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Mycopathologia. Author manuscript; available in PMC 2011 July 1.

Published in final edited form as:
Mycopathologia. 2010 July ; 170(1): 1–10. doi:10.1007/s11046-010-9297-2.

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An analysis of the Impact of *NRG1* Overexpression on the *Candida albicans* Response to Specific Environmental Stimuli

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Abstract

The ability of the opportunistic fungal pathogen *Candida albicans* to form filaments has been strongly linked to its capacity to cause disease in humans. We previously described the construction of a strain in which filamentation can be modulated both in vitro and in vivo by placing one copy of the *NRG1* gene under the control of a tetracycline-regulatable promoter. To further characterize the role of *NRG1* in controlling filamentous growth, and in an attempt to determine whether *NRG1* downregulation is a requirement for filamentation per se, or is only necessary under certain environmental conditions, we have conducted an analysis of the growth of the *tet-NRG1* strain under a variety of in vitro conditions. Through overexpression of *NRG1*, we were able to block filamentation of *C. albicans* in both liquid media and on solid media. Filamentation in response to the low-oxygen environment of embedded growth was also inhibited. In all of these conditions, normal filamentation could be restored by down regulating expression from the *tet-NRG1* allele. Interestingly, although elevated *NRG1* levels were able to inhibit the formation of true hyphae in response to a wide range of environmental stimuli, elevated *NRG1* expression did not affect the formation of pseudohyphae on nitrogen-limiting synthetic low ammonia dextrose (SLAD) medium. This work further illustrates the key role played by *NRG1* in the control of filamentation and suggests that, although *NRG1* repression plays a key role in regulating true hyphal growth, it apparently does not regulate pseudohyphal growth in the same fashion.

Keywords

Candida albicans; Filamentation; *NRG1*; Hyphae; Pseudohyphae

Introduction

Candida albicans is a commensal organism of humans, but is also an opportunistic fungal pathogen, capable of causing serious disease. *C. albicans* infections are most often associated with immuno-compromised patients such as those with HIV/AIDS, transplant recipients and chemotherapy patients. Mortality rates from *C. albicans* systemic infections range from 30 to 50% [1,2].

C. albicans can grow as yeast cells, pseudohyphae and true hyphae, and the ability to filament has been linked with the disease potential of this species. Filamentation is required for the formation of biofilms; these complex structures exhibit increased resistance to antifungal drugs and are important for *C. albicans* infections arising from implanted medical devices such as

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catheters. Further, strains that are locked either in the yeast form [3-5] or in the filamentous form [6,7] of growth are avirulent in the murine model of systemic candidiasis.

Filamentation is controlled by a complex network of signalling pathways, including the mitogen-activated protein kinase (MAPK) and cyclic AMP/protein kinase A pathways [8]. Although some components of these pathways have been identified, many aspects of the signalling process, including the initial signal transduction elements and the potential crosstalk between the different pathways remain unknown. Different environmental stimuli act to control filamentation through a number of different positive and negative factors at the base of these pathways. Expression of genes such as *EFG1, CPH1, RIM101* and *CZF1* promotes filamentation whereas expression of other genes including *RFG1, NRG1* and *TUP1* represses filamentous growth [9].

A key repressor of filamentation is the DNA binding protein Nrg1p, which acts in concert with the global transcriptional repressor Tup1p to suppress hypha formation. Elevated *NRG1* expression is known to repress the expression of a number of hypha-specific genes [10-14] while downregulation of *NRG1* is associated with the ability to form filaments [10,13,14]. In addition, putative Nrg1p response element (NRE) sequences have been identified in the upstream promoter regions of several hypha-specific genes including *ALS3* [15] and *HWP1* [16]. *NRG1* has also been identified as playing a key role in the regulation of chlamydospore formation in both *Candida dubliniensis* and *C. albicans* [17,18].

We previously described the construction of a novel *C. albicans* strain in which one copy of *NRG1* was placed under the control of a tetracycline-regulatable promoter [5]. In the absence of the tetracycline analogue doxycycline, this strain produces elevated levels of *NRG1* and is unable to filament, but in the presence of doxycycline forms true hyphae when grown under filament-inducing conditions. This *tet-NRG1* strain, when kept in the yeast form, is avirulent in the murine model of systemic candidiasis, even though the yeast cells are able to escape the bloodstream and penetrate the organs as efficiently as hyphal forms [5]. This strain is proving to be a valuable tool both in dissecting the role of *NRG1* in the control of filamentation in *C. albicans* and examining the host response to the yeast and hyphal forms. In an attempt to determine whether *NRG1* downregulation is a requirement for filamentation per se, or is only necessary under certain specific environmental conditions, we conducted an analysis of the response of the *tet-NRG1* strain grown under a variety of in vitro conditions.

