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Overexpression of *TUF1* restores respiratory growth and fluconazole sensitivity to a *Cryptococcus neoformans vad1*Δ mutant

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The yeast-like fungus *Cryptococcus neoformans* favours respiration as a mechanism of energy production, and thus depends heavily on mitochondrial function. Previous studies of a *C. neoformans vad1*Δ mutant revealed reduced expression of the mitochondrial elongation factor *TUF1* and defects in glycerol utilization, consistent with mitochondrial dysfunction. In this study, we found that *in trans* expression of *TUF1* in the *vad1*Δ mutant suppressed the mitochondrial defects, including growth on respiration-dependent carbon sources and fluconazole resistance, associated with *VAD1* deletion. Tetracycline, an inhibitor of mitochondrial translation, was found to confer resistance to fluconazole in the wild-type and *vad1*Δ mutant, whereas the fluconazole susceptibility of the *TUF1*-overexpressing strain was unaffected by tetracycline treatment. In the presence of fluconazole, the *vad1*Δ mutant exhibited increased activation of the global transcriptional regulator Sre1. *TUF1* overexpression failed to alter cleavage of Sre1 in response to fluconazole in the *vad1*Δ mutant, suggesting that *TUF1* repression in the *vad1*Δ mutant is distal to Sre1, or that it occurs through an independent pathway.

INTRODUCTION

Infections by the pathogenic fungus *Cryptococcus neoformans* are occurring with increasing frequency in regions of the world plagued by the HIV epidemic, especially in regions of Africa and South-East Asia, where *C. neoformans* has become the most common cause of community-acquired meningitis (Bicanic & Harrison, 2005). In addition, *C. neoformans* represents a significant threat to transplant recipients, as infections by *C. neoformans* can result in allograft loss or even death (Husain *et al.*, 2001; Singh *et al.*, 2005). The identification of novel therapeutic targets will aid in the treatment and prevention of cryptococcosis in these susceptible populations.

The basic mechanisms by which *C. neoformans* yeast cells generate energy differ significantly from those of the model yeast *Saccharomyces cerevisiae*. *C. neoformans* yeast cells rely heavily on glycolysis and aerobic respiration for energy production, with fermentation playing a minimal role (Akhter *et al.*, 2003). Both *S. cerevisiae* and *C. neoformans* utilize the highly conserved cytochrome oxidase pathway for electron transport. Three of the major constituents of this pathway are encoded by the mitochondrial genome in *C. neoformans*, and are translated using mitochondrial translational machinery (Litter *et al.*, 2005).

In addition to energy production, mitochondrial function has been linked to antifungal resistance in the pathogenic fungus *Candida glabrata*. Petite mutants of *C. glabrata* that lack functional mitochondria are resistant to azole antifungals (Brun *et al.*, 2004). Fluconazole is an important drug in anticryptococcal therapy, as it is the only azole antifungal with adequate central nervous system penetration for treatment of cryptococcal meningoencephalitis (Perfect & Durack, 1985). In addition, it is being used as monotherapy in regions of Africa where lack of infrastructure precludes the use of amphotericin B (Bicanic *et al.*, 2006).

In *C. neoformans*, fluconazole sensitivity is regulated by the sterol response element-binding protein Sre1 (Chang *et al.*,

Abbreviation: qRT-PCR, quantitative real-time PCR.

2007). Sre1 is required for the response to hypoxia in *C. neoformans*, and is activated by the accumulation of 4-methyl sterols (Hughes *et al.*, 2007). A *C. neoformans sre1*Δ mutant is severely attenuated in virulence, and exhibits hypersensitivity to fluconazole (Chang *et al.*, 2007; Chun *et al.*, 2007). A recent insertional mutagenesis study in *C. neoformans* that utilized the hypoxia mimetic $CoCl_2$ has demonstrated that Sre1, mitochondrial function and sterol metabolism are tightly linked (Ingavale *et al.*, 2008).

We have previously described a mutant of *C. neoformans* that lacks the DEAD-box RNA helicase Vad1; this mutant was identified in an insertional mutagenesis screen for genes involved in production of the virulence factor laccase (Panepinto *et al.*, 2005). The *vad1*Δ mutant exhibits a moderate deficiency in melanization due to reduced expression of laccase, but is severely attenuated in virulence. Forward genetic screening revealed that the *vad1*Δ mutant exhibited decreased expression of *TUF1*, a nuclear gene encoding a homologue of EF-TU, which is an elongation factor for translation within the mitochondrion (Rosenthal & Bodley, 1987). Associated with downregulation of *TUF1* was impaired growth on medium containing the respiration-dependent carbon source glycerol, suggesting a defect in mitochondrial function in the *vad1*Δ mutant (Panepinto *et al.*, 2005).

