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Investigating the role of Nrg1p and Tup1p during *Candida albicans* Chlamydospore Formation



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Introduction

Candida albicans exists and thrives on mucosal surfaces of the gastrointestinal tract and urogenital tract in healthy persons, but it can also cause severe and life-threatening infections, especially in immunocompromised patients (4), and thus has become a relevant topic in the medical community. A wide range of factors leads to various degrees of immunosuppression that predispose patients to infection by *Candida*. These factors include a severe underlying disease (e.g., AIDS or leukaemia), impaired phagocytic function (i.e., granulocytopenia or neutropenia), and exogenous factors (e.g., wide spectrum antibiotic treatment, i.v. drug use, transplantation medicine, trauma, abdominal surgery [5]). Also, candidiasis, a systemic yeast infection, now represents the third to fourth most frequent hospital acquired infection in the US and worldwide, and *C. albicans* remains the most frequent causative agent of candidiasis (1). These infections carry unacceptably high morbidity and

mortality rates and important economic repercussions with an estimated total direct cost of approximately 2 billion dollars in 1998 in US hospitals alone (14). Even treatments using available antifungal agents, mortality rates lie in the 30-50% range for these infected patients (13). Although several other *Candida* species are also human pathogens that have been found with increasing frequency, *C. albicans* remains by far the most medically important member of the genus (7).

C. albicans can transition between yeast cells, pseudohyphae or hyphae. Filamentation of yeast cells to hyphal growth is directly linked to its pathogenicity and cells that cannot make the transition are known to be avirulent. *C. albicans* is known to form chlamydospores when low levels of nutrients comprise its environment. Chlamydospores are thick-walled, spherical cells that are three to four times larger than normal yeast cells and are produced from suspensor cells at the end of what are likely pseudohyphal elements ([10] Figure 1).

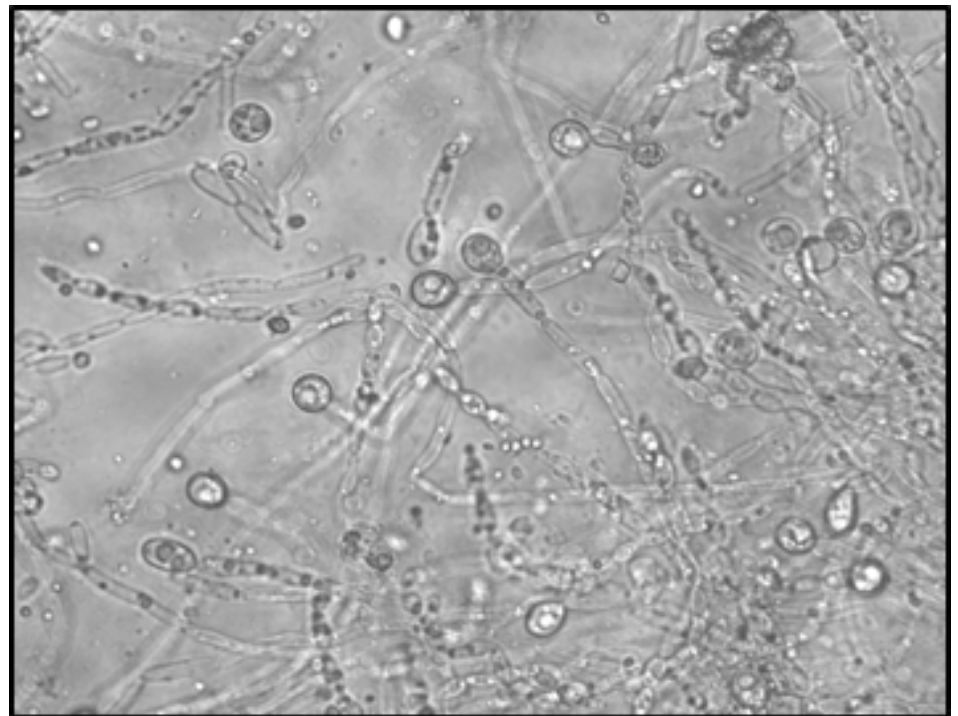


Figure 1: Chlamydospore formation of *C. albicans*

Because chlamydospores arise in conditions of nutrient depletion, they are believed to be dormant growth forms that can be induced to germinate by changing environmental conditions (6).

