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ABSTRACT

Drug resistance and cellular adhesion are two key elements of the worldwide dissemination and prevalence of the human fungal pathogen *Candida albicans*. Smi1 belongs to a family of hub proteins conserved among the fungal kingdom, whose functions in cellular signaling affect morphogenesis, cell wall synthesis and stress resistance. The data presented here indicate that *C. albicans SMI1* is a functional homolog of *Saccharomyces cerevisiae KNR4* and is involved in the regulation of the fungal cell wall synthesis. Expression of *SMI1* in the *S. cerevisiae knr4*Δ null mutant rescued its sensitivity to Caspofungin and to heat stress. Deletion of *SMI1* in *C. albicans* resulted in sensitivity to the cell-wall-perturbing compounds Calcofluor White and Caspofungin. Analysis of wild-type and mutant cells by Atomic Force Microscopy showed that the Young's Modulus (stiffness) of the cell wall was significantly reduced upon deletion of *SMI1*, while cell surface adhesion measured by Single Force Spectroscopy showed that the surface expression of adhesive molecules was also reduced in the mutant. Over-expression of *SMI1 gene*, on the contrary, drastically increased the cell surface adhesion phenotype *vs* the control strain. Finally, Smi1 cellular localization as cytoplasmic patches and concentrated spots at the sites of new cell wall synthesis is consistent with its role in the control of cell wall synthesis and appears conserved with the ones of its counterparts in *S. cerevisiae* and *Neurospora crass*a respectively for yeast cells and hyphae of *C. albicans.*

INTRODUCTION

Fungal infections are responsible of the death of about 1.5 million people per year worldwide. Hemiascomycetous yeasts of the genus *Candida* are the second most numerous agents of fungal infections worldwide, with a prominent contribution by *Candida albicans*, which causes over 400,000 cases of life-threatening systemic infections and 200,000 deaths per year (Brown et al., 2012). Only four classes of antifungal (AF) drugs are available for patient treatment and the emergence of resistance is becoming a serious concern. The fungistatic group of Azoles and the more recently-developed fungicidal group of Echinocandins constitute the two major classes of AF used to treat patients. Azoles block the biosynthesis of ergosterol - an essential sterol for fungal cell membranes - by targeting the cytochrome P450 14-α demethylase enzyme, Erg11, which catalyzes the conversion of lanosterol to ergosterol, thereby affecting membrane integrity and inhibiting fungal growth (Kathiravan et al., 2012; Vanden Bossche et al., 1995). On the other hand, Echinocandins, a class of compounds developed between 2001 and 2006, target the catalytic sub-unit of the β-1,3 glucan synthase protein complex, Fks1 (Odds et al., 2003). Studies of the molecular mechanisms of resistance to these two classes of AF compounds (recently reviewed in (Scorzoni et al., 2017)) have revealed that there are three major mechanisms leading to resistance: overexpression of multidrug efflux pump-encoding genes, notably CDR1, CDR2 and MDR1 (Sanglard and Odds, 2002; Sanglard et al., 1995), point mutations in the target proteins (ex: Erg11, Fks1) and alteration in the expression of proteins involved in sensitivity to the drug (ex: Erg3, Erg11). In addition, the formation of fungal biofilms can also be considered as a form of antifungal resistance due to the ability of the biofilm extracellular matrix (ECM) to bind and entrap antifungal compounds, particularly Azoles and Amphotericin B (Desai et al., 2014; Taff et al., 2013; Vediyappan et al., 2010; Zarnowski et al., 2014).

In this context, researchers have sought alternative antifungal targets and/or ways to improve the fungicidal effect of existing AFs. Such approaches notably involve targeting chaperones such as Hsp90 or components of stress signaling pathways, since these targets are more likely to simultaneously affect resistance to different classes of AF, morphogenesis mechanisms, cellular fitness and adaptation to changing environments. Key studies have been conducted in these areas by, for example, Brown and colleagues (Brown et al., 2010) and

Cowen and coworkers (Singh et al., 2009). Works of this latter group established the complex connections between Pkc1, Hsp90 and calcineurin suggesting interesting new strategies to treat fungal infections (LaFayette et al., 2010). However, these cellular targets suffer from a major drawback in that they are conserved in mammalian host cells, which make achieving fungal specificity a real challenge. Factors that regulate the pathogen's cell wall therefore remain a strong target for new, fungus-specific, therapeutic approaches.