In this study, we set out to determine whether overexpression of *TUF1* in the *vad1*Δ mutant was able to suppress *vad1*Δ mitochondrial deficiency. We determined that *TUF1* overexpression could restore respiratory growth and fluconazole sensitivity, and could suppress induction of fluconazole resistance by tetracycline treatment. However, hyperactivation of Sre1 in response to fluconazole treatment, and virulence defects of the *vad1*Δ mutant, were unaffected by *TUF1* overexpression. These data suggest that downregulation of *TUF1* contributes to the mitochondrial defects of the *vad1*Δ mutant, and that the influence of *VAD1* deletion on activation of Sre1 is either upstream of *TUF1* influence or through an independent pathway. These results also demonstrate that the fluconazole sensitivity of *C. neoformans* is dependent on mitochondrial function, and they highlight the potential for antibiotics that target bacterial protein synthesis to antagonize the antifungal activity of fluconazole towards *C. neoformans*.

METHODS

Construction of the *v*-K, *v-TUF1* and wt-*TUF1* strains. The coding sequence of the *TUF1* gene was amplified from genomic DNA of *C. neoformans* wild-type strain H99 (J. Perfect, Duke University, Durham, NC, USA) using primers TUFOE-F (5′-GGAGGATG-AATTCCATGCTCAGGAACGCCCTTCAGAG-3′) and TUFOE-R (5′-GGAGGAGAATTCTCCCTTATTCGTAGATCTCTGAAACG-3′) for cloning in-frame with the actin promoter in pORA-KUTAP to make p*TUF1*-KUTAP (Liu *et al.*, 2006). Once verified by sequencing, the expression construct and the empty vector were linearized with Sce1, transformed into a previously described *ura* derivative of the *vad1*Δ mutant, and selected on asparagine salts (ASN) agar (Panepinto *et al.*, 2005). Transformants were screened for episomal maintenance of the constructs by uncut Southern blotting. One vector control strain, *v*-K, and two *TUF1*-overexpression strains, *v-TUF1-1* and *v-TUF1-2*, were chosen for further study, and compared with a previously described wild-type strain harbouring the pORA-KUTAP vector (Panepinto *et al.*, 2009). Strains were maintained on ASN agar, unless otherwise indicated. As described above, the p*TUF1*-KUTAP vector was introduced into a *ura* derivative of the wild-type strain H99. Two strains were chosen for further study, wt-*TUF1-1* and wt-*TUF1-5*.

Spot-plate assay. Cells of each strain were grown on ASN agar for 2 days. A suspension of OD_{600} 1.0 of each strain was made in sterile deionized water. A 5 μl volume of this suspension, and five additional 1 : 5 dilutions, were spotted on ASN medium containing the indicated carbon source. Plates were incubated at 37 °C for 4 days, and photographed.

RNA isolation, Northern blot analysis and quantitative real-time PCR (qRT-PCR). Cells of each strain were grown to mid-exponential phase in yeast extract peptone dextrose (YPD) medium. Cells were harvested by centrifugation, and RNA was extracted using the Qiagen RNEasy mini kit, as described previously (Panepinto *et al.*, 2005). RNA was fractionated by formaldehyde agarose electrophoresis, and transferred to a Hybond-N+ nylon membrane (GE Healthcare). *TUF1* and *ACT1* expression was detected using ^{32}P-labelled probes that corresponded to the coding region of each gene. Hybridization was visualized by autoradiography using Kodak BIOMax-XR film. qRT-PCR for *TUF1* expression was performed using intron-spanning primers *TUF1*-qRT-F (5′-CACTGCCCATGTTGAATACG-3′) and *TUF1*-qRT-R (5′-CATATTTTTGATGTAATCGGC-3′), which correspond to an 85 bp region of the *TUF1* cDNA, and *ACT1*-qRT-F (5′-CTCTATGAAGTGTGATCTCG-3′) and *ACT1*-qRT-R (5′-CATA-CGGTCGGCAATACC-3′), which amplify a 100 bp region of the *ACT1* reference gene cDNA. Reverse transcription was performed on DNase-treated RNA using the iScript kit (Bio-Rad Laboratories), according to the manufacturer's protocol. PCRs were set up using iQ SyberGreen SuperMix (Bio-Rad Laboratories), according to the manufacturer's protocol. Briefly, a master mix was prepared to allow each reaction to contain 12.5 μl SyberGreen SuperMix, 10.5 μl diethylpyrocarbonate-treated water, 0.5 μl forward primer and 0.5 μl reverse primer. A 1 μl volume of cDNA was added to each reaction. qRT-PCR was performed using either a Bio-Rad iCycler or an iQ Real-Time PCR system. *TUF1* expression was calculated using the comparative C_t method, and normalized against *ACT1* expression (Livak & Schmittgen, 2001).

MIC determination. Fluconazole MICs were determined using broth microdilution and Etest (AB Biodisk). The broth microdilution assay was performed as recommended by the Clinical and Laboratory Standards Institute (CLSI), with the following modification: assays were performed in RPMI medium containing 2 % glucose, and buffered to pH 7 with 10 mM MOPS (Petrou & Shanson, 2000). The first non-turbid well was read as the MIC. Etests were carried out as described by the manufacturer, and were performed on SD agar [1 × yeast nitrogen base (YNB) without amino acids, 2 % glucose].