Methods

Strains and Media

The yeast strains used in the present study were the wild-type strain CAF2-1 *URA3/ura3::imm^{434}* [19], the transactivator-containing strain THE1 ade2::hisG/ade2::hisG ura3::imm^{434}/ura3::imm^{434} ENO1/eno1:: ENO1-tetR-ScHAP4AD-3×*HA-ADE2* [20], the *tet-NRG1* strain SSY50-B (derived from THE1) ade2::-hisG/ade2::hisG ura3::imm^{434}/ura3::imm^{434} ENO1/eno1::ENO1-tetR-ScHAP4AD-3×*HA-ADE2 NRG1/nrg1*::*tetO-NRG1-URA3* [5] and the Δ*tup1* and Δ*nrg1* null strains BCa2-10 ura3::imm^{434}/ura3::imm^{434} tup1::hisG/tup1::p405-URA3 and Bca23-3 ura3::imm^{434}/ura3::imm^{434} nrg1/nrg1::URA3 iro1::imm^{434}/iro1::imm^{434}, respectively [10] (derived from strain CAI4 [19]). These strains were routinely maintained as −80°C glycerol freezer stocks and retrieved on yeast extract-peptone-dextrose (YPD) medium as required. Expression from the *tet-NRG1* allele was abolished through the addition of 20 μlg ml^{-1} doxycycline to the growth medium as needed.

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RNA Isolation and Northern Blot Analysis

To verify the DOX regulation of the modified *tet-NRG1* allele, the wild-type CAF2-1 and modified strains were grown for 5 h in GlcNAc Medium or RPMI-1640 at 37°C. Total RNA was isolated using a previously published bead beater protocol [21], then separated through formaldehyde-containing agarose gels transferred to a nytran membrane (Whatmann) and subjected to Northern blot analysis as previously described [22]. The probe has the PCR product (NRGB), comprising the sequence −33 to +446 relative to the ATG start codon of the *NRG1* open reading frame previously described [5] and labelled with ^{32}P-dCTP using Ready-To-Go DNA labelling beads (GE Healthcare).

Liquid Assays

For filamentation assays in liquid media, strains were grown overnight in YPD at 25°C and diluted 1:20 into fresh RPMI-1640 supplemented with L-glutamine and buffered with morpholinepropanesulfonic acid (Angus Buffers and Chemicals), GlcNAc Medium [23], or Medium 199 (M199) buffered to pH 4.0 or pH 8.0 (Invitrogen) and incubated with shaking at 37°C. Samples were taken from these cultures at various time points, and their morphology was evaluated microscopically using a 40× objective lens and photographed using a digital camera.

Agar Invasion Assay

For the invasion assay, strains were streaked on YPD plates and incubated at 30°C for 48 h. The plates were then photographed with a digital camera both prior to and after surface cells were removed by gentle washing under running water.

Embedded Assays

Strains were grown in YPD overnight at 25°C, washed in sterile phosphate-buffered saline (PBS) and counted using a haemocytometer. Approximately 250 cells were mixed with molten YPD agar and poured onto YPD plates which were subsequently incubated at 30°C for 3 days. Embedded colonies were examined and photographed using a GL9-280 Stereo Zoom microscope (Jenco) equipped with a digital camera.

Spider Medium

For filamentation assays on solid Spider medium [24], strains were grown in YPD overnight at 25°C, washed in sterile PBS, counted using a haemocytometer and resuspended to a final concentration of 5×10^6 cells ml^{-1}. Aliquots of 2 μl (1×10^4 cells) were spotted onto Spider plates and the plates incubated at 37°C for 7 days. Colonies were examined and photographed using a GL9-280 Stereo Zoom microscope (Jenco) equipped with a digital camera. Cells removed from the colony were photographed using the 10× objective lens of a Micromaster Inverted Microscope equipped with a digital camera (Fisher Scientific).

SLAD Medium

For filamentation assays on SLAD medium [25], strains were grown in YPD overnight at 25° C, washed in sterile PBS and counted using a haemocytometer. Approximately 200 cells were spread onto SLAD plates. These plates were then incubated at 25°C for 7 days and colonies photographed using a digital camera. Cells removed from the colony were photographed using the 10× objective lens of a Micromaster Inverted Microscope equipped with a digital camera (Fisher Scientific).

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Results and Discussion

Deletion of *NRG1* has previously demonstrated the involvement of Nrg1p in the negative regulation of hypha-specific gene expression and its requirement in the control of filamentous growth [10,14]. We have sought to further understand the role of Nrg1p in regulating filamentation by constructing an engineered strain in which we can control *NRG1* expression. Using this novel strain, we previously demonstrated the ability to regulate *C. albicans* virulence in a murine model of disseminated candidiasis through the modulation of *NRG1* expression [5]. Many in vitro conditions are known to stimulate filament formation, but which of these is the predominant driver of hyphal development in vivo remains unknown. These filament-inducing stimuli are transmitted down their associated signalling pathways [8,9]. Each of these different pathways can be differentially stimulated by growing the fungus in several specific media. In an attempt to determine whether Nrg1p plays a preferential role in regulating any of the pathways, we present an in vitro characterization of the growth of the *tet-NRG1* strain in a variety of conditions which stimulate filamentation through distinct mechanisms.

