisG/arg4*::*hisG* *GRF10/grf10*Δ::*URA3*	This study
RAC117	*ura3*::λ*imm434/ura3*::λ*imm434* *his1*::*hisG/his1*::*hisG* *arg4*::*hisG/arg4*::*hisG* *grf10*Δ::*ARG4/grf10*Δ::*URA3*	This study
RAC120	*ura3*::λ*imm434/ura3*::λ*imm434* *his1*::*hisG/his1*::*hisG* *arg4*::*hisG/arg4*::*hisG* *grf10*Δ::*ARG4/GRF10*::*HIS1*:: *grf10*Δ::*URA3*	This study

MATERIALS AND METHODS

Strains

Strains of *C. albicans* used and generated in this study are listed in Table 1. *C. albicans* BWP17, a derivative of SC5314 (Fonzi and Irwin 1993), served as the parent strain for constructing the *GRF10* mutant strains RAC115, RAC117 and RAC120 using the PCR gene knockout method (Wilson, Davis and Mitchell 1999). Strain DAY185 was obtained from A. Mitchell (Davis *et al.* 2000), and strains SN152, OHWT and TF021 (Homann *et al.* 2009) were obtained from the Fungal Genetics Stock Center.

Media and growth conditions

Strains were grown on YPD or SC media (containing 0.17% yeast nitrogen base, 0.5 mM uridine, 0.15 mM adenine and CSM amino acids (Sunrise Science Products, MP Biologicals) (Sherman 1991). All stock strains were maintained at 4°C on YPD plates. Hyphal formation was monitored on the following liquid and solid agar media: Spider medium (Liu, Köhler and Fink 1994), 10% fetal calf serum, Medium-199 (Gibco-BRL) buffered with 155 mM HEPES, pH 7.5, SLAD medium (Gimeno *et al.* 1992) and Lee's medium (Lee, Buckley and Campbell 1975). The chlamydospore formation was monitored on the corn meal agar (CMA) medium (BD Scientific), supplemented with Tween 80 (1% v/v) and auxotrophic supplements. The final agar concentration for all solid media was 1.5% (w/v).

Hyphal induction

To induce hyphae, cells were grown overnight in YPD medium and washed twice with sterile water. For induction on solid medium, cell densities were adjusted to 2×10^7 cfu/ml, 5 μl of each strain was spotted and plates were incubated for 3 days at 37°C. For induction in liquid medium, cells were inoculated 1:50 into 2 ml of medium in 12-well tissue culture plates and the cultures were incubated at 37°C with mild shaking for 0, 4, 8 and 24 h. Each culture was examined microscopically using an Olympus BH2-RFCA microscope at 40× and photographed.

Quantitative macroscopic colony assay

Strains (DAY185, RAC117, OHWT and TF021) were grown overnight in YPD medium at 30°C in a tube roller. The macroscopic colony spot assay was performed as described (Homann *et al.* 2009): overnight cultures were diluted to $OD_{600} = 0.08$ in sterile water (1X) and a further 1:5 dilution was made in water (5X); 5 μL of these two samples was spotted onto Spider medium and the plates were incubated at 37°C up to 14 days. The macroscopic colony morphology was quantified by measuring the overall colony diameter (mm), central region (mm) and peripheral region (mm) using a regular ruler (Noble *et al.* 2010). The *p*-value was determined using the Student's t-Test.

Filamentous growth rate measurement

The macroscopic colony was grown in the same method as described above. The diameter size (mm), central region (mm) and peripheral region (mm) were measured by using a regular ruler daily for 12 days. Graphs and *p*-values for Student's t-Test were generated by using Excel. The filamentous growth rate was calculated from the slope of the graph, X-axis: time (day) and Y-axis: colony size (mm).

Chlamydospore and biofilm formation

To induce chlamydospore formation, overnight grown cultures or colonies were streaked deep into the surface of CMA plates and a coverslip was placed on top (Joshi, Solanki and Prakash 1993). Plates were incubated at 25°C for 3–5 days and chlamydospore formation was evaluated microscopically and photographed. Biofilm formation was assessed using the protocol as described (Krueger *et al.* 2004); briefly: overnight cultures were washed in phosphate buffered saline (PBS), diluted to an OD_{600} of ∼1 in PBS, and 100 μl was added to each well of a 96-well plate that had been coated with 10% fetal bovine serum and allowed to adhere for 90 min at 37°C. Non-adherent cells were removed by two washes with PBS. Biofilms were allowed to form for 48 h in supplemented YNB + glucose medium. After two washes with PBS, adherent cells were measured by absorbance at 595 nm in a spectrophotometric plate reader.

Virulence determination

C. albicans strains were grown in YPD broth at 30°C to stationary phase, washed twice with calcium- and magnesium-free PBS (from Biosource), and resuspended in PBS at a cell density of 5×10^6 cells ml^{-1} based on hemocytometer counts. Virulence in mice was assessed as described previously (Tsuchimori *et al.* 2000; Chauhan *et al.* 2005). Groups of 13 male BALB/c mice (20–22 g from Harlan) were formed and each mouse was injected through the lateral tail vein with a 200 μl inoculum containing 10^6 cells of wild-type control or mutant yeast. Mice were given food and water *ad libitum*. Survival of the mice was monitored twice daily and moribund mice were euthanized by asphyxiation with carbon dioxide, as recommended by American Veterinary Medical Association (AVMA 2001). Yeast was recovered from the kidneys of the first three mice from each group to die; PCR analysis showed that the recovered fungi had the same genotype as the inoculum (data not shown). The post-infection morphology of each strain was assessed microscopically from the homogenized kidney treated with 10% KOH (data not shown) (Odds, Van Nuffel and Gow 2000). In addition, three mice from each experimental group were sacrificed by CO_2 inhalation at 24, 48 and 72 h post infection; their kidneys were removed, weighed and homogenized in PBS. Serial dilutions of the homogenate were plated on YPD agar supplemented with 50 μg/ml streptomycin (to prevent bacterial growth), and were incubated at 30°C for 48 h. The number of colonies on each plate was determined, and a calculation was made of the number of colonies per gram of tissue. Statistical analyses were performed with the SPSS 15.0 software using the Kaplan–Meier survival analysis.