Chlamydospore-like structures have been detected occasionally in the human host, however, whether chlamydospores contribute to colonization or pathogenicity of *C. albicans* is unknown (4). Examining the regulation of the gene *NRG1p* and its direct relationship to filamentation (hyphal growth) and chlamydospore formation, will contribute greatly to the understanding of this virulence factor of *C. albicans* and what its biological role is. This study aims to fill in the gaps in our understanding of the mechanisms which underpin this important biological phenomenon, the genetic regulation of chlamydospore formation in relation to filament formation. The gene *NRG1* is a known inhibitor, or repressor, of the Hyphal Specific Genes (*HSG*) and has been strongly implicated in chlamydospore formation. It has been shown that differential regulation of the single gene *NRG1* in *C. albicans* and (closely related species) *Candida dubliniensis* is responsible for their species-specific response to environmental signals that induce chlamydospore development (12). Furthermore, morphogenesis (yeast to pseudohyphal/hyphal transition) is repressed by transcriptional inhibitors such as *Tup1p*, which associates with its DNA-binding partners *NRG1p* and *Rfg1p* (3). However, the nature by which this gene is regulated is in question; thus, in this study we intended to investigate (1) the hyphal specific genes regulated in chlamydospore formation and (2) the necessity or dependency of coupling with the gene *TUP1* during chlamydospore formation.

Materials and Methods

Strains. A comparison of four *C. albicans* strains and one *C. dubliniensis* strain were used. *C. albicans* wild-type strain SC5314 was used as a control along with *C. dubliniensis* wild-type strain. These strains were chosen based on their known performance in Staib medium, as published by Staib and Morschhäuser. Comparison strains used were *C. albicans* strains DPTY2 (*NRG1/NRG1 tet-NRG1*), SSY50b (*ΔNRG1/NRG1 tet-NRG1*), and

ICY49 (*ΔTup1/ΔTup1*).

Culture media and growth conditions for preparation of RNA analysis. All strains were grown overnight in liquid YPD (yeast, peptone, dextrose media) at 28°C. Cultures were then washed with phosphate buffered NaCl (PBS) x 2 (as described by Jansons and Nickerson, [6]). Various dilutions of each strain yielded a cell count of 10⁷-10⁶/mL. A 20:1 diluted subculture of each strain was added to liquid Staib media (syn. *Guizotia abyssinica*, creatinine, glucose, KH₂PO₄), (12). In addition, two subcultures of DPTY2 and SSY50b were made, one with the tetracycline analog, doxycycline, and the other without. These cultures were then grown at 32°C for 48 hours in a shaking water bath.

RNA isolation was achieved with our adapted methodology and the Masterpure yeast isolation kit (Epicentre™). 1µg of this RNA was used to generate cDNA using the first strand synthesis kit (Five-prime™), and the cDNA was quantitatively compared by real-time PCR using GoTaq™ Sybr™ green (Promega™) on an Mx3000P real-time machine (Stratagene™). The relative expression of the *ACT1* (actin) gene was used to normalize the data across samples.

Culture media and growth conditions for NRG1/Tup1 association assay. Initial steps up to and including PBS wash were the same as above. Each strain was inoculated to plates of Staib agar, with and without doxycycline and Rice Tween 80 agar (RTW), with and without doxycycline. RTW plates were housed in a candle jar. Plates were incubated at 28°C, in darkness, for 14 days. Cultures were analyzed by microscopy at 48 hours, 7 days and 14 days.