Effect of *NRG1* Overexpression on *C. albicans* Morphology in Liquid Media

We have previously shown that elevated expression of *NRG1* is able to inhibit filamentation when the cells are grown in YPD+ serum at 37°C [5]. To examine whether this inhibition was specific to a particular filamentation signal or a more general regulation, the *tet-NRG1* strain was grown in liquid media known to induce filamentation in *C. albicans* both in the presence and in the absence of doxycycline, and its growth was compared to that of the wild-type strain CAF2-1. Unlike the D*nrg1* null strain which is constitutively filamentous [10,14], the *tet-NRG1* strain grows as round free yeast cells in liquid YPD at 25°C and 30°C in the absence (elevated *NRG1* levels) or presence (reduced *NRG1* levels) of doxycycline (data not shown). Thus, even when expression from the *tet-NRG1* allele has been abolished, expression of *NRG1* from the remaining native copy of the gene is sufficient to prevent filamentous growth under yeast-growth conditions.

Growth in cell culture medium RPMI-1640 at 37°C stimulates formation of true hyphae in wild-type strains, and these conditions are typically used for in vitro biofilm formation on polystyrene surfaces [26]. When grown in RPMI-1640 medium for 3 h, the wild-type strain CAF2-1 grew, as expected, as long filaments in both the presence and absence of doxycycline (Fig. 1a). The *tet-NRG1* strain grown in the presence of doxycycline also grew as filaments. However, in the absence of doxycycline (elevated *NRG1* expression) the *tet-NRG1* strain grew almost exclusively as yeast cells (Fig. 1a).

Growth in liquid medium containing the amino sugar *N*-acetylglucosamine (GlcNAc) also stimulates *C. albicans* filamentation [23]. Cells were grown for 5 h in GlcNAc medium at 37°C and examined microscopically. Nearly all (>95%) of the CAF2-1 cells were filamentous, as were the cells of the *tet-NRG1* strain grown in the presence of doxycycline. In the absence of doxycycline, more than 90% of the *tet-NRG1* cells remained in the yeast form (Fig. 1a). To verify that *NRG1* transcription from the *tet-NRG1* allele was being properly modulated in these hypha-inducing media, as we previously demonstrated in YPD+ serum at 37°C [5], RNA was extracted and subjected to Northern blot analysis. In both RPMI-1640 and GlcNAc medium, transcription from the modified allele was entirely dependent on the presence or absence of doxycycline, and expression was higher than that from the wild-type alleles in the control strain CAF2-1 (Fig. 1b).

Elevated *NRG1* expression was sufficient to inhibit filamentation in liquid culture using cell culture medium RPMI-1640 or GlcNAc medium (Fig. 1) which are common conditions used by a number of investigators to induce filamentation in liquid cultures [9,23,27-30]. A role for

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Nrg1p regulation of filamentation in RPMI-1640 and GlcNAc media has not previously been demonstrated.

NRG1 Involvement in Alkaline pH-Driven Morphogenesis

The different host environments inhabited by *C. albicans* can vary widely in pH, especially the GI tract which ranges from the highly acidic stomach environment to the alkaline intestine. It has also been shown that the ability of *C. albicans* to respond to changes in pH is required for pathogenesis [32]. Filamentation is triggered in response to a shift from acidic growth conditions to a neutral or alkaline environment with signals related to pH transmitted through the conserved *RIM101*-mediated pathway [31-33]. While in *Saccharomyces cerevisiae* the transcription factor Rim101p acts through *NRG1*, this is not the case in *C. albicans* where a Δ*rim101* null strain fails to filament in response to neutral pH [34], but the Δ*nrg1* null strain is still able to form true hyphae under these conditions [14]. To further assess the role of *NRG1* expression in regulating *C. albicans* filamentation via the pH induction pathway, the *tet-NRG1* and CAF2-1 strains were grown in liquid M199 at pH 4 or pH 8. When grown for 5 h in M199 pH 4, cultures of both strains consisted of free-floating yeast cells or short chains of yeast cells (Fig. 2). In M199 at pH 8, CAF2-1 grew almost exclusively as filaments with very few free yeast cells observed irrespective of the presence of doxycycline (Fig. 2). Conversely, the *tet-NRG1* strain filamented normally only in the presence of doxycycline. Although Rim101p does not appear to regulate alkaline responses through Nrg1p in *C. albicans,* elevating *NRG1* expression was able to block the filamentation response to increased/alkaline pH. This indicates that Nrg1p functions in opposition to and downstream of the Rim101p pH response pathway.