RNA preparation, cDNA synthesis and quantitative real-time PCR

Strain SC5314 strain was grown in triplicate overnight in YPD medium at 30°C with shaking. The overnight culture was diluted 1:50 into 5 mL of SC + Uri medium, and grown with shaking at 30°C until mid-log phase (6 h, OD_{600} ∼0.5–1) or stationary phase (24 h, OD_{600} ∼8–9). To induce filamentation, the overnight culture was diluted 1:50, and cells were grown in 12-well dishes containing Spider medium with mild shaking at 37°C for 6 h; filamentation was confirmed by light microscopy. Cells were quickly cooling on ice and pelleted by centrifugation at 4°C for 5 min; the cell pellet was frozen at −80°C. RNA was extracted from the frozen pellets using the RiboPure Yeast Kit (Ambion) following the manufacturer's instructions, except that less RNA (3 μg) was treated with DNaseI. cDNA was synthesized from 200 ng of RNA using the SensiFAST cDNA synthesis kit (Bioline), following the manufacturer's instructions. The synthesized cDNA was diluted 1:5 in DEPC-treated water.

The RT-qPCR was performed on each cDNA sample following the manufacturer's instructions (SensiFAST SYBR No-ROX Kit, Bioline) using primers to *GRF10* (forward 5′-CAAACCGCTCAAATAGTCAAAG-3′ and reverse 5′-GCAAATTGTGGAGAGTTTGTAG-3′) and the reference gene *TEF1* (forward 5′-TTCGTCAAATCCGGTGATG-3′ and reverse 5′-CTGACAGCGAATCTACCTAATG-3′). The relative gene expression was calculated by the ΔC_T method using a reference gene as described by the Bio-Rad Laboratories qPCR manual. Student's t-test and statistical significance were calculated using Excel.

RESULTS

The *GRF10* gene is essential for normal filamentous growth

To determine if *GRF10* has a role in filamentation, we examined the effects of the *GRF10* gene deletion on *C. albicans* colony morphology on solid media. Homozygous and heterozygous strains were constructed with mutations in *GRF10* in the BWP17 background (Table 1). Morphological phenotypes of the *grf10*Δ mutant were examined on several different solid agar media. We observed striking defects in filamentation in strain RAC117 on 10% serum, M-199 and Spider media (Fig. 1) and found no difference in filamentation on SLAD or Lee's media (data not shown).

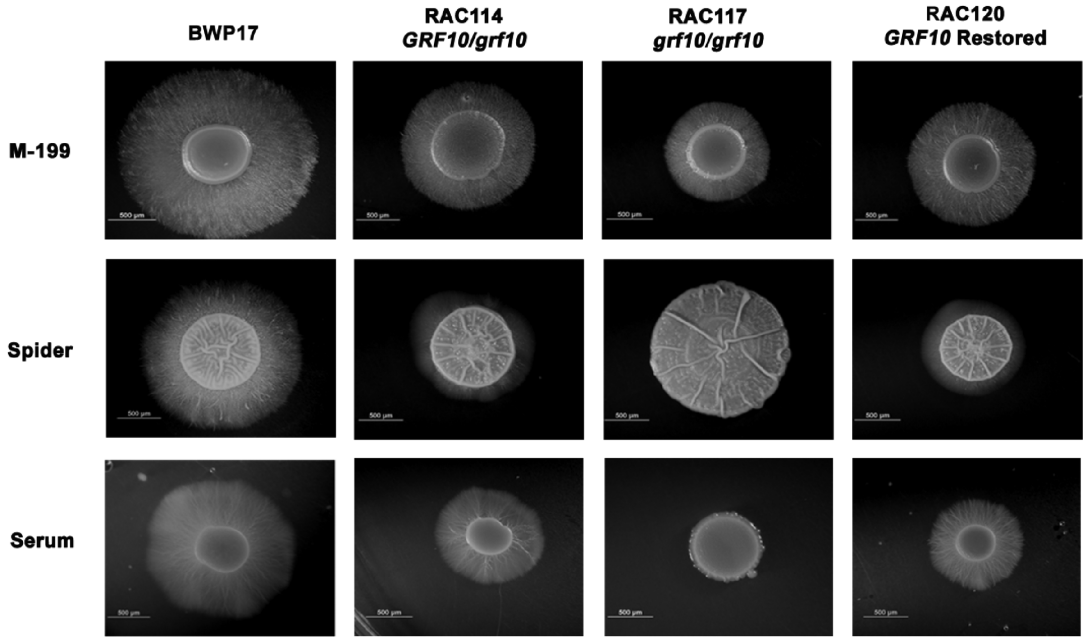

Figure 1. Disruption of GRF10 affects hyphal formation on serum, M-199 and Spider media. Cells (1×10^5) of parental strain BWP17 and mutant strains RAC114 (*GRF10/grf10*Δ), RAC117 (*grf10*Δ/*grf10*Δ) and RAC120 (*GRF10* restored) were inoculated on solid 10% serum, Spider medium and M-199, pH 7.5 medium. Plates were incubated at 37°C for 3 days and photographed. The induction of hyphae was performed at least three times, representative examples are shown. Bar, 500 mm.

