A conserved fungal hub protein involved in adhesion and drug resistance in the human pathogen Candida albicans

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ARTICLE INFO

Keywords:
Fungal cell wall
Adhesion
Caspofungin
Candida albicans
Atomic Force Microscopy

ABSTRACT

Drug resistance and cellular adhesion are two key elements of both 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 morphogenetics, 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 cell wall synthesis. Expression of SMI1 in S. cerevisiae knr4Δ null mutants rescued their 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 reduced by 85% upon deletion of SMI1, while cell surface adhesion measured by Force Spectroscopy showed that the surface expression of adhesive molecules was also reduced in the mutant. Over-expression of SMI1, on the contrary, increased cell surface adhesion by 6-fold vs the control strain. Finally, Smi1-GFP localized as cytoplasmic patches and concentrated spots at the sites of new cell wall synthesis including the tips of growing hyphae, consistent with a role in cell wall regulation. Thus, Smi1 function appears to be conserved across fungi, including the yeast S. cerevisiae, the yeast and hyphal forms of C. albicans and the filamentous fungus Neurospora crassa.

Introduction

Fungal infections are responsible of the death of an estimated 1.5 million people per year worldwide. Yeasts of the Candida genus are the second most numerous agents of fungal infections, 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 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 antifungals used to treat patients. Azoles block the biosynthesis of ergosterol – an essential sterol for fungal cell membranes – by targeting the cytochrome P450 14α 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 subunit of the β-1,3 glucan synthase protein complex (Odds et al., 2003). Studies of the molecular mechanisms of resistance to these two classes of antifungal compounds (recently reviewed in (Scorzoni et al., 2017)) have revealed that there are three major mechanisms leading to resistance in C. albicans: overexpression of multidrug efflux pump-encoding genes, notably CDR1, CDR2 and MDR1 (Sanglard and Odds, 2002; Sanglard et al., 1995), amino acid substitutions in the target proteins (ex: Erg11, Fks1) and alteration in the levels 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 mechanism due to the ability of the biofilm extracellular matrix (ECM) to bind and entrap antifungal...
comounds, particularly azoles and amphotericin B (Desai et al., 2014; Taff et al., 2013; Vediyappan et al., 2010; Zarnowski et al., 2014).

In this context, alternative antifungal targets and/or ways to improve the fungicidal effect of existing antifungals are being sought. 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 antifungals, 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 by 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 makes 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 Sm1, 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). Although its precise molecular mode of action is currently unknown, it has been shown that this conserved serine residues, S200 and S298, phosphorylated in vivo, are essential for Knr4 function in signal transmission (Ficarro et al., 2002; Basmaji et al., 2006). Knr4 is required for resistance to cell wall stress induced by elevated temperature or by 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, GSI1 protein, the homolog of Knr4 and Sm1 in the model filamentous fungus, Neurospora crassa, is also involved in the control of morphogenesis, caspofungin sensitivity and the synthesis of cell wall constituents, notably β-glucans (Endlerin and Selitrennikoff, 1994; Reshef-Eini et al., 2008; Seller and Plamann, 2003).

The C. albicans genome encodes two 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 the Cyr1 ade- nylate cyclase, a positive regulator of expression is induced in hyphal and planktonic cells by the Cyr1 ade

Plasmid construction and generation of epitope-tagged or mutant strains

C. albicans SMI1 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. cerevisiae expression vector YEplac195 PGK/CYC1 between the S. cerevisiae PGK1 promoter PPGK1 and the S. cerevisiae CYC1 terminator sequences (personal gift of Dr. J.L. Parrou, based on YEplac195 (Gietz and Sugino, 1988)). Thus, yielding the pSM11 plasmid. Plasmid pKNR4 expressing the S. cerevisiae KNR4 gene with its own promoter and terminator on a multicopy vector has been described previously (Martin et al., 1999).