Overexpression of *NRG1* Blocks Hypha Formation on Spider Medium

Spider medium is one of the strongest stimulators of filamentous growth in *C. albicans* [24]. It has previously been described that constitutively expressing *NRG1* using the *ACT1* promoter rescues the constitutively filamentous phenotype of the Δ*nrg1* null strain on both YPD and Spider medium [10]. We wanted to test whether a regulatable promoter could also control filamentation on Spider medium. When CAF2-1 was grown on Spider plates, highly wrinkled colonies were formed, indicating the presence of filamentous cells (Fig. 3). Material removed from one of the colonies and examined microscopically confirmed that the colony consisted almost entirely of clumps of filamentous cells. In the presence of doxycycline, the *tet-NRG1* strain also formed highly wrinkled colonies consisting primarily of filamentous cells. In the absence of doxycycline, however, the *tet-NRG1* strain formed flat, ruffled colonies. When a portion of one of these colonies was examined microscopically, it was found to be composed almost exclusively of yeast cells (Fig. 3). Thus, alterations in control of *NRG1* expression were able to influence filamentation on solid Spider medium.

Regulating *NRG1* Expression Modulates Agar Invasion

To examine whether Nrg1p also had an impact on filamentation and invasion, various strains were streaked onto YPD medium with and without doxycycline and incubated at 30°C for 2 days (Fig. 4a). After gentle washing, the constitutively filamentous Δ*tup1* and Δ*nrg1* null strains remained attached to the plate (Fig. 4b). Although most of the CAF2-1 cells washed off, some had invaded the agar. The behaviour of these three strains was not affected by the presence or absence of doxycycline. However, with respect to the *tet-NRG1* strain, invasion of the agar to similar extents as the CAF2-1 wild-type strain was seen only in the presence of doxycycline. In the absence of doxycycline, the *tet-NRG1* cells failed to invade the agar and were completely washed off the surface. We have previously demonstrated that a Δ*efg1* null strain, while impaired in its ability to filament, was nonetheless able to successfully transmigrate across an extracellular matrix-based substrate [35]. Similarly, the *tet-NRG1* strain

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has been shown to successfully escape the bloodstream and invade tissues in mice even when grown in the yeast form [5]. In contrast, the results presented here indicate that although yeast cells of the *tet-NRG1* strain are able to escape the bloodstream, they are severely impaired in their capacity to invade an agar plate.

NRG1 Overexpression Affects Embedded Growth Morphology

Growth of *C. albicans* cells embedded in solid medium is also known to stimulate filamentation. Studies of the transcriptional regulator *CZF1* have helped to characterize this response to growth under the increased physical pressure and low-oxygen environment encountered by *C. albicans* during growth in embedded conditions [36,37]. The transcription factor Czf1p is required for filamentous growth under embedded conditions, while *EFG1*, which normally promotes filamentation, appears to play a role in the repression of filamentation genes in these low-oxygen conditions [38]. The function of *NRG1* in regulating filamentous growth under embedded conditions has not previously been described. Wild-type CAF2-1 cells embedded in the solid medium began to filament after 48-h growth, and by 72 h all of the cells had formed filamentous colonies (Fig. 5). When the *tet-NRG1* strain was grown in the presence of doxycycline, it too formed filamentous colonies after 48 h and all colonies were filamentous after 72 h. However, unlike the wild-type strain which formed colonies with several large filamentous projections, the *tet-NRG1* colonies formed many thin filamentous projections giving the colonies a fuzzy appearance. This suggests that there may be a haploinsufficiency phenotype associated with the *tet-NRG1* strain which is effectively heterozygous for *NRG1* (on a transcriptional level) when grown in the presence of doxycycline. This is supported by the observation that the *tet-NRG1* parent strain THE1 displays the same phenotype on this medium as the CAF2-1 strain (data not shown). Conversely, when the *tet-NRG1* strain was grown embedded in medium lacking doxycycline, all the colonies formed after 48-h growth were smooth and after 72 h only very few colonies (<5%) showed evidence of filamentation, demonstrating a role for Nrg1p regulation in the control of the *C. albicans* response to the embedded/microaerophilic conditions. While the transcriptional regulator Efg1p switches from promoting to repressing filamentation when switched from aerobic to low-oxygen-embedded conditions, the observation that the *tet-NRG1* strain colonies remained smooth when *NRG1* expression was high but filamented readily when *NRG1* levels were low (Fig. 5), suggests that Nrg1p functions to suppress *CZF1*-mediated transcription directly rather than through its repression of Efg1p function. The lack of filamentation when *NRG1* levels are kept high in a low-oxygen environment surrounded by a solid matrix is reminiscent of the yeast cells observed in organs such as the kidney during a murine infection with the *tet-NRG1* strain in the absence of doxycycline [5].