The morphological defects we observed in the BWP17 genetic background were stronger than reported elsewhere for strains in the SN152 background (Homann *et al.* 2009), so this difference was examined more closely. We grew *grf10*Δ strains from both genetic backgrounds (RAC117 and TF021) on solid Spider medium, monitored the macroscopic appearance of the *C. albicans* colony (Fig. 2A and B), and measured the overall colony diameter as well as the central and peripheral regions for two weeks (Fig. 2C and D) (Noble *et al.* 2010). Regardless of the strain background, the *grf10*Δ mutant strains had an overall increase in the central region and a significant decrease in the peripheral region; this difference was more evident in strain RAC117 from the BWP17 strain background. The *grf10*Δ mutant exhibits a leaky adenine auxotrophy (Homann *et al.* 2009). To see if adenine affected filamentation, we examined morphology on Spider medium with and without adenine supplementation. The addition of adenine did not rescue the filamentation defect (Fig. S1, Supporting Information). Neither of the *grf10*Δ mutants differed in doubling times from their respective parental strains in SC medium (data not shown), indicating that the defect seen on Spider medium is in filamentation and not in the overall growth.

We quantitatively monitored the colony growth rate of the *grf10*Δ mutant TF021, which had the weaker phenotype, on solid Spider medium daily for up to two weeks (Fig. 3). During the first three days, both the mutant and the wild-type strain have the same appearance and colony size, with no visible hyphae extending from the edges of the spot; hyphae became visible at day 4. The overall rate of growth of the *grf10*Δ colony was slower by ~23% (Fig. 3A; 1.0 mm/day for TF021 and 1.3 mm/day for OHWT). This difference in overall colony growth rate comes from differences in the peripheral region; the peripheral region extension rate in the *grf10*Δ colony was slowed by ~30% relative to wild type (Fig. 3B; 0.9 mm/day for TF021 and 1.3 mm/day for OHWT), whereas the growth of the central region was constant in the two strains (Fig. 3C). Together these results show a defect in filamentation in the *grf10*Δ mutant, independent of the strain background.

The *grf10*Δ mutant is delayed in germ-tube formation

The morphological defects of the *grf10*Δ colony may be due to an inability to initiate germ-tube formation and/or maintain hyphal growth. To examine this, we monitored filamentation of the *grf10*Δ from the BWP17 strain background, because it had the stronger filamentation phenotype, during a 24-h time course in 10% serum, and in liquid M-199 and Spider media. In serum, the *grf10*Δ mutant was delayed in inducing filamentation at 4-h and it produced only germ tubes and pseudohyphae at 8 and 24 h (Fig. 4A). In M199 medium at 4 h, the *grf10*Δ mutant showed only minimal induction of germ tubes, detected in about 10–15% of the cells, and only 20–25% of the *grf10*Δ cells induced short pseudohyphae after 8- and 24-h incubation time (Fig. 4B). In Spider medium, the *grf10*Δ mutant exhibited only the formation of germ tubes, few pseudohyphae and no true hyphae (Fig. 4C). Overall, we see delays in initiating filamentation in broth that are consistent with the defects detected at the whole colony level on solid media.

The *grf10*Δ mutants are defective in chlamydospore formation

Given the defect that we observed in filamentation, we wondered if chlamydospore formation was also affected. We found that the *grf10*Δ strains from both strain backgrounds are defective in forming chlamydospores (Fig. 5A–F). We rarely saw hyphae projecting from the streaks in the *grf10*Δ mutant (Fig. 5B) and the chlamydospores were slow to form or did not form at all at the ends of the hyphal stalks in the null mutants (Fig. 5D and F). The defect in chlamydospore formation was also observed in the heterozygous mutant strains in the BWP17 background (Fig. 5G–H), indicating haploinsufficiency for this trait. In the