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

Construction of C. albicans original sm1 Δ/Δ and sm1B Δ/Δ knock-out mutants used PCR-generated ARG4 and HIS1 disruption cassettes flanked by 120 base pairs of target homology region (primer sequences are provided in Supplementary material Table 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 1.8 kb PCR product was purified and inserted into the GTW sequences of pEntry (Gateway™ system, Invitrogen). Recombination of pEntry-SMI1 plasmid and Clp10-P_TDΔ GTW plasmid (Chauvel et al., 2012) was performed also using the Gateway™ (system Invitrogen), Clp10-P_TDΔ GTW vector is a derivative of plasmid Clp10 (Murdad et al., 2000) that carries the sequence for integration at the RPS1 locus on C. albicans Chr1, the URA3 gene, and a Gateway™ cassette flanked by the attR sequences and preceded by the C. albicans P_TDΔ constitutive promoter (Delgado et al., 2003). The resulting Clp10-P_TDΔ-SMI1 construct was then used to transform by genome integration through targeted homologous recombination at the genomic RPS10 locus the host strain BW177, yielding SMI1OE strain, as well as the sm1 Δ/Δ original mutant, yielding sm1 Δ/Δ + P_TDΔ SMI1. To allow phenotype comparisons with the SMI1OE strain, we used as control strain BW177 AHU (Moreno-Ruiz et al., 2009) and the sm1 Δ/Δ original mutant was also transformed by the empty Clp10 vector, ensuring that all of these strains carry a functional URA3 allele (see Table 1 for full genotypes of the yeast strains used in this study).

GFP tagging of Sm1

Sm1 was C-terminally-tagged with GFP by amplifying GFP-NAT cassette from the pGFP-NAT plasmid (Milne et al., 2011) using primers gSM1_F and gSM1_R containing 100 bp of flanking homology to the SMI1 terminator and the C-terminus of the SMI1 ORF (C1_07870C_A), respectively (primer sequences are provided in Supplementary material Table S1). Transformants were selected on YPD agar containing 300 μg/ml nourseothricin (Sigma). Integration of the GFP-NAT cassette was confirmed by PCR using primers sSM1_F and sFP_R, which anneal to the chromosome outside the targeted region and with the cassette, respectively (primer sequences 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 (NH₄)₂SO₄ (Sigma Aldrich). Morphogenesis was induced at 37 °C for 3 h in 20% foetal calf serum (FCS), 2% glucose. Cells were concentrated by centrifugation and resuspended in sterile water to an density of 10⁶ cells ml⁻¹. Yeast cells were then deposited into the center of a µ-slide (Ibidi, Martinsried, Germany). Images of Smi1-GFP were then recorded in acetate buffer and the images were analyzed 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 OD₆₀₀ of 1 ± 0.1, then concentrated by centrifugation and resuspended in sterile water to an OD₆₀₀ 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 48 h of incubation at 30 °C, or at 37 °C for testing the sensitivity to elevated temperature.

Atomic Force Microscopy (AFM)

Sample preparation for AFM experiments: Yeast cells were concentrated by centrifugation, washed two times in acetate buffer (18 mM CH₃COONa, 1 mM CaCl₂, 1 mM MnCl₂, pH 5.2), resuspended in the same buffer, and immobilized on polydimethylsiloxane (PDMS) stamps prepared as described by (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 microstructured by convective (capillary) assembly.

AFM procedures: For imaging and force spectroscopy, we used an AFM Nanowizard III (JPK Instruments, Berlin, Germany). Force curves were then recorded in acetate buffer in quantitative-imaging mode (JPK Instruments, 2011, QITM mode-quantitative imaging with NanoWizard 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 maximal applied force was kept at 1 nN, the force curves length (Z-range) at 2 µm and the approach/retract speed at either 20 or 2 µm s⁻¹ for both imaging and force spectroscopy. 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 an area of 1–4 µm² on top of the cells, always avoiding any bud or budscar. 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, 1881). Results

Conservation of cellular function between S. cerevisiae Knr4 and C. albicans Smi1

The genome of the human fungal pathogen C. albicans contains two distinct homologs of the S. cerevisiae KNR4 gene, SM1 (C1_07870C_A) and SM1B (C3_05350C_A). Gene deletion mutants for each gene were generated and initial phenotypic analysis showed a strong phenotype (sensitivity to Calcofluor White (CFW) or SDS) for the smi1 Δ/Δ mutant but only a milder one for the smi1B Δ/Δ mutant (Supplementary material, Fig. S1). We therefore focused on the role of Smi1 in this study.