Here we describe the role of Smi1, a *C. albicans* protein homologous to the *Saccharomyces cerevisiae* hub protein Knr4 which interacts physically with both the Slt2 MAP kinase and calcineurin, thus connecting the two primary signaling pathways involved in cell wall maintenance during stress: the cell wall integrity pathway (CWI) and the calcineurin pathway (Dagkessamanskaia et al., 2010; Martin-Yken et al., 2016). Knr4 is required for resistance to cell wall stress induced by elevated temperature or the presence of antifungal compounds, including Caspofungin (Lesage et al., 2004; Markovich et al., 2004). Knr4 also plays a role in filamentous and pseudohyphal growth, mucin secretion and agar invasion (Birkaya et al., 2009). Similarly, GS1 protein, the homolog of Knr4 and Smi1 in the model filamentous fungi *Neurospora crassa,* is also involved in the control of morphogenesis, Caspofungin sensitivity and new cell wall synthesis notably β-glucans (Enderlin and Selitrennikoff, 1994; Resheat-Eini et al., 2008; Seiler and Plamann, 2003).

The *Candida albicans* genome encodes 2 homologs of *KNR4: SMI1 and SMI1B*. Previous studies have shown that deletion of *SMI1* affects cell wall β-glucan synthesis, biofilm formation and biofilm extracellular matrix production, as well as biofilm-associated resistance to Fluconazole (Nett et al., 2011). Global transcriptomic studies indicate that *SMI1* expression is induced in hyphal and planktonic cells by Cyr1 and is also biofilm-induced, while it is repressed by the Hap43 regulatory protein and Caspofungin (Liu et al., 2005). Much less is known about *SMI1B*, although it appears to be the closest homolog of *S. cerevisiae KNR4* according to phylogeny.

In this paper we further characterize the function of *SMI1* in *C. albicans*. We provide evidence that Smi1 is a functional homolog of *S. cerevisiae* protein Knr4, that its accurate expression is critical for the regulation of fungal cell wall integrity and biophysical properties, and that the cellular localization of Smi1-GFP in yeast and hyphal cells is consistent with the ones observed for its counterparts, *i.e.* Knr4 in *S. cerevisiae* yeasts and GS1 in the hyphae of *N. crassa.*

Material and Methods

Strains and growth media

The *C. albicans* and *S. cerevisae* strains used in this study are listed in Table 1.

Depending on experimental conditions, yeast strains were grown in YPD (1 % (W/V) yeast extract, 2 % (W/V) peptone, and 1 % (W/V) dextrose), YP (1 % (W/V) yeast extract, 2 % (W/V) peptone) supplemented with 10 % Fetal Bovine Serum (FBS), or SD (synthetic dextrose, 0.67 % (W/V) yeast nitrogen base (YNB; Difco) with 2 % glucose) supplemented as necessary with arginine, histidine or uridine (20 mg/l). Agar (2 %) was used for growth on solid medium. *Escherichia coli* strains TOP10 (Invitrogen) or DH5α were used for DNA cloning and maintenance of the plasmid constructs.

Plasmid construction and generation of epitope-tagged or mutant strains:

C. albicans SM1 gene was PCR amplified with primers: Sense SMI1 New start and Antisense SMI1 (sequences in Supplementary Material Table S1). The PCR product was then cloned in the *S. cerevisae* expression vector YEplac195 *PGK/CYC1* between *PGK* promotor P_{PGK1} *and CYC1* terminator sequences (personal gift of Dr J.L. Parrou, based on YEplac195 (Gietz and Sugino, 1988)), thus yealding p*SM1* plasmid. Plasmid p*KNR4* expressing the *S. cerevisae KNR4* gene with its own promotor and terminator on a multicopy vector has been described previously (Martin et al., 1999).

S. cerevisiae cells were transformed according to the lithium acetate method (Gietz and Woods, 2006). *C. albicans* cells were transformed using the lithium-acetate protocol of Walther and Wendland (Walther and Wendland, 2003), followed by selection of transformants for uridine, arginine or histidine prototrophy when using the *URA3, ARG4* or the *HIS1* markers, respectively.

Construction of *C. albicans smi1* Δ/Δ and *smi1B* Δ/Δ knock-out mutants (Table 1) used PCR-generated *ARG4* and *HIS1* disruption cassettes flanked by 120 base pairs of target homology region (primer sequences are provided in supplementary material S1) as described by (Gola et al., 2003) and (Schaub et al., 2006). Independent transformants were produced and the gene replacements were verified by PCR on whole yeast cells as described previously (Gola et al., 2003; Schaub et al., 2006).

The *SMI1* (C1_07870C_A) gene was amplified using primers: SMI1 Forward and SMI1 Reverse (sequences provided in supplementary material Table S1). The resulting 1.6Kb PCR product was purified and inserted in the GTW sequences of pEntry (Gateway system™,

Invitrogen). Construction of the Smi1 over-expressing strain was performed by recombination of pEntry-SMI1 plasmid and pCIp10-P_{TDH3}-GTW plasmid (Chauvel et al., 2012) also using the Gateway system™ (Invitrogen), followed by genome integration through targeted homologous recombination at the genomic *RPS10* locus in the host strain BWP17.