Sterol scan analysis. Sterol levels were measured as described by Arthington-Skaggs *et al.* (1999), with the following alterations. Strains were inoculated at a density of OD_{600} 0.2 in 30 ml SD medium from an overnight starter culture. Cells were grown in a shaking incubator at 30 °C and 250 r.p.m. for 72 h. The OD_{600} of the cultures was measured, and approximately 80 OD units of cells [the concentration of cells (as OD units per ml) multiplied by the culture volume in ml] was subjected to heptane extraction. Extracted sterols were diluted 1 : 1 with ethanol and scanned spectrophotometrically from 230 to 300 nm on a SpectraMax M5 plate reader/spectrophotometer

(Molecular Devices). Absorbance values were normalized to pellet weight.

Western blot analysis. Cells of the wt-K, *v*-K and *v-TUF1-1* strains were grown to mid-exponential phase in YPD broth. Following the addition of 4 μg fluconazole ml^{-1}, cells were harvested at 0, 2 and 4 h. Total protein was extracted by mechanical disruption for 5 min, alternating vortexing and chilling every 30 s. The lysates were cleared by centrifugation at 15 000 ***g*** for 5 min, after which proteins were quantified using the Bio-Rad Protein Assay reagent. A 15 μg quantity of total protein from each time point was run on a 4–12 % SDS-PAGE gel and transferred to a nitrocellulose membrane. Full-length and cleaved Sre1 was detected using polyclonal antiserum against Sre1 (1 : 250; a generous gift from Peter Espenshade, Johns Hopkins University School of Medicine, Baltimore, MD, USA), and a peroxidase-conjugated anti-rabbit secondary antibody (1 : 1000; Pierce). Antibody reactivity was visualized using Super Signal West Pico reagent, as recommended by the manufacturer (Pierce).

Virulence studies. wt-K, *v-K* and *v-TUF1* strains were inoculated into the lateral tail vein of Swiss albino mice (National Institutes of Health), using 1×10^6 cells for each inoculation. A total of 10 mice were inoculated per group, and mouse health was monitored over 50 days. Moribund mice were sacrificed by CO_2 narcosis. All animal studies were performed in accordance with the University of Illinois at Chicago Institutional Animal Care and Use Committee regulations. Melanization was assayed on ASN agar without glucose, pH 6.5, and with 50 μg ml^{-1} L-3,4-dihydroxyphenylalanine (L-DOPA) as a laccase substrate. The degree of melanization was observed after 24 h at 30 °C.

RESULTS AND DISCUSSION

Overexpression of *TUF1* suppresses *vad1*Δ respiratory growth defects

Our previous studies have demonstrated that the *C. neoformans vad1*Δ mutant exhibits reduced expression of the mitochondrial translation elongation factor *TUF1*, and that it is defective in utilization of respiration-dependent carbon sources (Panepinto *et al.*, 2005). In addition, knockout of *TUF1* in wild-type *C. neoformans* by RNA interference completely inhibits growth on glycerol, leading us to hypothesize that the respiratory defects exhibited by the *vad1*Δ mutant are due to *TUF1* downregulation (Panepinto *et al.*, 2005). To further test this hypothesis, we constructed *v-TUF1*, which was a *C. neoformans* Δ*vad1* strain harbouring an episomal vector expressing *TUF1* under the control of the *ACT1* promoter in pORA-KUTAP. For the following studies, *v-TUF1* was compared with a wild-type strain (wt-K) described previously (Panepinto *et al.*, 2009) and a *vad1*Δ strain (*v*-K), harbouring the empty vector (this study). The level of *TUF1* overexpression achieved by introduction of the episomal construct was measured by Northern blotting (Fig. 1a). Independent assessment of *TUF1* overexpression in these strains by qRT-PCR revealed that *TUF1* was overexpressed 3.5- and 4.0-fold in *v-TUF1-1* and *v-TUF1-2*, respectively, relative to *v*-K, when normalized to the *ACT1* reference gene. To determine whether overexpression of *TUF1* could restore growth on respiration-dependent carbon sources, we compared the growth of *v-TUF1-1* and *v-TUF1-2* with that of wt-K and *v*-K on glycerol, pyruvate and lactate in a spot-plate assay. As demonstrated in Fig. 1(b), overexpression of *TUF1* restored growth of the *vad1*Δ strain on respiration-dependent carbon sources. This is consistent with data from *S. cerevisiae*, in which overexpression of *TUF1* is able to suppress respiratory defects of mitochondrial tRNA mutants (De Luca *et al.*, 2006). These data suggest that the respiratory defects of the *vad1*Δ mutant are linked to repression of *TUF1* expression.