Elevated *NRG1* Expression does not Inhibit Pseudohyphal Formation

Many of the regulators for hyphal growth in *C. albicans* are conserved in *S. cerevisiae*, but whereas *S. cerevisiae* filamentous growth occurs only as pseudohyphal cells, *C. albicans* is able to form both pseudohyphae and true hyphae. Although Park and Morschhauser demonstrated that *NRG1* was required for *C. albicans* hypha formation on SLAD when grown at elevated temperatures [39], this medium was originally designed to stimulate pseudohyphal formation at low temperatures [25]. When the wild-type CAF2-1 strain was grown on SLAD medium at 25°C for 7 days, it formed colonies that were generally smooth with some short, ruffled projections and some long individual filaments (Fig. 6). Similarly, the *tet-NRG1* strain showed a halo of filamentous growth around a central smooth colony when grown in the presence of doxycycline, although the length of the extended filaments was increased compared to those see in the wild-type strain (note scale bars Fig. 6). Strikingly, the filamentous phenotype was also observed when this strain was grown in the absence of doxycycline. Although the *tet-NRG1* strain lacked the longer filaments when *NRG1* was highly expressed, it was still able to form colonies with a halo of pseudohyphal growth (Fig. 6). The presence of

pseudohyphal cells was confirmed by removing some cells from the colonies and examining them microscopically (Fig. 6). Unlike all the other conditions tested, elevated *NRG1* expression was not sufficient to block filamentation on SLAD medium, suggesting that formation of pseudohyphae may not be regulated by Nrg1p. Interestingly, it has been observed previously that the Δ*cek1* null mutant was unable to form true hyphae on SLAD medium, but was still able to form pseudohyphae [40], indicating that blocking hyphal induction, either by impairing the MAP-kinase signalling pathway or through increased *NRG1* expression, fails to block pseudohyphal formation on SLAD medium. Taken together, these results suggest that there are important differences between the regulation of hypha and pseudohypha formation in *C. albicans*, even though many of the genes controlling filamentation are conserved between *S. cerevisiae* and *C. albicans*.

Summary

C. albicans is a common commensal organism of humans and under the right conditions can become a successful pathogen. It can be found in several different environments within the host, including the oral and vaginal cavities, the gastrointestinal tract, the bloodstream, and deep within internal organs. These locations vary greatly in environmental conditions including oxygen levels, pH, physical stress and nutrient availability. The ability of *C. albicans* to grow in this variety of conditions indicates a capacity to adapt its growth in response to these differing external stimuli [8,41]. Filamentation is controlled by a complex network of regulatory pathways that converge onto specific transcriptional regulators. Modulation of the expression of the negative regulator, *NRG1*, is sufficient to control the ability to filament and to cause disease in the murine model of disseminated candidiasis, and is here demonstrated to be able to control the ability to filament in response to a variety of specific environmental signals in vitro. Elevated *NRG1* expression was able to block filamentation in liquid media such as RPMI-1640 and YPD containing serum [5] or media containing GlcNAc and was also able to regulate the invasion of solid medium.

The specific environmental conditions used here were designed to examine the involvement of Nrg1p in blocking filamentation stimulated through the activity of the four major regulatory transcription factors. Increased *NRG1* expression blocked filamentation on solid Spider medium, which induces filamentation in a Cph1p dependent fashion. *NRG1* overexpression was also able to block the filamentation response to increased pH by the Rim101p-mediated pathway and also filamentation in response to low oxygen, and embedded conditions mediated by the transcription factors Czf1p and Efg1p. Taken together with our previously published observations using the *tet-NRG1* strain in vivo, it is clear that *NRG1* is of fundamental importance for regulating the filamentation response in *C. albicans* and that this response can be controlled through modulation of *NRG1* expression. Perhaps most significantly, the ability to form pseudohyphae on nitrogen-limiting SLAD medium was not impaired by elevated *NRG1* levels and is so far the sole filamentation response that could not be blocked in this strain. This suggests that while regulation of *NRG1* expression is pivotal to the induction of true hyphae by a wide range of environmental conditions, it does not play such a key role in pseudohyphal formation.

Acknowledgments

We thank Professor A.D Johnson (UCSF) for supplying the Δ*tup1* (Bca2-10) and Δ*nrg1* (BCa23-3) null strains used in this study. The work presented here was funded in part by National Institutes of Health Grant award RO1 A1063256 from the National Institute of Allergy and Infectious Diseases to SPS.

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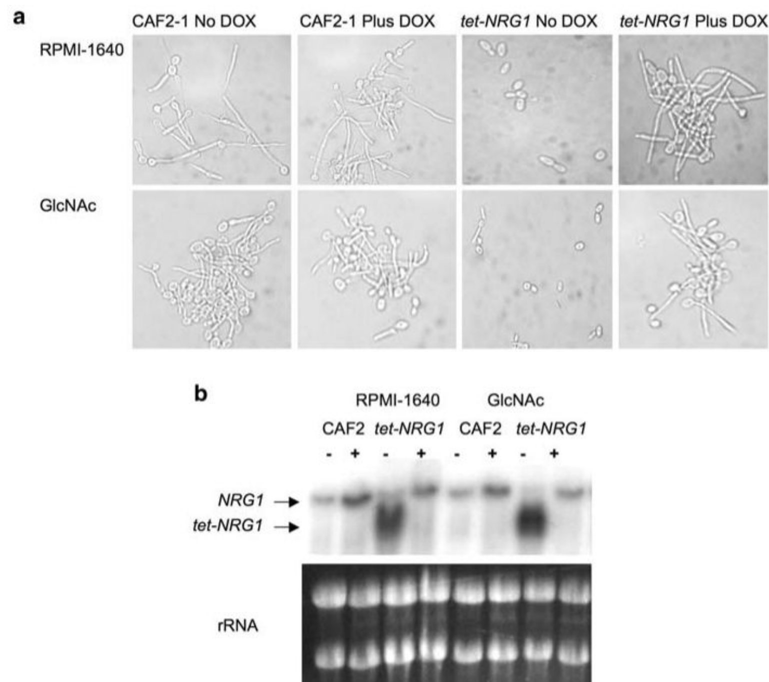