GFP tagging of Smi1

Smi1 was C-terminally-tagged with GFP by amplifying GFP-NAT cassette from the pGFP-NAT1 plasmid (Milne et al., 2011) using primers gSmi1 _F[a] and gSmi1_R[a] containing 100 bp of flanking homology to the *SMI1* terminator and C-terminal of the *SMI1* ORF (C1_07870C_A), respectively (Figure X –primer table). Transformants were selected on YPD agar containing 300 µg/ml nourseothricin (Sigma). Integration of the GFP-NAT cassette was confirmed by PCR using primers sSmi1_F and sFP_R, which anneal to the chromosome outside the targeted region and within the cassette, respectively (primer sequences are provided in Supplementary material Table S1).

Microscopy of Smi1-GFP

Yeast were grown for 2 h at 30 °C in YNB medium containing amino acids and $(NH_4)_2SO_4$ (Sigma Aldrich). Morphogenesis was induced at 37 °C for 3 h in 20 % FCS, 2 % glucose. Cells were imaged on µ-slides (Ibidi, Martinsried, Germany). Images of Smi1-GFP localization were captured on an UltraVIEW® VoX spinning disk confocal microscope (Nikon, Surrey, UK), using a 488 nm laser. Multiple Z-stack images were acquired and Z-stack projections at maximum intensity were created using Volocity 6.3 software (Perkin Elmer).

Phenotypic Sensitivity tests:

Drop tests to evaluate the sensitivity of different strains and mutants to cell wall affecting drugs were performed as previously described (Ram et al., 1998) with minor modifications (Martin et al., 1999). Briefly, yeast cells were grown in liquid YPD to OD_{600} of 1+/- 0.1, then concentrated by centrifugation and resuspended in sterile water to an $OD_{600\ of}$ 8. Serial dilutions of 1/1, 1/10, 1/100 and 1/1000 were then spotted on solid media containing either calcofluor white or Caspofungin at the indicated concentrations. Growth was scored and photographs taken after 48h of incubation at 30 °C, or at 37 °C for testing the sensitivity to elevated temperature.

Atomic Force Microscopy:

Sample preparation for AFM experiments.

Yeast cells were concentrated by centrifugation, washed two times in acetate buffer (18 mM CH3COONa, 1mMCaCl2, 1mMMnCl2, pH5.2), resuspended in the same buffer, and immobilized on polydimethylsiloxane (PDMS) stamps prepared as described by Dague et al. (Dague et al., 2011; Formosa et al., 2014a). Briefly, freshly oxygen-activated microstructured PDMS stamps were covered with a total of 100 µl of cell suspension and allowed to stand for 15 min at room temperature. Yeast cells were then deposited into the stamps microstructures by convective (capillary) assembly.

Force curves were then recorded in acetate buffer in quantitative-imaging mode (JPK Instruments, 2011, QITM mode-quantitative imaging with Nano-Wizard 3 AFM),(Chopinet et al., 2013; Formosa et al., 2014b; Smolyakov et al., 2016) with MLCT AUWH cantilevers (nominal spring constants: 0.01, 0.1, and 0.5 N/m). For imaging, cantilevers with a spring constant of 0.01 N/m were used. For force spectroscopy experiments, cantilevers with spring constants of 0.1 and 0.5 N/m were used. The applied force was kept at 1 nN for both imaging and force spectroscopy. For imaging and force spectroscopy, we used an AFM Nanowizard III (JPK Instruments, Berlin, Germany). The spring constant of each cantilever was determined by the thermal-noise method (Hutter and Bechhoefer, 1993).

For elasticity measurements, force maps of 32 by 32 or 64 by 64, hence either 1024 or 4096 force curves were recorded on a 1 µm x 1 µm square area on top of the cells. The force-distance curves recorded were transformed into force-indentation curves by subtracting the cantilever deflection on a solid surface. The indentation curves were then fitted to the Hertz model (Hertz, H., 1881).

Fluorescence Microscopy:

Yeast were grown for 2 h at 30 °C in YNB medium containing amino acids and (NH4)2SO4 (Sigma Aldrich). Morphogenesis was induced at 37 °C for 3 h in 20 % FCS, 2 % glucose. Cells were imaged on µ-slides (Ibidi, Martinsried, Germany). Images of Smi1-GFP localization were captured on an UltraVIEW® VoX spinning disk confocal microscope (Nikon, Surrey, UK), using a 488 nm laser. Multiple Z-stack images were acquired and Z-stack projections at maximum intensity were created using Volocity 6.3 software (Perkin Elmer).