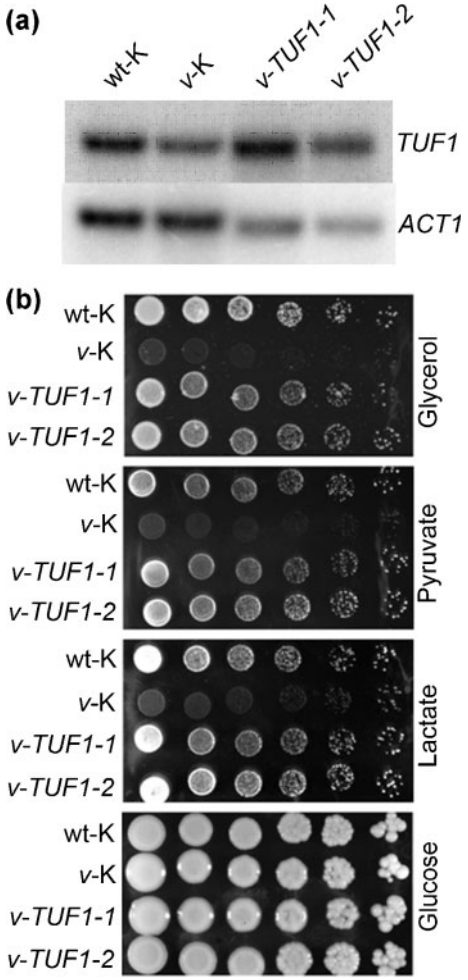

Fig. 1. (a) Northern blot analysis of *TUF1* expression in wt-K, *v*-K and *v-TUF1* strains. RNA was harvested from cultures grown to mid-exponential phase in YPD broth at 30 °C. RNA was hybridized with *TUF1* and *ACT1* probes. (b) Spot-plate assay demonstrating restoration of growth on respiration-dependent carbon sources by overexpression of *TUF1* in the *vad1*Δ mutant. Plates contained the indicated carbon sources at 2 % in ASN agar, pH 6.5. Plates were incubated at 37 °C for 4 days and then photographed.

To determine the effect of *TUF1* overexpression on carbon source utilization in wild-type *C. neoformans*, we compared the growth of two independent isolates of wild-type

C. neoformans harbouring the *TUF1*-overexpression construct with that of the vector control strain, as described above, by spot-plate assay. Both wt-*TUF1* strains exhibited a severe growth defect on minimal medium with glucose as the carbon source (Fig. 2a). Surprisingly, the growth of both wt-*TUF1* strains appeared more robust in medium with glycerol as the carbon source compared with growth in medium containing glucose, with overall growth still less than that of the wt-K strain (Fig. 2b). The level of *TUF1* overexpression as measured by qRT-PCR was found to be 1.5-fold for wt-*TUF1-1* and 1.6-fold for wt-*TUF1-5*, relative to wt-K, at 30 °C in YPD medium using *ACT1* expression for normalization. These results suggest that although *TUF1* overexpression is able to compensate for mitochondrial defects in a *vad1*Δ mutant, even modest inappropriate overexpression of *TUF1* in wild-type *C. neoformans* has detrimental effects on growth, especially at 37 °C in the presence of glucose.

Overexpression of *TUF1* suppresses fluconazole resistance in the *vad1*Δ mutant

A recent study has demonstrated that inhibition of mitochondrial protein synthesis with tetracycline induces fluconazole resistance in *Candida albicans* and *C. neoformans*, with the effect on *C. neoformans* being much greater than on *Candida albicans* (Oliver *et al.*, 2008). This led us to hypothesize that downregulation of *TUF1* in the *vad1*Δ mutant could confer fluconazole resistance that would be suppressed by *TUF1* overexpression. To test this hypothesis, the fluconazole sensitivity of wt-K, *v*-K, *v-TUF1-1* and *v-TUF1-2* was assessed by Etest. As demonstrated in Fig. 3, comparison of fluconazole MICs by the Etest method revealed an increase in MIC in *v*-K compared with the wt-K strain, with the *v-TUF1-1* and *v-TUF1-2* strains exhibiting decreased fluconazole MICs compared with the parent *v*-K strain. The fluconazole MIC was also measured by broth microdilution, and the MICs for the *v*-K and wt-K strains were found to be 8 and 2 μg ml^{-1}, respectively, in each of three replicate assays, demonstrating further that *VAD1* deletion results in fluconazole resistance. The fluconazole MICs of the *v-TUF1-1* and *v-TUF1-2* strains, as measured by broth microdilution, were equivalent to that of the wt-K strain, with each strain exhibiting a MIC value of 2 μg ml^{-1} in three replicate experiments. This is consistent with the ability of *TUF1* overexpression to suppress the fluconazole resistance of the *vad1*Δ mutant. Disparities in the MIC results between the two methods are likely to stem from differences in growth medium. However, both assays demonstrate that the *v-K* strain exhibits resistance to fluconazole relative to wt-K, and that overexpression of *TUF1* restores fluconazole sensitivity to the *vad1*Δ mutant. These data demonstrate that overexpression of *TUF1* in the *vad1*Δ mutant is able to suppress the fluconazole-resistant phenotype. Because the effect of *TUF1* overexpression in *vad1*Δ had the same consequences in both *v-TUF1-1* and *v-TUF1-2*, only *v-TUF1-1* was assayed in the following studies.