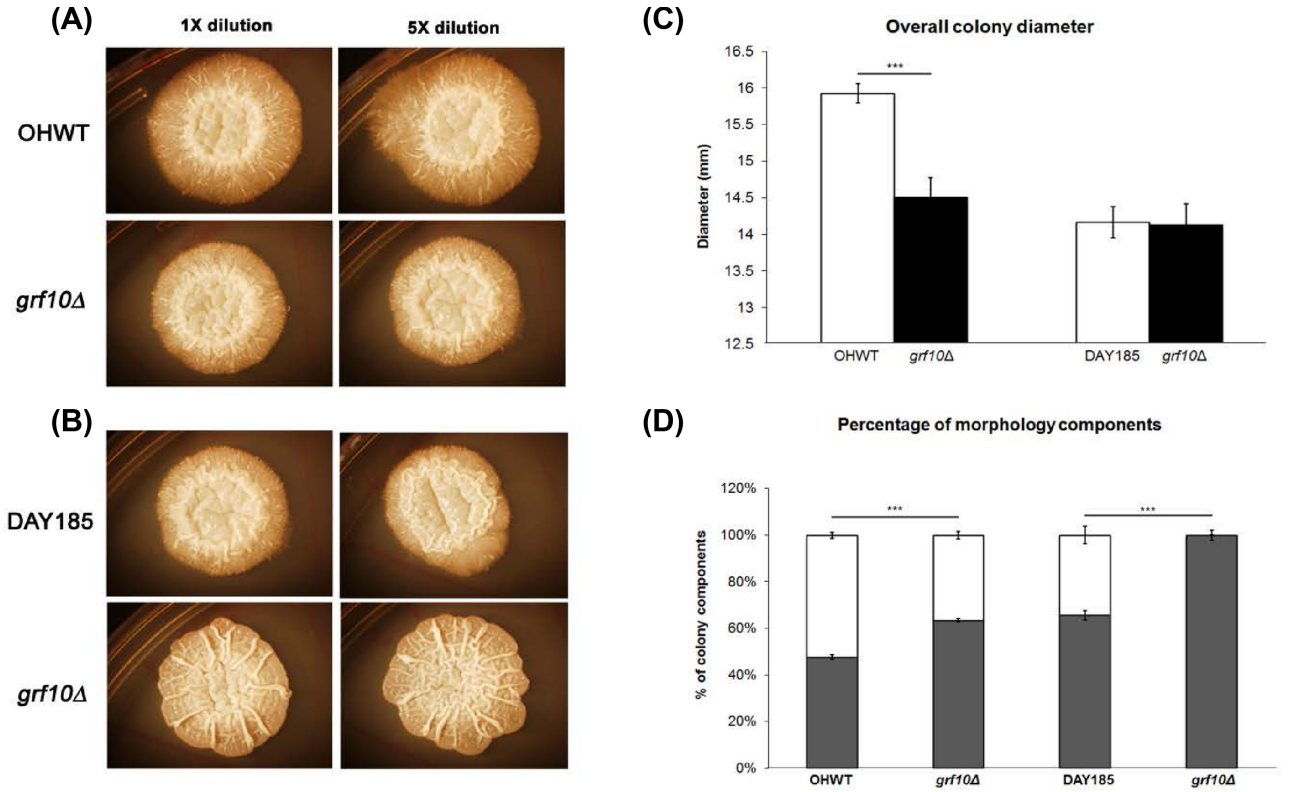

Figure 2. The *grf10*Δ mutant in two genetic backgrounds exhibits filamentation and colony defects. Cells (0.08 OD_{600}) were spotted onto Spider medium; photos of the macroscopic colonies were taken on day 10: (**A**) strains OHWT and TF021 (SN152 background), (**B**) strains DAY185 and RAC117 (BWP17 background). (**C**) Quantification of the overall colony diameter (mm) at day 10. (**D**) Distribution of the overall colony diameter by percentage into the central region (dark bar) and peripheral region (white bar). All measurements were averaged from six biological samples with two technical replicates (n = 12). Student's t-test was calculated using Excel. *** *p*-value < 0.0001.

SN152 background, we observed unusually large, round cells lacking a thick cell wall (Fig. S2, Supporting Information).

The *grf10*Δ mutants are deficient in biofilm formation

Filamentation is required for the formation of the *Candida* biofilm (Blankenship and Mitchell 2006). Since the *grf10*Δ mutant exhibited a filamentation defect, we investigated whether the ability to form a biofilm was also affected in the mutants. We compared biofilm formation in the null and heterozygous mutants with the parental strain BWP17 using a spectrophotometric assay (Krueger *et al.* 2004). As shown in Fig. 6, the parental strain BWP17 produced a dense biofilm (OD_{595} of 0.58 ± 0.02). We observed a significantly reduced biofilm formation in the *grf10*Δ mutant (OD_{595} of 0.2 ± 0.016, $p = <0.001$) as well as in the heterozygote strains RAC114 (OD_{595} of 0.39 ± 0.01, $p = <0.001$) and RAC120 (OD_{595} of 0.4 ± 0.02, $p = 0.009$), indicating a gene dosage effect for Grf10.

The *grf10*Δ mutation attenuates mortality in a mouse model of infection

To determine whether *GRF10* is required for virulence, we used a mouse model of disseminated candidiasis (Tsuchimori *et al.* 2000), comparing the survival of mice infected with the wild-type, *grf10*Δ null and *grf10* heterozygous mutant strains. Mice infected with either of the two heterozygous *GRF10* mutants (RAC114 and RAC120) survived to 12 days, whereas the wild-type strain (DAY185) caused mortality in 8 days (Fig. 7A). Remarkably, 80% of mice infected with the *grf10*Δ mutant (RAC117) survived for 4 weeks post infection, at which point the experiment was terminated. We repeated this result by infecting 10 additional mice with the *grf10*Δ mutant; again, we found that 70% of the mice survived for 4 weeks post infection. We note that these strains differ in their auxotrophies for arginine and histidine (see Table 1). We think that these differences are unlikely to have affected virulence because the two heterozygotes RAC114 and RAC120 had similar virulence profiles in spite of their auxotrophic differences, and secondly, Noble and Johnson found that neither the *arg4*Δ or *his1*Δ mutations had an effect on virulence in the mouse systemic infection model (Noble and Johnson 2005). The survival of the *grf10*Δ null infected mice is statistically different from the survival of the DAY185-infected mice, with a *p*-value of <0.0001.

To examine the ability of the mice to clear the infection, we prepared kidney homogenates from the mice sacrificed during the first three days after infection, and plated serial dilutions of the homogenate to determine the extent of colonization. For mice infected with DAY185, we found a high colony count (5.6×10^5 cfu/gm tissue) with a trend that suggested an increase in colony number (Fig. 7B). Interestingly, the two *grf10*-heterozygous strains (RAC114 and RAC120) displayed a cell count that was about half of DAY185 (2.5×10^5 cfu/gm tissue) and that was the same finding in the animals sacrificed at 48 and 72 h. Infection with the *grf10*Δ strain (RAC117) displayed the same lower cfu/gm tissue at 24 h as found in the heterozygous mutant strains, but the cell counts decreased from the mice sacrificed during the next two days. These findings are consistent with the attenuation of virulence that we observed.