Fig. 1.

Growth in filament-inducing liquid media. **a** Samples were taken from cultures grown in RPMI-1640 or GlcNAc medium at 37°C for 5 h in the presence or absence of doxycycline, and their morphology was examined microscopically. **b** Total RNA was prepared from the cultures in (**a**) and subjected to Northern blot analysis to determine *NRG1* expression levels. As seen in our previous study [5], the CAF2-1 wild-type strain contains one hybridizing band while the *tet-NRG1* strain contains two hybridizing bands: one identical in size to the wild-type allele and a second, smaller band whose expression is entirely dependent on the presence or absence of doxycycline. Ethidium bromide-stained rRNA bands are shown to confirm equal loading of all samples

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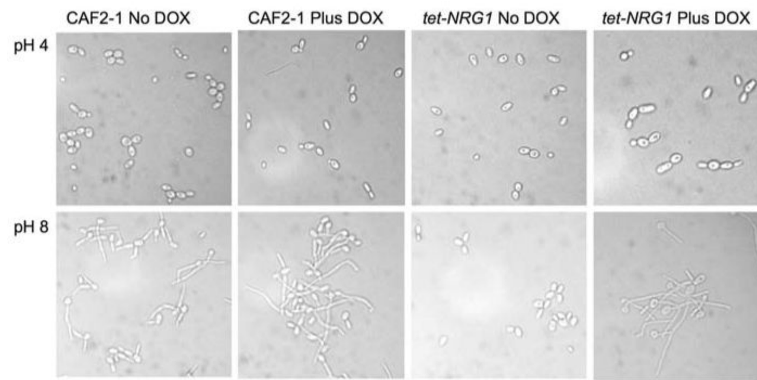

Fig. 2.
Induction of filamentation in response to neutral pH. Samples were taken from cultures grown for 5 h at 37°C in M199 buffered to either pH 4 or pH 8, in the presence or absence of doxycycline, and their morphology was examined microscopically

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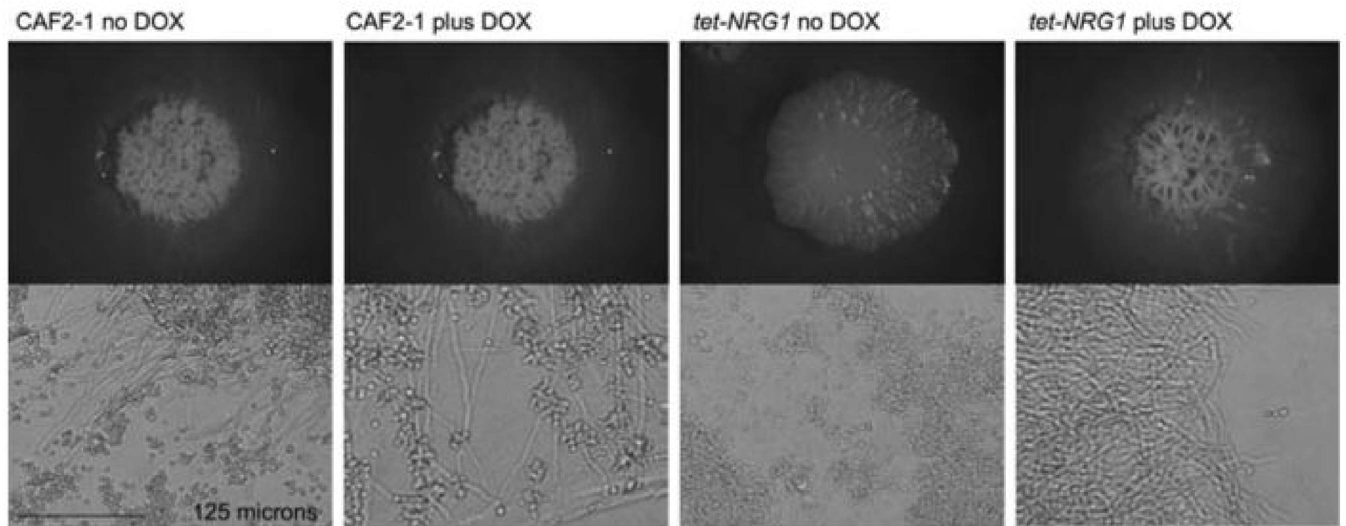