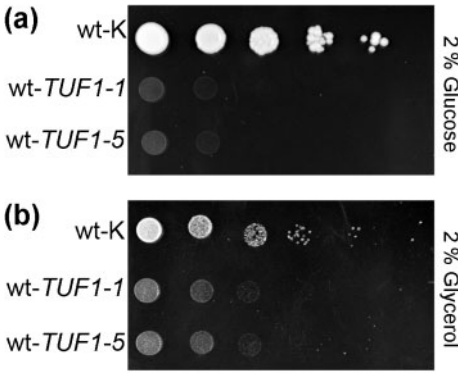

Fig. 2. Overexpression of *TUF1* in wild-type *C. neoformans* is growth-inhibitory. Growth of two independently derived *TUF1*-overexpression strains was compared with that of a wild-type strain harbouring the empty vector (wt-K) by spot-plate assay at 37 °C on minimal medium containing either glucose (a) or glycerol (b) as the carbon source.

TUF1 overexpression abrogates tetracycline-induced fluconazole resistance

Tetracycline is thought to affect mitochondrial function through inhibition of mitochondrial translation, similar to the expected consequence of *TUF1* downregulation (Zhang *et al.*, 2005). If *TUF1* overexpression restores fluconazole sensitivity through upregulation of mitochondrial translation, we would expect the *v-TUF1-1* strain to be insensitive to tetracycline-induced fluconazole resistance. To test this hypothesis, we determined fluconazole MICs by Etest analysis on wt-K, *v*-K and *v-TUF1-1* in the presence of increasing concentrations of tetracycline (Fig. 4). As previously reported for wild-type *C. neoformans*, tetracycline increased the fluconazole MIC of both wt-K and *v*-K (Oliver *et al.*, 2008). However, tetracycline had little effect on the fluconazole MIC of *v-TUF1-1*, even at 400 μg tetracycline ml^{-1}. This suggests that overexpression of *TUF1* renders mitochondrial translation less sensitive to inhibition by tetracycline. In addition, it confirms that the effect of tetracycline on fluconazole sensitivity is a result of its activity as an inhibitor of mitochondrial translation.

The mechanism by which mitochondrial translation can influence fluconazole sensitivity is unknown. Studies in *Candida albicans* demonstrate only a modest increase in fluconazole MIC in the presence of tetracycline relative to the drastic effect seen in *C. neoformans* (Oliver *et al.*, 2008). Likewise, sterol profiles of *Candida albicans* in the presence of tetracycline show only a modest difference from untreated controls. To determine whether sterol content was affected by tetracycline treatment, sterols were extracted from wt-K, *v*-K and *v-TUF1-1* grown in the presence and absence of 200 μg tetracycline ml^{-1}, and a sterol profile was obtained for each strain. As demonstrated

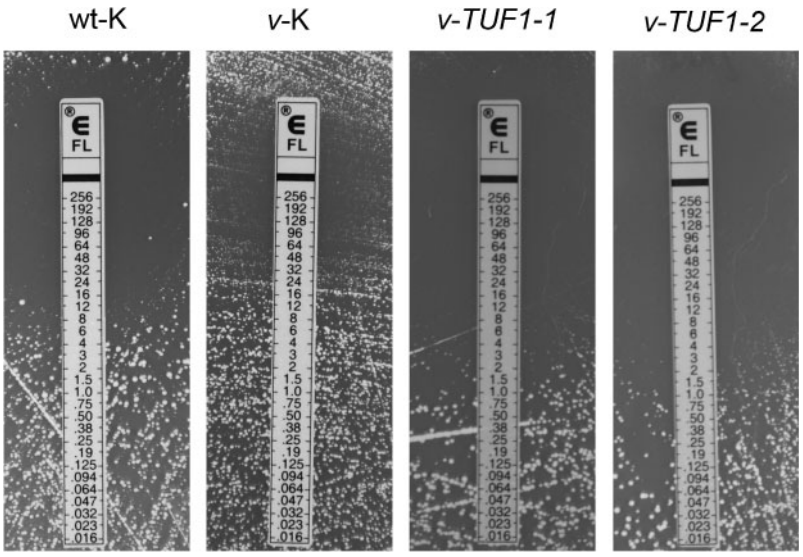

Fig. 3. Etest analysis demonstrating an increased fluconazole MIC in the *v*-K strain compared with the wild-type control (wt-K). Fluconazole sensitivity was restored by over-expression of *TUF1* (*v-TUF1-1* and *v-TUF1-2*).