The SM1 open reading frame was amplified and cloned into a S. cerevisiae expression vector, under the control of the strong and constitutive ADH1 promoter. The SM1 coding sequence is 1863 bp and contains two CTag codons at positions 1717 and 1762, which have a 97% chance of translation as a Serine instead of a Leucine in this fungus, compared to a Leucine in S. cerevisiae expression vector, under the control of the strong and constitutive ADH1 promoter. The SM1 coding sequence is 1863 bp and contains two CTag codons at positions 1717 and 1762, which have a 97% chance of translation as a Serine instead of a Leucine in S. cerevisiae expression vector, under the control of the strong and constitutive ADH1 promoter. The SM1 coding sequence is 1863 bp and contains two CTag codons at positions 1717 and 1762, which have a 97% chance of translation as a Serine instead of a Leucine in S. cerevisiae expression vector, under the control of the strong and constitutive ADH1 promoter. The SM1 coding sequence is 1863 bp and contains two CTag codons at positions 1717 and 1762, which have a 97% chance of translation as a Serine instead of a Leucine in S. cerevisiae expression vector, under the control of the strong and constitutive ADH1 promoter. The SM1 coding sequence is 1863 bp and contains two CTag codons at positions 1717 and 1762, which have a 97% chance of translation as a Serine instead of a Leucine in S. cerevisiae expression vector, under the control of the strong and constitutive ADH1 promoter. The SM1 coding sequence is 1863 bp and contains two CTag codons at positions 1717 and 1762, which have a 97% chance of translation as a Serine instead of a Leucine in S. cerevisiae expression vector, under the control of the strong and constitutive ADH1 promoter. The SM1 coding sequence is 1863 bp and contains two CTag codons at positions 1717 and 1762, which have a 97% chance of translation as a Serine instead of a Leucine in S. cerevisiae expression vector, under the control of the strong and constitutive ADH1 promoter. The SM1 coding sequence is 1863 bp and contains two CTag codons at positions 1717 and 1762, which have a 97% chance of translation as a Serine instead of a Leucine in S. cerevisiae expression vector, under the control of the strong and constitutive ADH1 promoter. The SM1 coding sequence is 1863 bp and contains two CTag codons at positions 1717 and 1762, which have a 97% chance of translation as a Serine instead of a Leucine in S. cerevisiae expression vector, under the control of the strong and constitutive ADH1 promoter.
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 (Fig. S2).

Deletion of SMI1 renders C. albicans sensitive to cell wall targeting drugs

We further investigated the role of Smi1 in maintaining cell wall integrity using a medically-relevant β-glucan synthase-targeting drug, the echinocandin caspofungin. The influence of Smi1 on the sensitivity to CFW was also retested in parallel. Deletions of both alleles of SMI1 in the C. albicans BWP17 strain background (but bearing a functional URA3 allele) led to a marked increase in sensitivity to caspofungin and CFW at 30 °C (Fig. 2). These phenotypes are consistent with those observed for the S. cerevisiae knr4Δ mutant and the proposed role of these proteins in stress signaling pathways (See Discussion). Re-integration of the SMI1 open reading frame under the constitutive promoter PTDH3 in the smi1Δ/Δ deletion mutant restored the wild-type phenotype (Fig. 2).

Cell wall biophysics

Cell wall strength/elasticity

Atomic Force Microscopy (AFM) under liquid conditions can be used to investigate the nanomechanical properties of live wildtype and mutant cells (Dague et al., 2010; Ene et al., 2015; Formosa et al., 2013; Liu et al., 2015). Here we first measured the cell surface elasticity of three C. albicans strains: the control strain, BWP17 AHU, the homozygous deletion mutant smi1Δ/Δ and the strain over-expressing the SMI1 from the PTDH3 promoter in the BWP17 genetic background, SMI1-OE. Using the Atomic Force Microscope in the Force Volume mode, we collected between 1024 and 4160 force curves per cell on a minimum of 12 cells from 4 independent cultures for each strain. The elasticity of the cells was quantified from these curves by calculating the Young’s modulus.