Results

Analysis of genetic expression of Hyphal Specific Genes in tet-regulated NRG1 strains. Quantitative real-time PCR analysis of DPTY2 and SSY50b, with and without doxycycline, reveals a difference in expression of the major hyphal-specific genes on chlamydospore formation. This data shows that when complete repression of *NRG1* (DPTY2) occurs within the first 48hrs, the major hyphal-specific genes are

affected differently from the filamentation pathway, as seen by the control effects of SSY50b (Fig. 2).

Analysis of dependency of NRG1 in chlamydospore formation. In order to determine whether the association of *NRG1p* and *Tup1p* is involved in the development of chlamydospores in *C. albicans*, we used two types of media. Using staib agar, which is known to stifle chlamydospore formation in *C. albicans* but not in *C. dubliniensis*, we created one plate with the addition of doxycycline and the other plate without. Since comparisons of *C. albicans* and *C. dubliniensis* have been found to regulate *NRG1p* differently (12), we took advantage of the wild-type strains SC5314 (*C. albicans*) and *C. dubliniensis* as controls to our variable strains DPTY2 (*ΔNRG1/tet-NRG1*), SSY50b (*NRG1/tet-NRG1*) and ICY49 (*ΔTup1/ΔTup1*). Another way we controlled the experiment was to use a nutrient-poor media, Rice Tween 80 agar, in a candle jar to limit the oxygen availability, which supports chlamydospore formation in *C. albicans*. The RTW plates were made with and without doxycycline.

Each strain was inoculated onto the plain Staib and RTW plates, with the tet-regulateable strains DPTY2 and SSY50b added to the doxycycline plates. All cultures were grown at 28°C in darkness for 14 days. Comparison assessments were documented via microscopy at 48 hours, 7 days and 14 days (Fig. 3).

In the Staib agar, we were able to induce chlamydospore formation in *C. albicans* that is indistinguishable from the *C. dubliniensis* wild-type. However, for SSY50b, which has one regulateable allele of *NRG1* and one wild-type allele, we were unable to induce chlamydospore formation. SSY50b acts the same as SC5314 (wild-type), which grows a negligible amount of chlamydospores (1-5% 10µL⁻¹). This data shows that expression of *NRG1* has a significant negative repression on chlamydospore formation in *C. albicans*, and when it is inhibited, chlamydospore formation is induced.

If *TUP1* is a crucial binding partner to *NRG1* to induce chlamydospore formation, deleting the gene should show similar results to the complete repression of *NRG1*. Our results show the contrary.

When ICY49 is grown in the same conditions described above on Rice Tween 80 agar plus doxycycline, chlamyospore formation is no more significant than the wild-type expression of SC5314. This leads to the conclusion that TUP1 is not a significant participant in chlamyospore formation. Further investigations will focus on if NRG1 alone regulates chlamyospore formation or if it binds with any other gene during this regulation.