Results:

1. Conservation of cellular function between* Saccharomyces cerevisiae *Knr4 and* Candida albicans *Smi1:

The genome of the human pathogen *Candida albicans* contains two distinct homologs of the *Saccharomyces cerevisiae KNR4* gene, *SMI1* (C1_07870C_A) and *SMI1B* (C3_05350C_A). Gene deletion mutants for each gene were generated and initial phenotypic analysis showed a strong phenotype (sensitivity to CFW or SDS) for the *smi1*Δ/Δ mutant but not for the *smi1B* Δ/Δ mutant (Figure S1). We therefore focused on the role of Smi1 in this study.

The *SMI1* open reading frame was amplified and cloned into a *S. cerevisiae* expression vector, under the control of the strong and constitutive *ADH1* promoter. The *SMI1* coding sequence is 1,863 bp and contains two GTC codons at positions 1717 and 1762, which have a 97 % chance of translation as a Serine in *C. albicans* through non-canonical codon usage in this fungus, compared to a Leucine in *S. cerevisiae* (White et al., 1995), although these usage frequencies appear to be sensitive to environmental stressors (Simões et al., 2016). These two codons are located within the C-terminal of Smi1, which is less conserved than the central domains among members of the fungal Knr4/Smi1 super-family of proteins. The complete sequence alignment of several fungal protein members of this super-family is shown in Figure S2. The C-terminal part of Knr4 is largely unstructured and not directly necessary for protein function in *S. cerevisiae* (Dagkessamanskaia et al., 2010; Martin-Yken et al., 2016). Hence, we tested the ability of the *C. albicans SMI1* gene, retaining its two ambiguous codons, to complement the cell wall-related phenotypes of *S. cerevisiae knr4* null mutants. Our phenotypic screen included sensitivity to Calcofluor White (CFW), SDS, caspofungin and elevated temperature in haploid and diploid *S. cerevisiae* genetic backgrounds. CFW is a compound that binds to nascent chitin fibrils and has been used since 1994 to identify fungal cell wall mutants (Ram et al., 1994). Expression of *SMI1* gene in *S. cerevisiae* was able to complement these phenotypes (Figure 1A) and also complemented the *smi1*Δ growth defect at elevated temperature (Fig.1B), a specific defect in this organism that is linked to cell cycle progression through START (Fishel et al., 1993, Martin-Yken et al., 2003). Hence, the function of the Smi1/Knr4 proteins appears to be conserved between the two species despite their phylogenetic distance and the differences between the two protein sequences, which share only 34% identity and 49% similarity (Figure S1).

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280 ***2. Deletion of* SMI1 *renders C. albicans sensitive to cell wall targeting drugs:***

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282 We further investigated the role of Smi1 in maintaining cell wall integrity using a
283 medically relevant β-glucan synthase-targeting drug, the echinocandin Caspofungin. The
284 influence of Smi1 on the sensitivity to CFW was also retested in parallel. Homozygous
285 deletions of the two alleles of *SMI1* gene in the *C. albicans* BWP17 genetic background lead
286 to a marked increase in the sensitivity towards both Caspofungin and CFW at 30 °C (Figure
287 2). These phenotypes are consistent with the ones observed for the *S. cerevisiae knr4*Δ mutant
288 as well as with the role proposed for these proteins in stress signaling pathways (See
289 discussion). Re-integration of *SMI1* open reading frame under the constitutive promoter P_{TDH3}
290 in the *smi1*Δ/Δ deletion mutant restored the wild-type phenotype, while integration of the
291 empty *URA3*-bearing vector did not (Fig. 2).

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294 **3. Cell Wall Biophysics**

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296 **3.1 Cell Wall Strength / Elasticity**

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298 Atomic Force Microscopy under liquid conditions can be used to investigate the
299 nanomechnical properties of live wildtype and mutant cells (Dague et al., 2010; Ene et al.,
300 2015; Formosa et al., 2013; Liu et al., 2015). Here we first measured the cell surface
301 resistance *vs* the elasticity of three *C. albicans* strains: the control strain, BWP17 AHU (which
302 carries the functional *URA3* marker), the homozygous deletion mutant *smi1*Δ/Δ AHU and a
303 strain over-expressing the *SMI1* gene from the strong constitutive promoter P_{TDH3} in the same
304 BWP17 genetic background, SMI1-OE. Using the Atomic Force Microscope in the Force
305 Volume mode, we collected between 1024 and 4160 force curves per cell on a minimum of
306 12 cells from 4 independent cultures for each strain. The elasticity of the cells was quantified
307 from these curves by calculating the Young's Modulus (YM) as previously described (Dague
308 et al., 2010), with YM=1 / Elasticity. The higher the Young's Modulus, the stiffer the cells
309 are. As can be seen on Figure 3, the Young's Moduli measured for the control strain BWP17
310 AHU showed a mean value of 782 +/-158 kPa, significantly higher than the ones measured
311 for the homozygous deletion mutant which had a mean value of 93 +/-34 kPa. The Young's