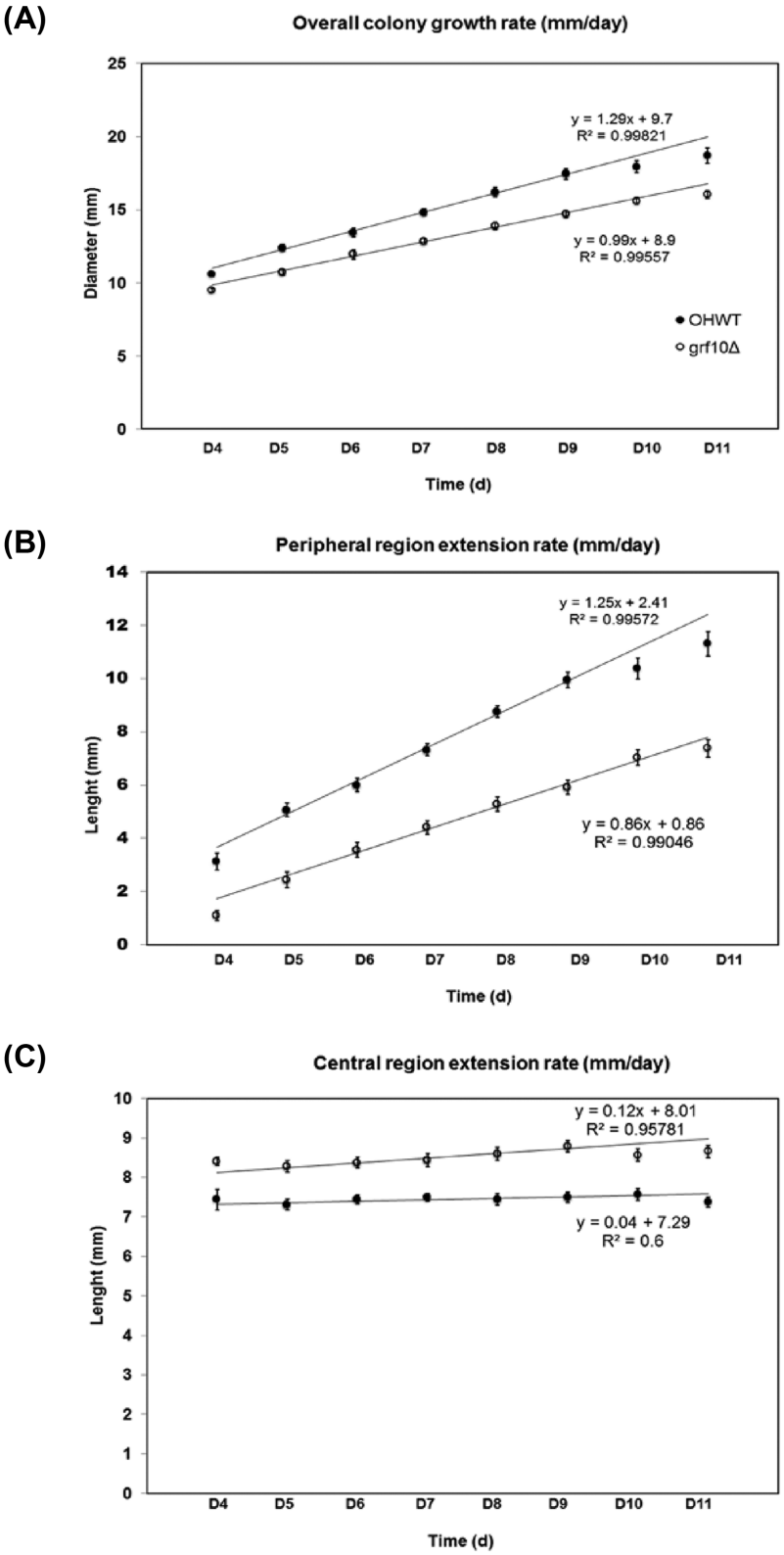

Figure 3. The *grf10Δ* mutant was deficient in the growth of hyphae. Strains (OHWT and TF021) were grown on solid Spider medium and the radial growth was monitored during a time-course of 11 days (D1–D11). Diameter of the overall colony **(A)**, the peripheral region **(B)** and the central region **(C)** were measured. The wild-type strain OHWT is represented by the solid circles and the *grf10Δ* mutant (TF021) is represented by the open circles. The line represents the best fit to the data from D5-D9; the radial rate is the slope of of this line. Data were collected from four biological isolates with two technical replicates (n = 8).