Fig. 1. C. albicans SMI1 gene expression suppresses the cell wall associated phenotypes of S. cerevisiae knr4Δ mutants. A) Transformed haploid control strain BY4741a and mutant strain knr4Δ with either empty plasmid YEpplac195 PGK/CYC1, pSMI1 (YEpplac195 bearing C. albicans SMI1 gene under PPGK1) or pKNR4 bearing S. cerevisiae KNR4 gene, were grown in liquid SD medium lacking uracil at 30 °C to an OD600 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 diploid control strain W3032N and mutant strain HM1315 knr4Δ/Δ with either empty YEpplac195 PGK/CYC1 plasmid, pSMI1 bearing C. albicans SMI1 gene or pKNR4, were grown overnight in liquid SD medium lacking uracil at 30 °C and concentrated to OD600 8 ± 0. Serial dilutions of yeast cultures were spotted on YPD plates. Growth was scored after 2 days at 30 °C and 37 °C.

Fig. 2. Calcofluor White and caspofungin sensitivity of the C. albicans smi1Δ/Δ mutant. The control strain BWP17 AHU, the mutant strain smi1Δ/Δ and the deletion mutant with SMI1 gene re-integrated smi1Δ/Δ + PTDH3-SMI1 were grown in liquid YPD medium at 30 °C to an OD600 of 1, and concentrated to OD600 8 ± 0. Serial dilutions of yeast cultures were spotted on YPD plates in the absence or presence of 40 μg of CFW or 150 ng caspofungin ml⁻¹. Growth was scored after 2 days at 30 °C.
Fig. 3. A: Elasticity maps recorded on independent cells of BWP17 AHU, smi1 Δ/Δ and SMI1-OE strains. Maps of Young’s Moduli (YM = 1/Elasticity) measured by Atomic Force Microscopy on independent cells of control strain BWP17 AHU, smi1 Δ/Δ mutant and SMI1-OE strain. YM scales are shown (bright yellow: maximum at 500 kPa; dark red: minimum at 0.0 kPa). The corresponding topography map is presented below each elasticity map, also with scale (bright yellow: maximum at 500 nm; dark red: minimum at 0.0 nm). Analyzed areas cover squares of 1 × 1 to 2 × 2 µm². B: Young’s Moduli of smi1 Δ/Δ mutant and SMI1-OE vs control strain BWP17 AHU. 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 and the SMI1-OE strain. The Young’s Moduli quantified from these curves 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. C: Representative Approach Force Curves of BWP17 AHU, smi1 Δ/Δ and SMI1-OE. Forces measured by AFM in nN as a function of the indentation (tip position) in nm, for the three strains. BWP17 AHU: red curves, smi1 Δ/Δ: black curves and SMI1-OE: blue. These force curves are obtained upon approaching the AFM tip towards the cell surface (horizontal part), touching the cell (contact point: where the curve starts to bend), further indenting into the cell surface and facing distinct resistance levels. The slope of the second part of each curve corresponds to the cell surface resistance against the tip progression.
Young's Modulus represents the cell's stiffness: the higher the YM, the stiffer the cell. In the control strain, BWP17 AHU, the mean value ± SEM of the YM was 782 ± 70 kPa (Fig. 3, Table 2), significantly higher than that of the homozygous deletion mutant (mean ± SEM = 93 ± 33 kPa). The YM of the over-expression strain (mean ± SEM = 298 ± 62 kPa) lay between the control strain and the deletion mutant, suggesting either a gene dosage effect for SMI1 expression or an effect of uncoupling the expression of this gene from the cell cycle.