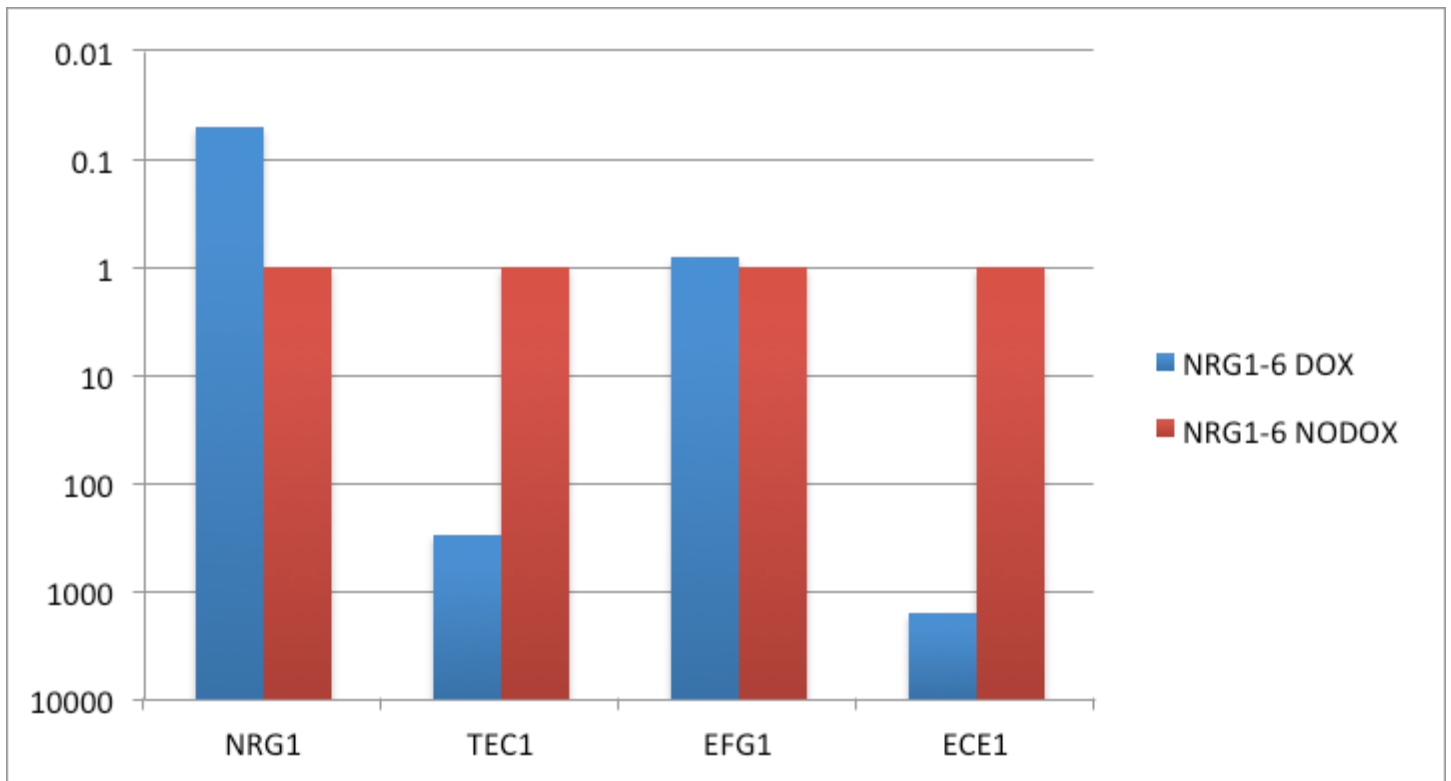
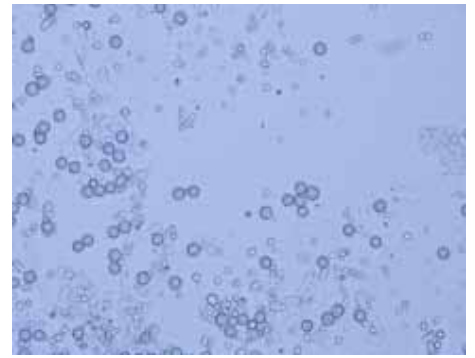
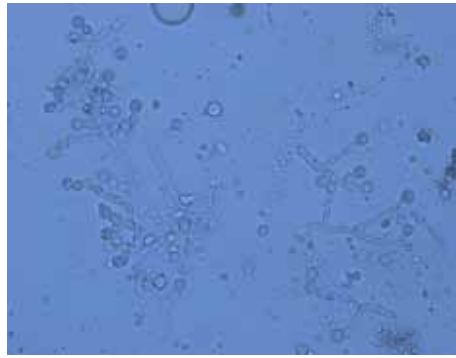
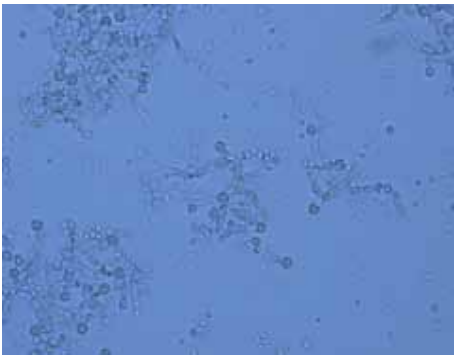
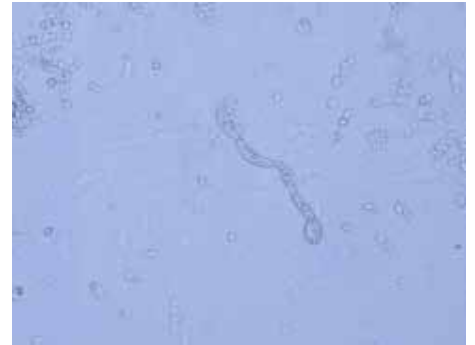
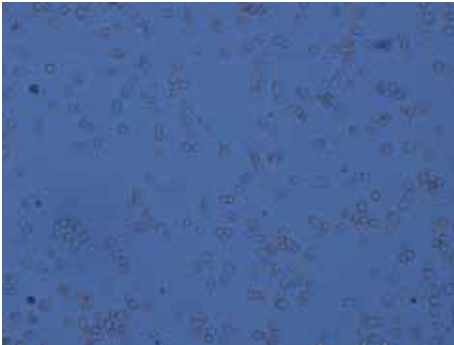