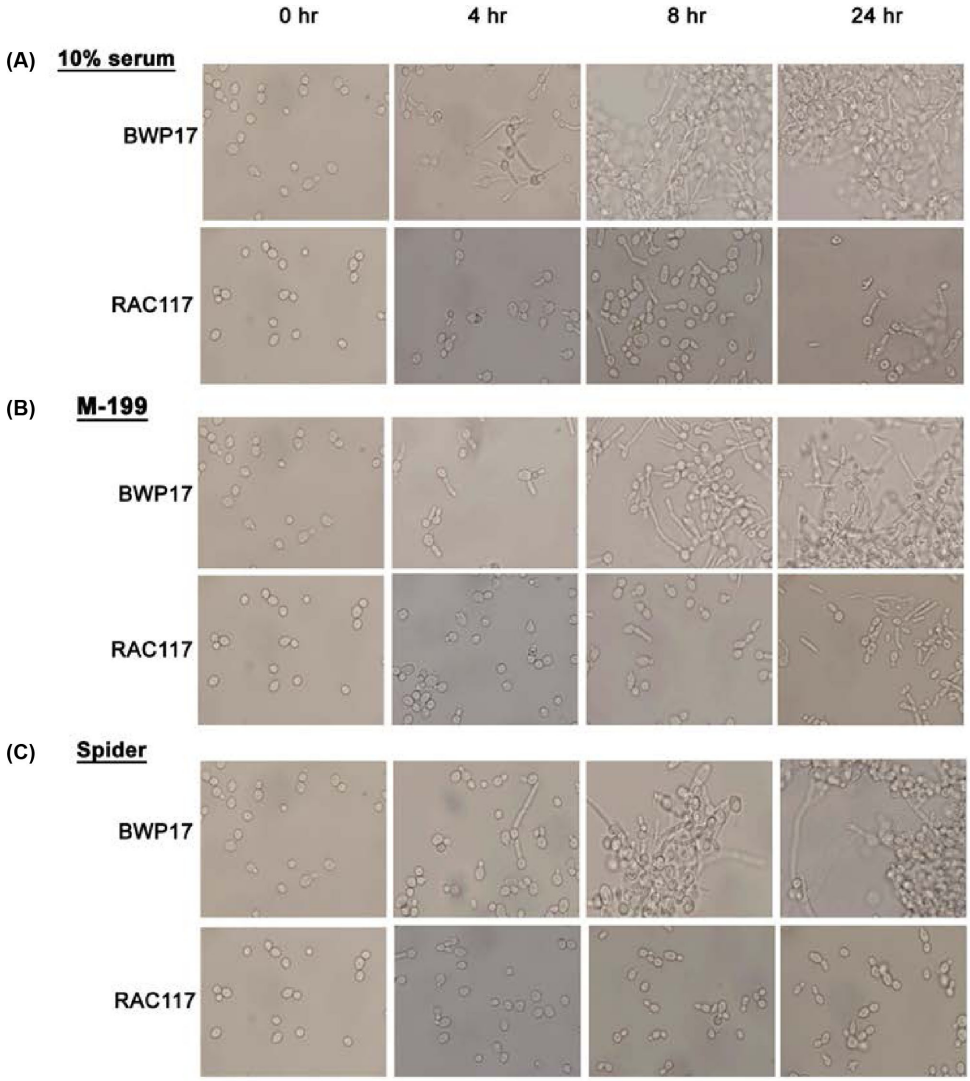

Figure 4. The *grf10*Δ mutant is delayed in germ-tube formation. Strains BWP17 (parental wild-type) and RAC117 (*grf10*Δ) were inoculated in (**A**) 10% fetal calf serum, (**B**) M199 medium, and (**C**) Spider medium, and were grown at 37°C; aliquots were removed microscopy analysis at 0, 4, 8 and 24 h. Cellular morphology was examined at 40× magnification. *Note*—the photos of the cells at time 0 are the same, and are reproduced here for ease of comparison across the time course.

Expression of *GRF10* gene increases during stationary phase and filamentation

Given the important role for Grf10 during filamentation, we wondered whether its gene expression changed during filamentation. To investigate this, we examined expression of *GRF10* gene during planktonic yeast growth in log phase and during stationary phase in SC medium, and after hyphal induction on Spider medium. In this analysis, strain SC5314 strain was used as it was parental to both parental strains SN152 and BWP17, and the derived *grf10* mutants. We found that expression of *GRF10* was induced approximately 4-fold during stationary phase and filamentous growth (Fig. 8).

DISCUSSION

In eukaryotes, the evolutionarily conserved homeobox genes play critical roles in pattern formation and morphology in animals, plants and fungi. Filamentation and spore formation are crucial developmental steps in the life cycle of most fungi. Strikingly, these developmental processes are controlled by homeobox genes in many diverse fungal species (Berben, Legrain and Hilger 1988; Torres-Guzmán and Domínguez 1997; Arnaise *et al.* 2001; Colot, Park and Turner 2006; Kim, Park and Kim 2009; Liu, Xie and Zhao 2010; Antal *et al.* 2012; Kim, Kwon and Roe 2012; Lee *et al.* 2014). The absence of the homeobox *GRF10* gene leads to defects in filamentous growth in *C. albicans*. Although it was reported that the *grf10*Δ mutant did not impair filamentation (Homann *et al.* 2009), we found morphology defects in both the SN152 background as well as in the BWP17 background. The involvement of *GRF10* in *C. albicans* morphology is supported by the finding that overexpression of *GRF10* triggers filamentous growth both on solid and in liquid media under conditions that normally support yeast growth (Chauvel, Nesseir and Cabral 2012).

Consistent with a defect in filamentation, we found that processes dependent upon filamentation were also affected in the *grf10*Δ mutant. These included chlamydospore development (Fig. 5), biofilm formation (Fig. 6), and virulence in two different mouse infection models (Fig. 7 and Romanowski, Zaborin and