Fig. 3.
Growth on solid Spider medium. Cells from an overnight culture were washed in sterile PBS, counted and spotted onto solid Spider medium in the presence or absence of doxycycline and incubated at 37°C for 7 days. The morphology of the colonies and of cells taken from those colonies was examined microscopically

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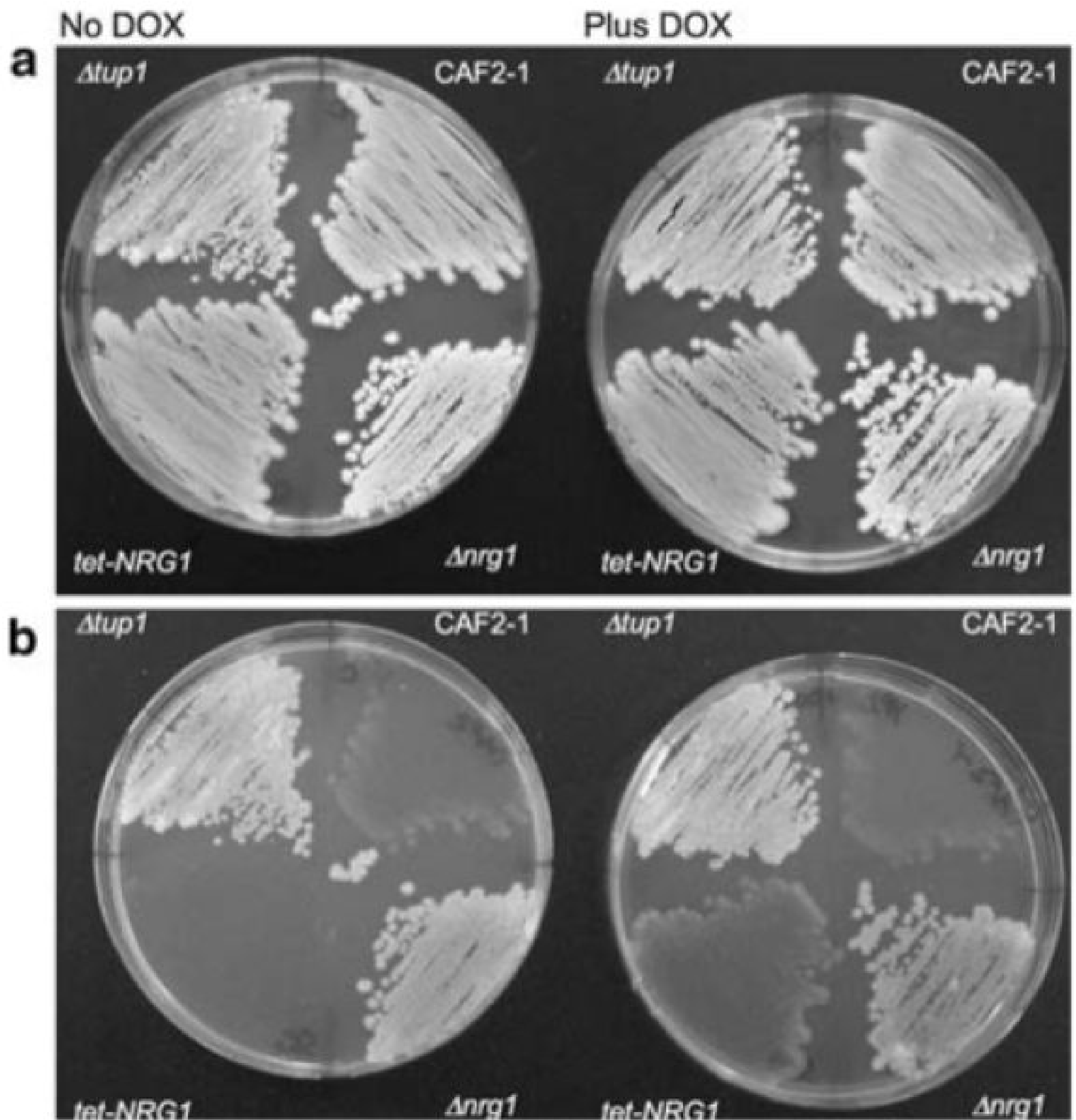

Fig. 4.