Moduli measured on the cells of the over-expression strain lay between the control strain and the deletion mutant, with a mean of 298 +/- 62 kPa, suggesting either a gene dosage effect for *SMI1* expression or possibly an effect of uncoupling the expression of this gene from the cell cycle. These values are represented for each cell individually on Figure 3 since to allow visualization of the cell-to-cell variability. The mean values ± SEM of the Young Moduli obtained for these three strains are also reported in Table 2. These measurements show that the deletion of both alleles of *SMI1* resulted in a reduction of the cell surface stiffness by eight to ten fold, indicating a very soft cell wall for this mutant.

3.2 Adhesion:

Another cell surface feature that can be easily and very precisely measured by AFM is the ability to adhere to surfaces, using Single Molecule Force Spectroscopy (Axner et al., 2010; Benoit et al., 2000; Formosa et al., 2014a; Hinterdorfer et al., 1996; Neuman and Nagy, 2008). Here, adhesion between the cell surface and the AFM bare tips, constituted of Si_3N_4 was measured by scanning square areas on the top of yeast cells. We recorded force curves whose retract parts were then used to generate adhesion maps where the intensity of each pixel corresponds to the force required to dissociate the AFM tip from the sample, *i.e.* the adhesion force, expressed in picoNewtons pN (see Figure 4). It can be seen immediately from these heat maps that the *smi1*Δ/Δ mutant cell surface is almost not adhesive compared to the control strain, while the *SMI1* overexpressing cells on the other hand display a marked increase in their surface adhesion. In addition to the visual representation by these adhesion maps, the cellular adhesion was also evaluated by three quantitative parameters: the mean adhesion force and specific energy of each adhesion event observed and the overall proportion of these adhesive events among all the recorded force curves. In order to quantify these values we defined as an adhesive event any force curve showing an adhesion force above 50pN. With this threshold level we calculated the percentage of adhesive events for each cell type and measured the area situated below the retract curves, which represent the adhesion energy of the event (Table 2). These values indicated that adhesive events were encountered more frequently at the surface of the over-expressing strain SMI1-OE (63% of the recorded 21,500 force curves) than on the control strain (46% of over 13,300 force curves), and were rare on the deletion mutant surface (19% of over 12,300 force curves). The average adhesion force measured on the control strain was of 127 +/- 52 pN, calculated on 6256 adhesive force curves. This has to be compared with an average force of 70 +/-16 pN

(hence barely above the threshold) on 2300 force curves for the *smi1*Δ/Δ mutant. For the *SMI1* OE-strain on the other hand, not only were the adhesive events more frequent (63 %) but they were also much stronger, with forces measured up to 2176 pN and an average value of 712 +/- 260 pN calculated on 12,257 adhesive force curves. The specific energy of these adhesive events also differed; with adhesion energies measured for the over-expressing strain an order of magnitude stronger that for the control strain, while they were about seven times lower on the surface of the deletion mutant. Hence, the homozygous deletion of *SMI1* gene abrogates almost entirely the ability of the mutant cell to adhere using the chemistry described here, while *SMI1* over-expression (or constitutive expression) leads to a highly adhesive phenotype.

4. Cellular Localization:

The homolog of Smi1 and Knr4 in the model filamentous fungus *Neurospora crassa*, GS-1 localizes at the growing tip of hyphae as a sphere positioned at the "Spitzenkörper" (Verdin et al., 2009). The Spitzenkörper (or apical body) is a fungal structure specific to true hyphae, located at the hyphal tip. It is comprised of the secretory vesicles that are required for continuous polarized growth (Girbardt, 1957; Harris et al., 2005). GS-1-GFP and Knr4-GFP have been imaged respectively at the tip of *N. crassa* hyphae (Riquelme et al., 2011; Sánchez-León et al., 2011) and at the tip of elongated shmoos in *S. cerevisiae* ((Martin-Yken, 2012) and our own unpublished data). To test whether the *C. albicans* homolog would be similarly positioned, a GFP-tag was integrated at the C-terminus of the Smi1 protein at its chromosomal locus and its cellular localization was visualized by confocal fluorescent microscopy in yeast and hyphal cells.