![Fig. 3](image)

**Table 2**

| Cell Type   | Young Modulus (kPa) | % of Adhesive Events | Mean Adhesion Force (pN) | Adhesion Energy (Area below the force curve) (J) |
|-------------|---------------------|----------------------|--------------------------|-----------------------------------------------|
| BWP17 AHU   | 782 (± 70)          | 46.1                 | 127 (± 33)               | 1.77 × 10⁻¹⁷                                  |
| smi1 Δ/Δ    | 93 (± 33)           | 19.4                 | 70 (± 16)                | 0.26 × 10⁻¹⁷                                  |
| SMI1-OE     | 298 (± 62)          | 62.9                 | 712 (± 102)              | 15.82 × 10⁻¹⁷                                 |

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

†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 50 pN on the retraction curve.

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

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

The Young's Modulus represents the cell's stiffness: the higher the YM, the stiffer the cell. In the control strain, BWP17 AHU, the mean value ± SEM of the YM was 782 ± 70 kPa (Fig. 3, Table 2), significantly higher than that of the homozygous deletion mutant (mean ± SEM = 93 ± 33 kPa). The YM of the over-expression strain (mean ± SEM = 298 ± 62 kPa) lay between the control strain and the deletion mutant, suggesting either a gene dosage effect for SMI1 expression or an effect of uncoupling the expression of this gene from the cell cycle. Fig. 3A shows maps of the recorded YM on square areas on the surfaces of three characteristic cells for each cell type, together with the corresponding topography maps. In this figure, the first line of maps (YM values of the control strain) is to compare with the third line (YM values of the homozygous deletion mutant) and the fifth line (YM values of the SMI1 over-expressing strain). In addition, all the YM values recorded are represented for each cell individually on Fig. 3B, to allow visualization of cell-to-cell variability. Finally, Fig. 3C shows selected representative individual force curves for the three strains. These curves represent the force encountered while approaching the AFM tip vertically toward the cell surface. Before the tip touches the cell it does not
encounter any resistance and the curve is simply horizontal. When the tip touches the cell (contact point), the curve starts to bend. Moving the tip further down results in indenting into the cell surface, where distinct resistance levels can be met. Hence, the slope of the second part of each curve represents the cell surface resistance against the ongoing progression of the tip: the steeper the slope, the harder the surface. These results therefore demonstrate that the deletion of both alleles of \( \text{SMI1} \) resulted in a reduction in cell wall stiffness by eight to ten folds, indicating that a cell wall integrity is compromised in this mutant.

**Adhesion**

Another cell surface feature that can be easily and 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 \( \text{Si}_3\text{N}_4 \), was measured by scanning areas of 1 \( \mu \text{m}^2 \) on the top of individual yeast cells. We recorded force curves whose retraction values were 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 (Fig. 4). These data, represented as two-dimensional matrixes, show that the adhesion between the probe and the cell surface of the \( \text{smi1} \Delta/\Delta \) mutant was minimal compared to the control strain, while the cells of the \( \text{SMI1} \) over-expression strain displayed a marked increase in their surface adhesion. Cellular adhesion was also evaluated by three quantitative parameters: the mean adhesion force, the specific energy of each adhesion event observed, and the overall frequency 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. Using this threshold level, we calculated the percentage of adhesive events for each cell type and measured the area situated below the retraction 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 \( \text{SMI1-OE} \) (63% of the recorded 21,500 force curves) than on the control strain (46% of over 13,500 force curves), and less frequently on the deletion mutant surface (19% of over 12,300 force curves). The mean adhesion force measured for the control strain was 127 ± 33 pN, calculated from 6200 adhesive force curves. This is to be compared with a mean ± SEM force of 70 ± 16 pN (barely above threshold) for 2300 force curves for the \( \text{smi1} \Delta/\Delta \) mutant. For the \( \text{SMI1-OE} \)-strain, adhesive events were more frequent (63%) and they were also much stronger, with forces measured up to 2176 pN with a mean value ± SEM of 712 ± 102 pN, calculated over 13,000 adhesive force curves. The specific energy of these adhesive events also differed; with adhesion energies for the over-expressing strain an order of magnitude stronger than that for the control strain, while they were approximately seven times lower on the surface of the deletion mutant. Hence, the homozygous deletion of \( \text{SMI1} \) gene abrogates almost entirely the ability of the mutant cell to adhere using the chemistry described here, while \( \text{SMI1} \) over-expression leads to a highly adhesive phenotype.