Invasion of solid medium. The wild-type CAF2-1, Δ*tup1* null, Δ*nrg1* null and the *tet-NRG1* strains were streaked onto YPD agar and incubated at 30°C. Plates were photographed after 2 days growth (**a**). The plates were subsequently washed under running water and photographed again (**b**). Cells that had not invaded the solid medium washed away while those cells that had invaded remained

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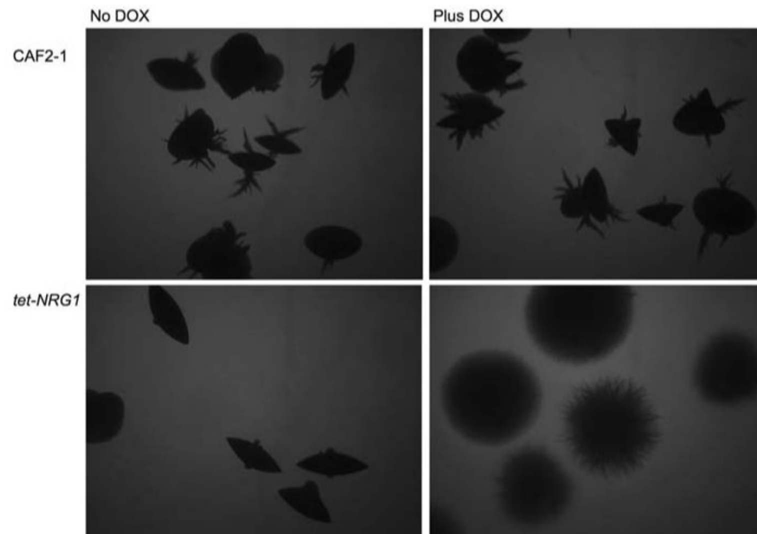

Fig. 5.

Growth under embedded conditions. Cells from an overnight culture were washed in sterile PBS, counted and were embedded in molten YPD agar in the presence or absence of doxycycline and incubated for 72 h at 30°C. The morphology of the colonies formed in the medium was examined microscopically

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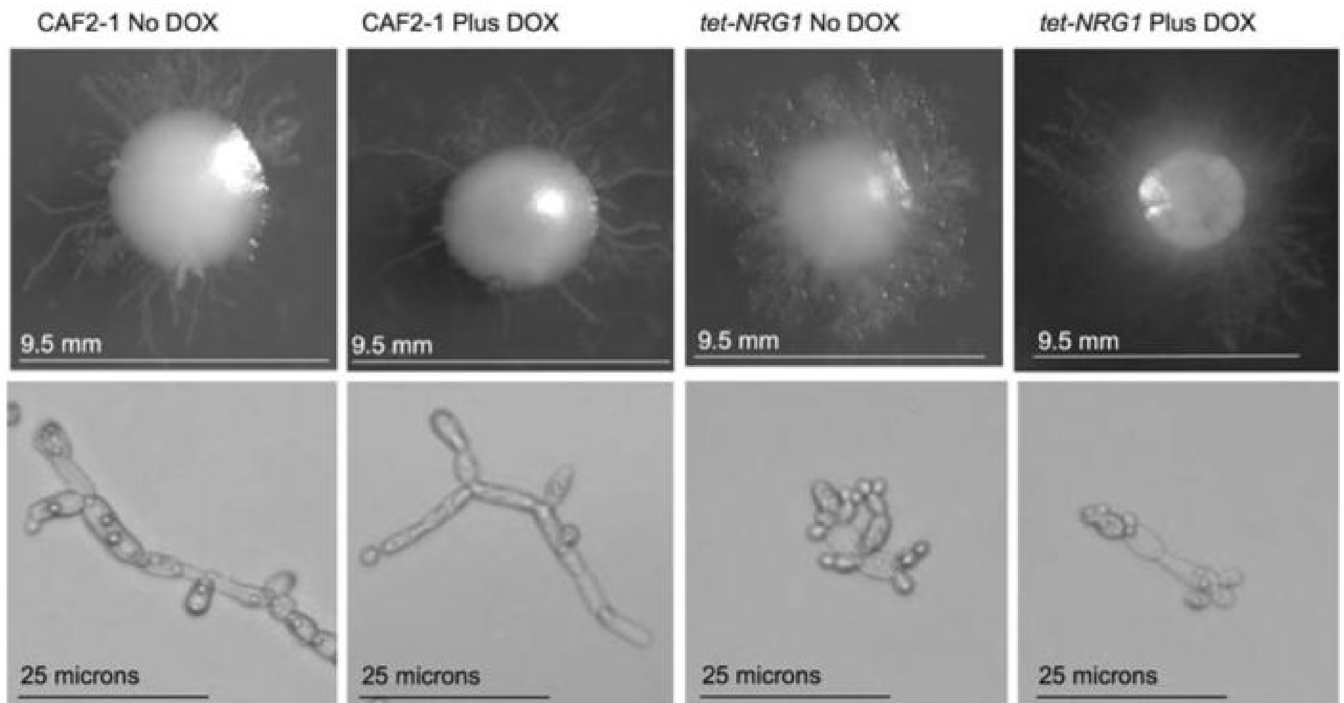

Fig. 6.

Growth on solid SLAD medium. Cells from an overnight culture were washed in sterile PBS and spread onto solid SLAD medium in the presence of absence of doxycycline and incubated at 25°C for 7 days. The morphology of the colonies (*upper panels*) and of cells removed from these colonies (*lower panels*) was examined microscopically