In yeast cells, Smi1-GFP appeared both as punctate patches in the cytoplasm and localized transiently to nascent buds. This localization is similar to that reported for Knr4 (Dagkessamanskaia et al., 2010; Martin et al., 1999). Punctate patches and a more diffuse cytoplasmic distribution were observed in *C. albicans* hyphae, but, unlike in yeast, Smi1-GFP was consistently retained at the growing hyphal tip throughout the cell cycle. This is reminiscent of *N. crassa* GS-1 localization (see above and discussion). Smi1-GFP was occasionally observed at hyphal septa as a dim signal, but this presence did not reflect specific stages of the cell cycle. Hence, Smi1 in *C. albicans* appears to associate with intracellular

organelles and localizes to sites of new cell wall growth, a pattern which partially reflects those observed for homologs of Smi1 in *S. cerevisae* in yeast cells and the hyphae of *N. crassa*.

DISCUSSION

Our results establish a significant conservation of Knr4/Smi1 function between *S. cerevisiae* and *C. albicans* and demonstrate the role of Smi1 in resistance to Caspofungin, regulation of cell wall integrity and cell surface adhesion properties of this major human fungal pathogen. These features suggest that Smi1 might be relevant as a new drug target for combination therapies. Previous work by Nett and colleagues identified a role for Smi1 in the production of extracellular matrix upon biofilm formation and hence the associated resistance to Fluconazole (Nett et al., 2011). Their results indicated that these effects were linked to the cell wall integrity pathway but were in fact regulated by Smi1 independently of the CWI pathway, suggesting a control pathway for Smi1 distinct from that of the PKC pathway. Lafayette and colleagues dissected the mechanisms through which PKC regulates resistance to both azoles and echinocandins in the two yeast models *C. albicans* and *S. cerevisiae* (LaFayette et al., 2010). They showed that, in *C. albicans*, Pkc1 and calcineurin signaling pathways independently regulate antifungal resistance *via* a common unknown target, which they designed as "X" (see (LaFayette et al., 2010), Fig 9B thereof). Considering the knowledge accumulated on Knr4 in budding yeast together with the data obtained for *C. albicans* and presented here, we propose that Smi1 might constitute this previously unidentified "X", a common target of the Pkc1 and calcineurin signaling pathways. In addition, another result of this work is the very low stiffness of the *smi1*Δ/Δ mutant cell wall indicated by its Young Modulus (Fig. 3, Table 2). The severity of this phenotype compared to the milder one observed for *S. cerevisiae knr4*Δ mutant suggests a more important (central) role of Smi1 in the network of signaling pathways controlling cell wall integrity than that of Knr4 in the baker yeast, which is in agreement with these two proteins being the hypothetical "X" in the work of LaFayette and colleagues (LaFayette et al., 2010). The very soft cell wall phenotype observed for the deletion mutant *smi1*Δ/Δ is similar to, although more severe, than the one observed for the *S. cerevisiae knr4*Δ mutant whose Young's Modulus in stationary phase was reduced three-fold compared to the control strain (Dague et al., 2010). This could

result from the reduced level of β-glucan in the cell wall of this mutant previously reported (Nett et al., 2011). The fact that the homozygous deletion mutant *smi1*Δ/Δ is so strongly affected, despite the presence in this strain of both functional alleles of *SMIB*, argues for a major role for the *SMI1* gene, at least in the conditions tested (30°C, liquid rich medium, yeast form of *C. albicans* cells).

Our results show that homozygous deletion of *SMI1* gene leads to a marked increase in the sensitivity of *C. albicans* to both CFW and Caspofungin at 30 °C. Since Caspofungin tolerance has been reported in *C. albicans* mutants with elevated cell-wall chitin (Perlin, 2015; Plaine et al., 2008; Walker et al., 2013; Yang et al., 2017) while elevated chitin is known to induce hypersensitivity to CFW (Elorza et al., 1983; Roncero and Durán, 1985), this finding may appear puzzling at first glance. However, this is consistent with the phenotypes observed for the *S. cerevisiae knr4*Δ mutant (Lesage et al., 2004; Martin et al., 1999). Moreover, as our cell wall stiffness measurements, these phenotypes indicate a severely modified cell wall organization of the deletion mutant *smi1*Δ/Δ compared to the control strain, coherent with a possible upstream role of Smi1 in cellular signaling pathways allowing this protein to influence simultaneously the expression level of different cell wall synthesis genes. Consistent with this, early results obtained in the bakers' yeast established a role for Knr4 in the transcriptional control of all *S. cerevisiae* chitin synthase genes (Martin et al., 1999). The complex interplay of cellular signaling pathways controlling *C. albicans* susceptibility to echinocandins has been described by Munro and colleagues (Walker et al., 2010), and it now seems possible that Smi1 is one piece of this puzzle.