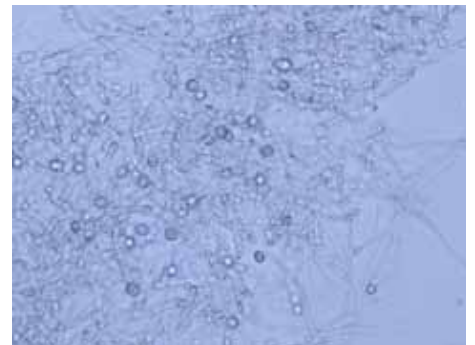
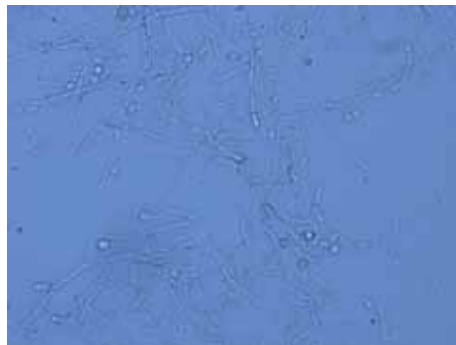
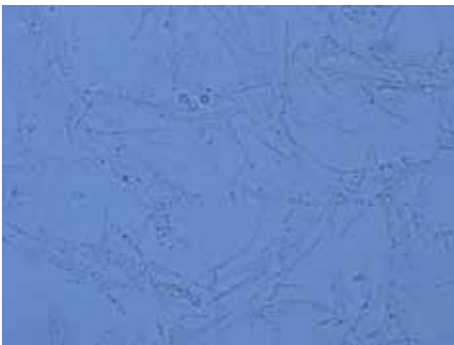
Figure 2: Differential expression of hyphal specific genes on NRG1p



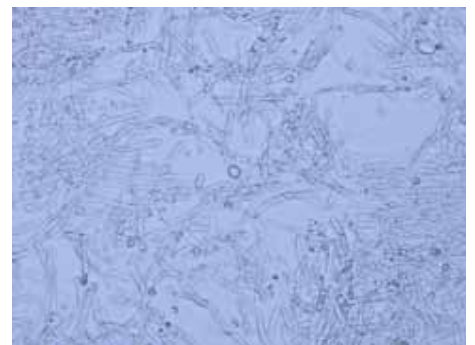
A



B



C



D

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