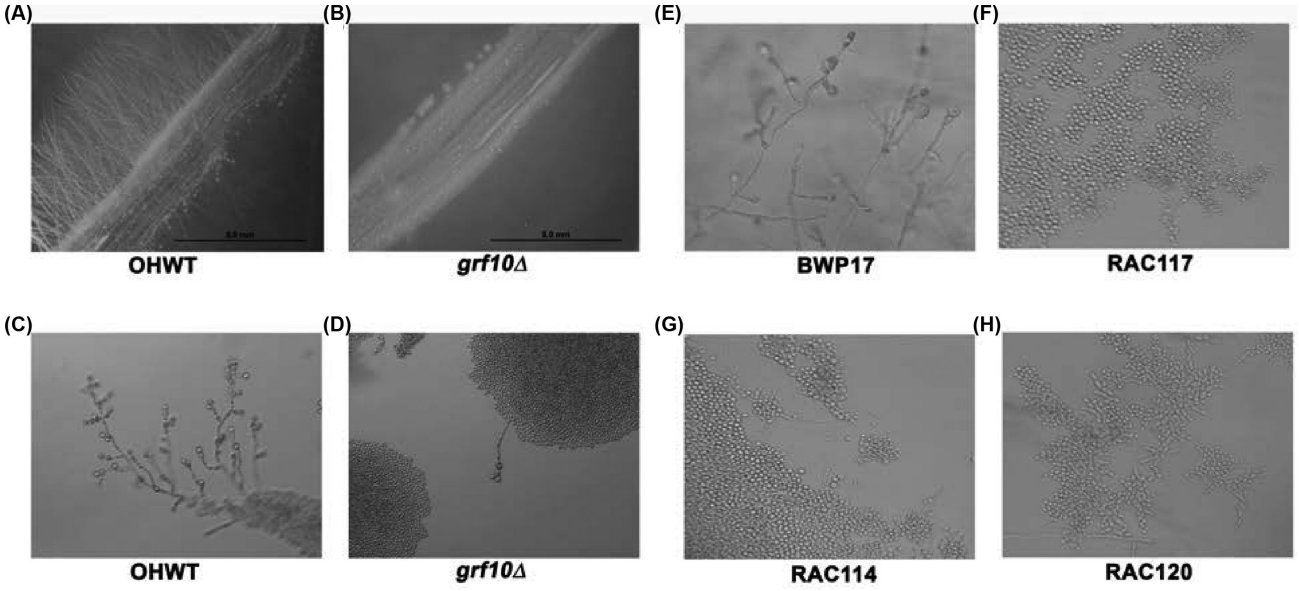

Figure 5. The *grf10*Δ mutant is defective in forming chlamydospores. Chlamydospore formation was induced on corn meal agar medium. View of the edge of a streak showing filamentation from (**A**) OHWT and (**B**) the *grf10*Δ mutant at low magnification (1.25× magnification). Representative chlamydospores or individual cells observed from (**C**) strain OHWT, (**D**) the *grf10*Δ mutant TF021, (**E**) BWP17, (**F**) the *grf10*Δ mutant RAC117, (**G**) the *GRF10/grf10*Δ heterozygous strain RAC114, (**H**) and the *GRF10* restored strain RAC120. Panels (C) and (D), 20× magnification; panels (E–H), 40× magnification.

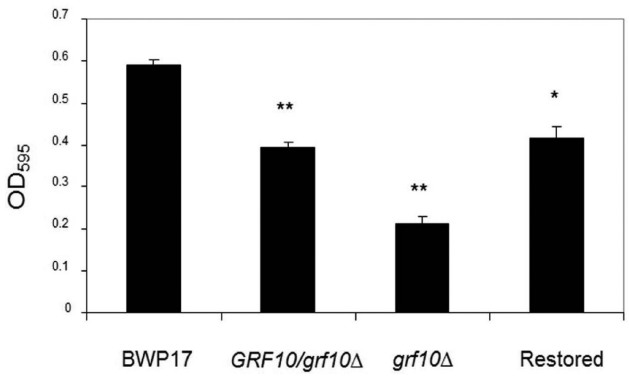

Figure 6. *grf10*Δ mutants were reduced in biofilm formation. Homozygous and heterozygous mutant and wild-type strains were allowed to form a biofilm, and the extent of biofilm formation was assessed by measuring the OD_{595}. Assays were performed in triplicate, the mean and standard deviation of the OD_{595} measurements are plotted. *, p-value = 0.009; **, p-value < 0.001 relative to BWP17.

Valuckaite 2012). The chlamydospore defect, which had not been reported previously in either strain background, was a very robust phenotype and exhibited haploinsufficiency. The role for chlamydospores is currently unknown, but they may represent a specialized growth form (Citiulo *et al.* 2009). Chlamydospore formation is generally induced when the cells are grown on nutrient-poor and under oxygen-limited conditions, suggesting that the Grf10 transcription factor may control hyphal development under specific environmental cues.

Expression of the *GRF10* gene is upregulated during filamentation and stationary phase. It was previously shown to be upregulated during *C. albicans* biofilm formation (Yeater, Chandra and Cheng 2007; Nobile, Fox and Nett 2012; Desai, Bruno and Ganguly 2013) and it was identified as one of eight core target genes whose expression was regulated by the biofilm master regulators (Nobile, Fox and Nett 2012). Five of the six biofilm master regulators—Efg1, Bcr1, Brg1, Ndt80 and Tec1—bind to the promoter of *GRF10*. As the overexpression of *GRF10* can rescue the biofilm defects in only the *tec1*Δ and *bcr1*Δ strains, Nobile and colleagues suggest that it acts downstream of Tec1p and Bcr1p (Nobile, Fox and Nett 2012). Tec1p is required for hyphal formation whereas Bcr1p regulates the expression of genes related to adherence (Nobile and Mitchell 2005). The upregulation of *GRF10* gene during stationary and filamentous phases may be required to support the additional roles for the Grf10 transcription factor during filamentation.

In many fungal species, entry into stationary phase by environmental stress or nutrient starvation can trigger mating, meiotic division and spore formation, and/or invasive growth by pseudohyphae and true hyphae (Gimeno *et al.* 1992; Chu *et al.* 1998; Mata *et al.* 2002). Our results reveal that the *GRF10* gene is induced by at least 4-fold during stationary phase and in response to hyphal-inducing conditions. Interestingly, *Phx1* gene, the *GRF10* homolog in the fission yeast *S. pombe*, is upregulated during stationary phase; Phx1 induces expression of pyruvate decarboxylase and genes for the biosynthesis of its cofactor thiamine (Kim, Kwon and Roe 2012; Kim *et al.* 2014). These gene products are proposed to shift carbohydrate metabolism from respiration to fermentation, which the authors suggest decreases the reactive oxygen species (ROS) and promotes long-term survival. It still remains unclear if Grf10 transcription factor plays a role in metabolic control in addition to filamentation during *C. albicans* stationary phase.