A previous study investigating the effects of Caspofungin on the yeast cell wall by Atomic Force Microscopy did reveal that treatment by this echinocandin increased *C. albicans* cell wall stiffness and at the same time enhanced cell surface adhesion (Formosa et al., 2013). With this in mind, together with the remarkable sensitivity of the *smi1*Δ/Δ deletion mutant to this drug, it is tempting to postulate that *SMI1* gene product is required for the establishment of the cell's response to Caspofungin. It has however not been possible to test the effect of Caspofungin treatment on the deletion mutant by AFM, precisely due to its extreme sensitivity and lethality in the conditions of the test.

The cellular localization observed for Smi1 in yeast cells and hyphae, as cytoplasmic patches and concentrated spots at the polarized growth sites, is coherent with the idea of cellular function conservation between Smi1 and *S. cerevisiae* Knr4. Indeed, similar cellular

localization has been reported for the MAP kinase Slt2 and for Calmodulin, the phosphatase which activates Calcineurin (Brockerhoff and Davis, 1992; van Drogen and Peter, 2002). In the model filamentous fungus, *N. crassa*, GS1, the homolog of Smi1, localizes at the Spitzenkörper within the hyphal tip (Verdin et al., 2009; Sánchez-León et al., 2011). This localization is conserved in another ascomycete filamentous fungus, *A. nidulans* (also called *Emericella nidulans*), (Schultzhaus et al., 2015). The results presented here indicate that Smi1 also localizes in or around the Spitzenkörper of *C. albicans* hyphae in a similar manner to that observed for GS-1 in *N. crassa*. Finally, given the cell wall related phenotypes reported for GS-1 mutants of *N. crassa* (Enderlin and Selitrennikoff, 1994; Resheat-Eini et al., 2008; Seiler and Plamann, 2003), it is tempting to speculate that the function of these proteins is not only conserved between *C. albicans* and *S. cerevisiae*, but also, to some extent at least, in filamentous fungi. The role of Smi1 in the control of cell wall synthesis, cellular adhesion and drug resistance is of great interest with regard to finding new antifungal targets. The decisive advantage of Smi1 as a drug target over Hsp90, calcineurin, Pkc1 or other MAP kinases is the fact that the Knr4/Smi1 superfamily of proteins is specific of the Fungal Kingdom and absent from host cells (Martin-Yken et al., 2016). In addition, since this protein family is conserved among fungi, including other fungal pathogens of mammals (*C. glabrata* and *Aspergillus species* notably) and also plants (ex: *Magnaporthe grisea*), developing drugs that target Smi1 might lead to broader antifungal applications in domains such as agriculture.

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Strain name	Genotype	References
Candida albicans strains :		
BWP17	*ura3*Δ*::λimm434/ura3*Δ*::λimm434, arg4*Δ*::hisG/arg4*Δ*::hisG, his1*Δ*::hisG/his1*Δ*::hisG*	(Wilson et al., 1999)
CEC161	Isogenic to BWP17 but *arg4*Δ*::hisG/ARG4, his1*Δ*::hisG/HIS1*	(Firon et al., 2007)
BWP17 AHU	Isogenic to CEC161 but *RPS1/rps1::*CIp10-*URA3*	(Chauvel et al., 2012)
***smi1*Δ/Δ**	Isogenic to BWP17 but *smi1*Δ*::HIS1*/*smi1*Δ*::ARG4*	This study
***smi1*Δ/Δ - AHU**	Isogenic to *smi1*Δ/Δ but *RPS1/rps1::*CIp10-*URA3*	This study
smi1*Δ/Δ + P_{TDH3} *SMI1	Isogenic to *smi1*Δ/Δ but *RPS1/rps1::*CIp10-P_{TDH3}-*SMI1*	This study
***smi1B*Δ/Δ**	Isogenic to BWP17 but *smi1B*Δ*::HIS1*/*smi1B*Δ*::ARG4*	This study
SMI1-OE	Isogenic to CEC2908 but *RPS1/rps1::*CIp10-P_{TDH3}-*SMI1*	This study
DAY185	*ura3::imm434/ura3::imm434 his1::hisG::HIS1/his1::hisG arg4::hisG::ARG4-URA3/arg4::hisG*	Davis *et al*., 2000
SMI1-GFP	DAY185 *SMI1*::*SMI1-GFP-NAT*	This study
Saccharomyces cerevisiae strains :		
BY4741a	MAT **a**; *his3*Δ*1 leu2*Δ*0; met15*Δ*0; ura3*Δ*0*	(Brachmann et al., 1998)
***knr4*Δ**	BY4741**a** *YGR229c::KanMX4*	YKO Collection (Open Biosystems)
W303-2N	MAT **a**/α *ura3-1/ura3-1 leu2-3,112/leu2-3,112 trp1-1/trp1-1 his3-11,15/his3-11,15 ade2-1/ade2-1 can1-100/can1-100*	
HM1315	W303-2N *YGR229c::KanMX4/ YGR229c::KanMX4*	This study.