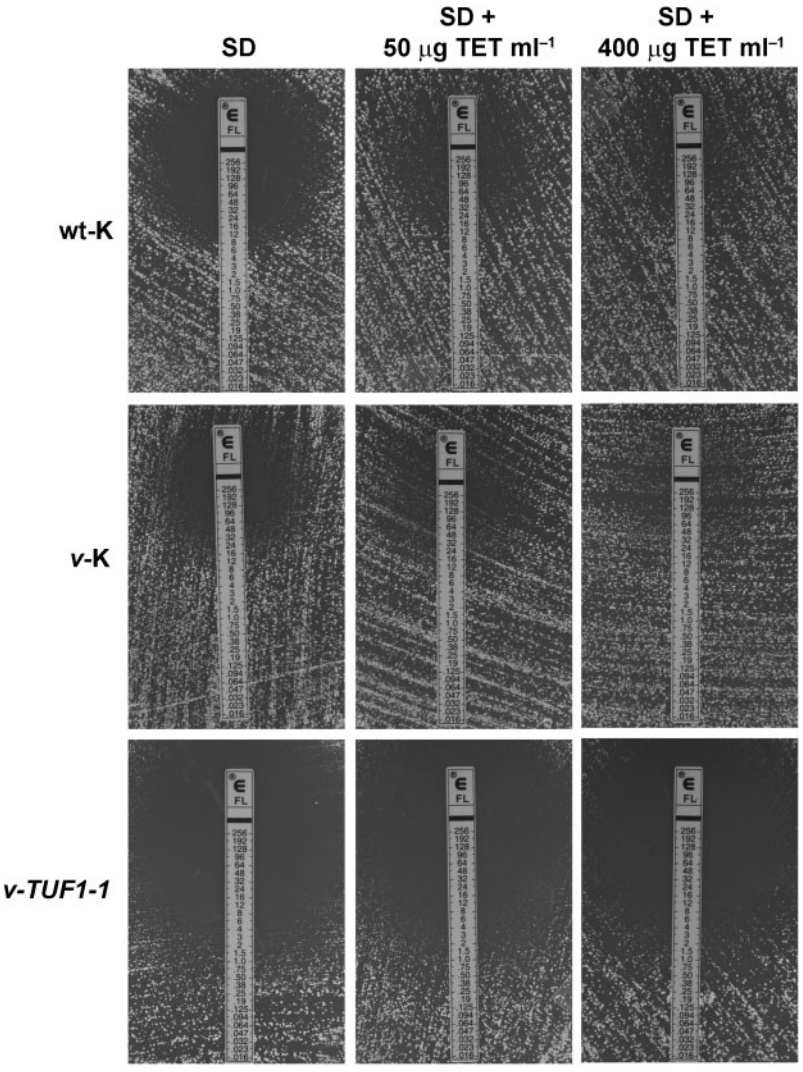

Fig. 4. Etest analysis showing fluconazole MICs in the presence of tetracycline (TET) at 50 and 400 μg ml^{-1} in wt-k, *v*-K and v-*TUF1-1* strains. Etests were performed on SD agar, with and without the indicated concentration of tetracycline, incubated at 30 °C for 3 days, and then photographed.

in Fig. 5, the height of the weight-corrected sterol profiles (±SEM) for each strain correlated with the fluconazole MIC, with the *v*-K strain exhibiting the highest-profile peaks and fluconazole resistance, and the *v-TUF1* strain the lowest-profile peaks and fluconazole sensitivity. *wt*-K was intermediate in profile peaks and fluconazole MIC. Treatment with 200 μg tetracycline ml^{-1} was found to increase peak height in all three strains (Fig. 5), with the effect being most significant in the wt-K strain. It should be noted, however, that the increase in peak height in the tetracycline-treated *v-TUF1-1* strain did not reach the height of the untreated wt-K profile, consistent with the lack of tetracycline-induced fluconazole resistance observed in the *v-TUF1-1* strain. The magnitude of the profiles in tetracycline-treated wt-K and *v*-K was similar, and this might suggest that these strains were approaching a maximum in sterol synthesis. In addition, we would expect the effect of tetracycline on the *v*-K strain to be less, as overall mitochondrial translation is likely to be reduced owing to *TUF1* repression, resulting in an increased sterol profile height in the absence of tetracycline. Taken together, these data suggest that *TUF1* repression, or inhibition of mitochondrial translation with tetracycline, confers fluconazole resistance, at least in part, through an increase in sterol content. Future studies will investigate the mechanism by which sterol content is upregulated in these strains. A possible explanation for the differences in tetracycline-induced fluconazole resistance between *C. neoformans* and *Candida albicans* may stem from fundamental metabolic differences between these two pathogenic fungi. Consistent with this interpretation is that *TUF1* appears to be an essential gene, since our previously described attempts to generate a deletion mutant were unsuccessful (Panepinto *et al.*, 2005). In contrast, the *S. cerevisiae TUF1* orthologue is not essential, with the deletion mutant exhibiting respiratory growth defects (Chiron *et al.*, 2005). The pronounced effects of tetracycline on *C. neoformans* fluconazole susceptibility observed previously, and the essential nature of *TUF1* in *C. neoformans*, are consistent with the minimal fermentative capacity of *C. neoformans* compared with the ascomycetous yeast. The reduced capacity of *C. neoformans* to ferment is likely to result in strong reliance on mitochondrial function, whereas *Candida albicans* and *S. cerevisiae* favour fermentative pathways to produce ATP.