Homeodomain proteins commonly interact with another protein partner to regulate target genes (Mann, Lelli and Joshi 2009). Pho2, the *GRF10* orthologue from *S. cerevisiae*, interacts with at least three transcription factor partners, Bas1, Pho4, and Swi5 (Bhoite, Allen and Garcia 2002). Orthologues of these protein partners are conserved in *C. albicans* (Bas1, Pho4 and Ace2). *C. albicans BAS1* mutants have an adenine auxotrophy (Homann *et al.* 2009), and we have data showing that Bas1 regulates purine biosynthetic genes with Grf10 (Wangsanut et al. manuscript in preparation). In *C. albicans*, the Pho4 transcription factor plays a role in the phosphate response and its role in filamentation has

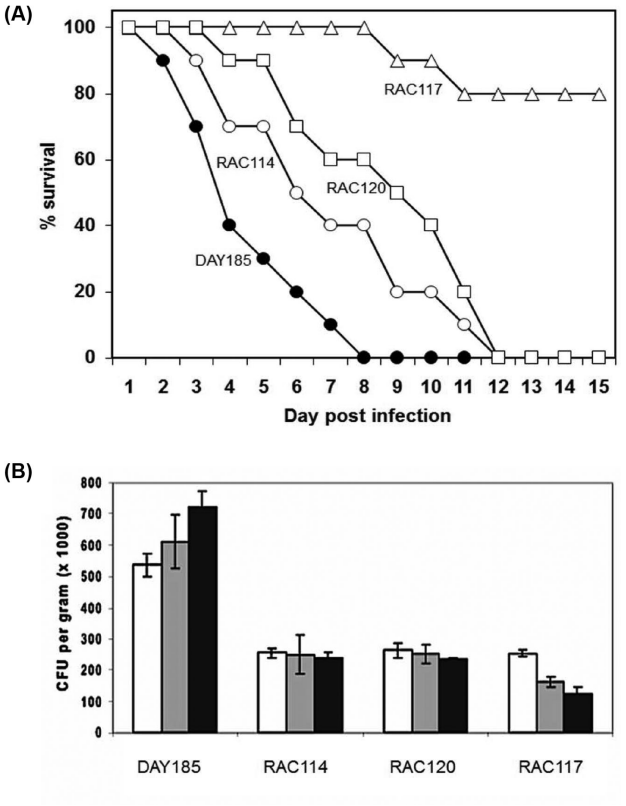

Figure 7. The *grf10*Δ mutants are less virulent in a mouse model of infections. (**A**) Ten Balb/c mice were injected with 1×10^6 cells through the lateral tail vein for each strain and survival was monitored for 15 d post-infection. •, wild type (DAY185); °, heterozygote (RAC114); Δ, homozygous null (RAC117); □, restored strain (RAC120). (**B**) Mice were sacrificed during the first three days after infection, and kidney homogenates were prepared and plated to determine the extent of colonization (colony forming units per gram; CFU/g). Histogram color: white, 24 h after infection; grey, 48 h after infection; black, 72 h after infection. The *GRF10* genotype of the strains used in both panels are wild-type DAY185 (*GRF10*/*GRF10*), RAC114 (*GRF10*/*grf10*Δ), RAC120 (*grf10*Δ/*grf10*Δ::*GRF10*) and RAC117 (*grf10*Δ/ *grf10*Δ).

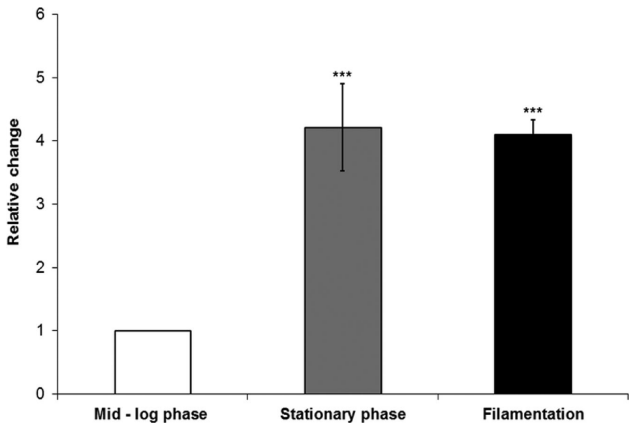

Figure 8. The *GRF10* gene is induced during stationary phase and filamentation. Strain SC5314 was grown in SC + Uri medium to mid-log phase, stationary phase, or induced for filamentation in Spider medium. *GRF10* expression was quantified by qRT-PCR and normalized to *TEF1* (*EFT3*). Three biological samples and two technical replicates (n = 6) were performed. Student's t-test was performed using Excel. ***p-value < 0.0001.

been reported (Romanowski, Zaborin and Valuckaite 2012); however, it does not require Grf10 to upregulate phosphate metabolic genes (unpublished data and (Homann *et al.* 2009; Kerwin and Wykoff 2009)). In *S. cerevisiae*, *SWI5* and *ACE2* are paralogs, arising from the whole genome duplication; Swi5 cooperatively binds with Pho2 to activate *HO* gene to initiate mating type switching while Ace2 activates its target genes independently from Pho2 cooperation (Dohrmann, Voth and Stillman 1996). *C. albicans* possesses a single orthologue Ace2 (Butler *et al.* 2004; Kelly *et al.* 2004) that plays a direct role in morphogenesis through the RAM pathway (Regulation of Ace2 Morphogenesis). Ace2 is required for cell separation, adherence and virulence in animal studies and is a positive regulator of biofilm formation (Kelly *et al.* 2004; Finkel, Xu and Huang 2012). We do not know if Grf10 interacts with Ace2 or another transcription factor to regulate its target genes. Further experiments on protein–protein interaction and identification of specific target genes during morphogenesis are needed to enlighten the functions of the homeobox *GRF10* gene in *C. albicans*.

SUPPLEMENTARY DATA

Supplementary data are available at FEMSYR online.

ACKNOWLEDGEMENTS

Thank you to Jessica Quick and Lilli Seabol for technical assistance.

FUNDING

This work was supported by NIH grant no. 1R03AI075226 and Georgetown University Pilot Project funds to RJR.

Conflict of interest. None declared.

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