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484 **Table 1. Yeast strains used in this study.**

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Cell Type	Young Modulus (kPa)	% of Adhesive events	Mean Adhesion Force (pN)	Adhesion Energy *(=Area below the force curve*) (J)

BWP17 AHU	**782 (+/- 158)**	**46.1**	**127 (+/- 52)**	**1.77 x10^{-17}**
***smi1*Δ/Δ AHU**	**93 (+/- 34)**	**19.4**	**70 (+/- 26)**	**0.26 x10^{-17}**
SMI1-OE	**298 (+/- 62)**	**62.9**	**712 (+/- 445)**	**15.82 x10^{-17}**

Table 2: Summary of Atomic Force Microscopy measurements for BWP17 AHU, *smi1*Δ/Δ AHU, and SMI1-OE strains.

Column 1: Mean values with standard deviation of Young's Moduli calculated from force curves obtained as described above (3.1).

Column 2: Percentage of adhesive events measured by AFM, calculated from at least 12,000 force curves for each cell type, with a threshold level for the definition of an adhesive event as 50pN on the retraction curve.

Column 3: Mean values of Adhesion forces for each cell type, calculated from adhesive force curves obtained as described above.

Column 4: Mean values of the Adhesion Energy for each strain, calculated from the area below the force curves presenting an adhesion event.

LEGENDS TO FIGURES

Figure 1. *C. albicans SMI1* gene expression suppresses the cell wall associated phenotypes of *S. cerevisiae knr4* Δ mutants.

A) Transformed control strain BY4741a and mutant strain *knr4*Δ with either empty plasmid YEplac195 *PGK/CYC1* or p*SM1* bearing *C. albicans SM1* gene or p*KNR4* bearing *S. cerevisiae KNR4* gene were grown in liquid SD medium lacking uracile at 30°C to an OD_{600} of 1, and concentrated to OD600 8±0. Serial dilutions of yeast cultures were spotted on YPD plates in the absence or presence of 150 ng caspofungin ml−1. Growth was scored after 2 days at 30°C.

B) Transformed control strain W3032N and mutant strain HM1315 *knr4*Δ/Δ with either empty YEplac195 *PGK/CYC1* plasmid or p*SM1* (Yeplac195 bearing *C. albicans SM1* gene under P_{PGK1}) or p*KNR4* (Yeplac195 bearing *S. cerevisiae KNR4* gene under P_{PGK1}) were grown overnight in liquid SD medium lacking uracile at 30°C and concentrated to OD_{600} 8±0. Serial dilutions of yeast cultures were spotted on YPD plates. Growth was scored after 2 days at 30°C and 37°C.

Figure 2. Calcofluor White and caspofungin sensitivity of the *C. albicans smi*1Δ/Δ mutant.

Control strain BWP17 and mutant strain ***smi1* Δ/Δ** were grown in liquid YPD medium at 30°C to an OD_{600} of 1, and concentrated to OD600 8±0. Serial dilutions of yeast cultures were spotted on YPD plates in the absence or presence of 40mg of CFW or 150 ng caspofungin ml−1. Growth was scored after 2 days at 30°C.

Figure 3. Young Moduli of *smi*1Δ/Δ mutant and SMI1-OE *vs* control strain.

Atomic Force Microscope was used to collect over 12,300 force curves for each strain on the control strain BWP17 AHU, the *smi1*Δ/Δ mutant AHU and the SMI1-OE strain over-expressing the *SMI1*. The Young's Moduli (YM =1 / Elasticity) were quantified from these curves as described and are presented here as a dot on the mean YM value, with SEM for each cell. The bar represents the mean of the YM values with each SEM. Statistical analysis was done using the One-way ANOVA test, **** = p value < 0.0001.

Figure 4. Adhesion Maps recorded on the cellular surfaces of *smi* Δ/Δ mutant and SMI1-OE *vs* control strain.

Adhesion force measurements performed on single *C. albicans* cells by Atomic Force Microscopy. The adhesion maps presented have been recorded on three independent and representative *C. albicans* cells for each cell type. Each analyzed area covers 1×1 µm2. Adhesion scales are shown and read as follows: bright yellow = maximum adhesion force at 2 nN; dark red = minimum at 0.0 nN.

Figure 5. Smi1-GFP localizes to apical growth sites in yeast and hyphae. Cells were grown on Ibidi µ-slides in YNB medium at 30°C for 2h (yeast) and 20 % FCS, 2 % glucose at 37°C for 3h (hyphae). Smi1-GFP localised transiently to emerging bud tips in yeast (arrows) and to septa in hyphae (asterisks) but was maintained consistently at hyphal/branch tips (arrows). Punctate fluorescence patches were also observed throughout yeast and hyphal cells. Images are maximum projections of individual z-stacks.

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