The *vad1*Δ mutant hyperactivates Sre1 in response to fluconazole

The response of *C. neoformans* to azole antifungal drugs includes activation of Sre1 (Chang *et al.*, 2007; Chun *et al.*, 2007). Sre1 responds to the accumulation of 4-methyl sterols that occurs during antifungal drug treatment and hypoxia, and deletion of *SRE1* confers profound fluconazole sensitivity (Chang *et al.*, 2007; Hughes *et al.*, 2007). Microarray experiments have demonstrated that the hypoxic response in *C. neoformans* involves downregulation of *TUF1*, similar to that seen in the *vad1*Δ mutant (Chun *et al.*, 2007). These similarities in gene expression, coupled with fluconazole resistance and increased sterol content, led us to hypothesize that the *vad1*Δ mutant would hyperactivate Sre1 in response to fluconazole treatment. As demonstrated in Fig. 6(a), the *vad1*Δ mutant exhibited increased activation of Sre1 after fluconazole treatment for 2 h, whereas wild-type Sre1 activation was barely visible after drug treatment for 2 h. However, overexpression of *TUF1* in the *vad1*Δ mutant had no effect on the hyperactivation of Sre1 (Fig. 6b), suggesting that

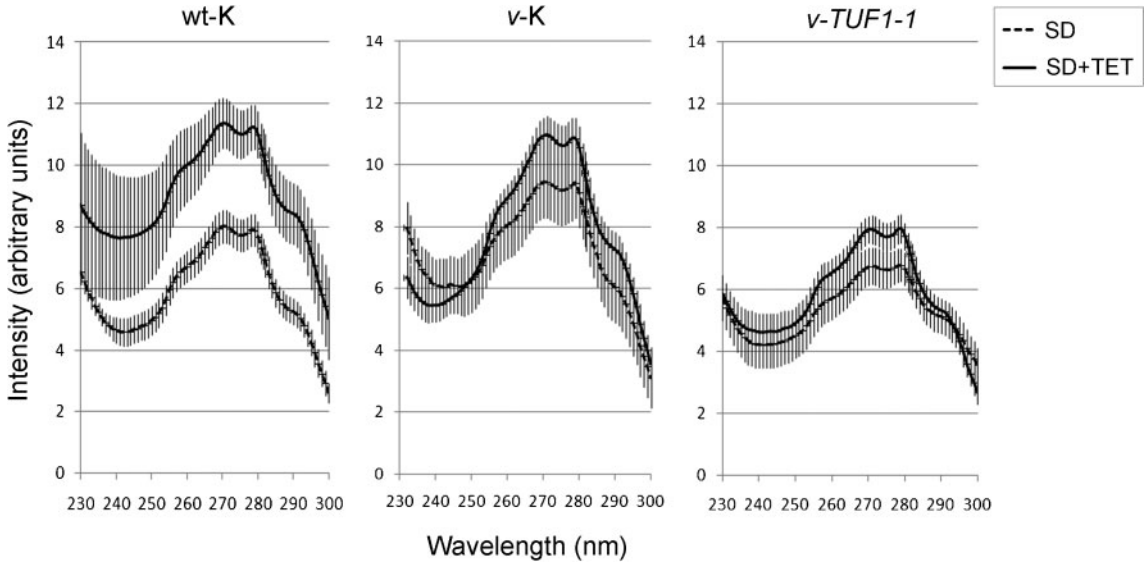

Fig. 5. (a) UV scans of heptane-extracted sterols from cultures grown in the presence and absence of tetracycline. Data shown are representative of three independent assays; vertical bars, SEM. TET, tetracycline.

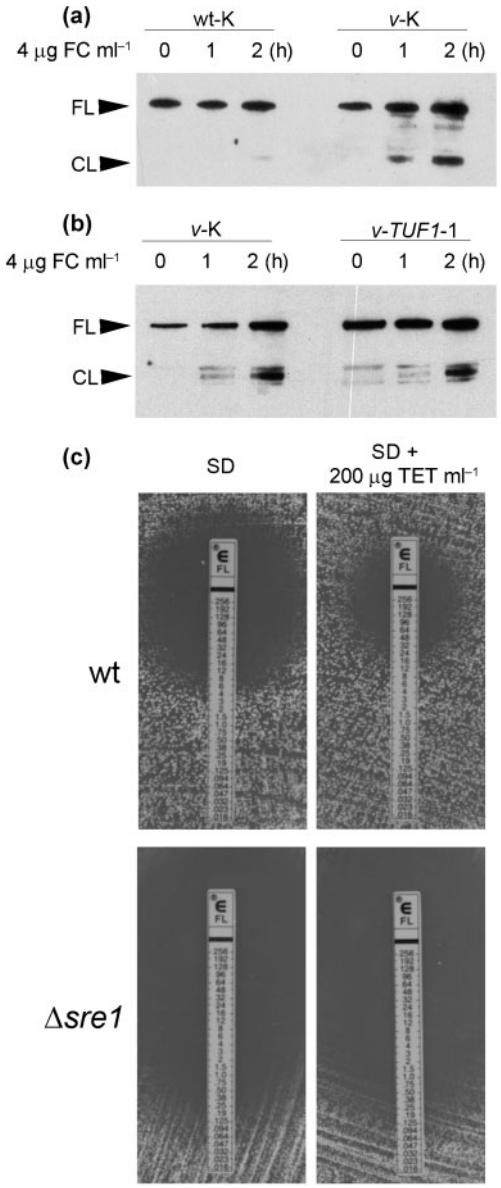

Fig. 6. Western blot analysis of Sre1 activation in response to fluconazole treatment. (a) Time course of Sre1 activation in the presence of 4 μg ml^{-1} fluconazole (FC) comparing the wt-K and *v*-K strains. Activation of Sre1 is indicated by the appearance of the cleavage fragment (CL) in addition to the inactive, full-length Sre1 band (FL). (b) Time course of Sre1 activation in the presence of 4 μg fluconazole ml^{-1}, comparing the *v*-K and *TUF1*-K strains. (c) Etests comparing the fluconazole sensitivity of wild-type strain H99 (wt) with that of a *sre1*Δ mutant in the presence and absence of 200 μg ml^{-1} tetracycline (TET).

Sre1 regulation is upstream of *TUF1* in *C. neoformans*, or that it functions in an independent pathway. This is also supported by the inability of tetracycline to increase resistance to fluconazole in the *sre1*Δ mutant (Fig. 6c). Taken together, these data support a model in which *TUF1* downregulation in a *vad1*Δ mutant may be a direct result of Sre1 dysregulation. Future studies will test this model, and investigate a regulatory role of Vad1 in Sre1 activation.

Overexpression of *TUF1* in the *vad1*Δ mutant does not restore virulence

Our previous studies have demonstrated that severe virulence attenuation accompanies deletion of *VAD1* (Panepinto *et al.*, 2005). To determine the contribution of *TUF1* downregulation to *vad1*Δ virulence attenuation, the ability of a *TUF1*-expressing *vad1*Δ strain to cause mortality in a murine model of cryptococcosis was compared with that of the wild-type and *vad1*Δ mutant derivatives bearing an empty vector. Cells (1×10^6) of each strain were injected into the lateral tail vein of Swiss albino mice, and mortality was monitored for 7 weeks. All of the wild-type mice succumbed to infection in the first 2 weeks, whereas none of the mice infected with the *vad1*Δ mutant succumbed to the infection. One mouse infected with the *TUF1*-expressing strain succumbed to infection on day 21, with the remaining nine mice surviving to 7 weeks, at which time the experiment was terminated (data not shown). This suggests that restoration of *TUF1* expression in the *vad1*Δ mutant is not sufficient to restore virulence, and that the virulence defects of the *vad1*Δ mutant are most likely due to effects on multiple genes, as Vad1 is a global regulator.

Because *VAD1* was initially discovered in a screen for *C. neoformans* mutants defective in melanization, we assayed the effect of *TUF1* overexpression on the melanin deficiency of the *vad1*Δ mutant. Overexpression of *TUF1* did not restore wild-type melanization to the *vad1*Δ mutant (data not shown), consistent with the inability of *TUF1* overexpression to restore virulence traits to the *vad1*Δ mutant.

In this study, we demonstrated that overexpression of *TUF1*, encoding the mitochondrial translation elongation factor, was able to suppress several aspects of the *vad1*Δ phenotype related to mitochondrial function. These aspects included rescuing growth on respiration-dependent carbon sources and restoring fluconazole sensitivity. This study supports a role for mitochondrial protein synthesis in the regulation of fluconazole sensitivity, demonstrating that inhibition of mitochondrial protein synthesis by tetracycline confers resistance to fluconazole in *C. neoformans* and increases overall sterol content. These results not only provide insight into possible mechanisms of drug resistance in *C. neoformans*, but also highlight potential interactions of antimicrobial agents in patients undergoing treatment for polymicrobial infections. This study raises the possibility that antibacterial agents that target protein synthesis antagonize the activity of azole antifungal drugs. Future studies using animal models of cryptococcal infection will address this question *in vivo*.

The study also points to the unique reliance of *C. neoformans* on mitochondrial respiration. Perturbation of

mitochondrial function by *TUF1* repression, as in the *vad1*Δ mutant, affects carbon metabolism, sterol metabolism and susceptibility to fluconazole. Similar effects on sterol metabolism and fluconazole sensitivity were recapitulated by treatment with tetracycline. In addition, overexpression of *TUF1* in wild-type *C. neoformans* appears to impair glucose utilization, favouring the use of respiration-dependent glycerol under the conditions tested herein. Further studies of mitochondrial function in this important pathogen could identify targets for antifungal therapy that may confer fluconazole sensitivity by activating, rather than impairing, aspects of mitochondrial function.

Although overexpression of *TUF1* was able to act as a phenotypic suppressor of *vad1*Δ mitochondrial defects, it was unable to restore virulence to the *vad1*Δ mutant. Therefore, the regulatory defects underlying the complex phenotype of the *vad1*Δ mutant likely arise from modulation of multiple targets, as Vad1 is a regulator of many genes and pathways. The mechanism by which *TUF1* is downregulated in the *vad1*Δ mutant is unclear, and a possibility that *TUF1* repression is secondary to defects in another regulator, such as Sre1, remains. Further studies delineating the role of Vad1 as a regulator of mRNA fate will determine the specific target transcripts regulated by Vad1, and lead to a mechanistic understanding of the *vad1*Δ phenotype.

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