**Cellular localization**

GS-1, the homolog of Smi1 and Knr4 in the model filamentous fungus, \( \text{N. crassa} \), 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 composed 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 at the tip of \( \text{N. crassa} \) hyphae (Riquelme et al., 2011; Sánchez-León et al., 2011; Verdin et al., 2009) and at the tip of elongated shmoos in \( \text{S. cerevisiae} \), respectively ((Martin-Yken, 2012) and our 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. This Smi1-GFP fusion protein is functional, as attested by its ability to complement the caspofungin and CFW hypersensitivity phenotypes of the smi1 Δ/Δ mutant (not shown).

In yeast cells, Smi1-GFP appeared both as punctate patches in the cytoplasm and localized transiently to nascent buds (Fig. 5). This localization is similar to that reported for Knr4 in S. cerevisiae (Dagkessamanskaia et al., 2010; Martin et al., 1999). Punctate patches and a more diffuse cytoplasmic distribution were observed in C. albicans hyphae (Fig. 5), but, unlike in yeast, Smi1-GFP signal was consistently retained as a bright spot at the growing hyphal tip throughout the cell cycle, reminiscent of N. crassa GS-1 localization at the tip of growing hyphae (Verdin et al., 2009). 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 reflects those observed for homologs of Smi1 in S. cerevisiae in yeast cells and the hyphae of N. crassa.

**Discussion**

Our results indicate that there is significant conservation of Knr4/Smi1 function in cell wall regulation between S. cerevisiae and C. albicans and demonstrate the role of Smi1 in tolerance to caspofungin, regulation of cell wall integrity and cell surface adhesions 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 during 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 is a candidate for this previously unidentified “X”, a common target of the Pkc1 and calcineurin signaling pathways. The marked reduction in cell-wall stiffness of the smi1 Δ/Δ cell wall, as indicated by its Young’s Modulus (Fig. 3, Table 2), compared to the milder phenotype observed for the S. cerevisiae knr4Δ mutant (Dague et al., 2010), suggests a more central role for Smi1 in the cell-wall integrity signaling network in C. albicans than that of Knr4 in the baker’s yeast, which is in agreement with the hypothesis of Lafayette and colleagues (LaFayette et al., 2010). 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 SM11 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 SM11 leads to an increase in the sensitivity of C. albicans to both CFW and caspofungin at 30 °C. Caspofungin tolerance has been reported in C. albicans mutants that have elevated cell-wall chitin (Perlin, 2015; Plaine et al., 2008; Walker et al., 2013; Yang et al., 2017), yet others report that elevated chitin can induce hypersensitivity to CFW (Elorza et al., 1983; Roncero and Durán, 1985). However, this dual sensitivity is consistent with the phenotype observed for the S. cerevisiae knr4Δ mutant (Lesage et al., 2004; Martin et al., 1999). Our cell wall stiffness measurements

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**Fig. 5.** Smi1-GFP localizes as patches concentrated to apical growth sites in yeasts and hyphae. Cells were grown on Ibidi µ-slides in YNB medium at 30 °C for 2 h (yeast) and 20% FBS, 2% glucose at 37 °C for 3 h (hyphae). Smi1-GFP localized transiently to emerging bud tips in yeasts (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.
indicated that organization of the cell wall is significantly modified in the deletion mutant smi1 Δ/Δ, consistent with a possible upstream role of Smi1 in cell-wall regulatory pathways. Early results obtained in bakers’ yeast established a role for Knr4 in the transcriptional control of all S. cerevisiae chitin synthase genes (Martin et al., 1999), so the role of Smi1 in C. albicans may also include control of expression levels of cell wall biosynthesis genes. 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. Indeed, a previous study investigating the effects of caspofungin on the cell walls of S. cerevisiae and C. albicans by AFM revealed that treatment with this echinocandin increased C. albicans cell wall stiffness and at the same time enhanced cell surface adhesion (Formosa et al., 2013). Although the high level of sensitivity to caspofungin of the C. albicans smi1Δ/Δ mutant meant that it was not possible to test this in this fungus, we speculate that Smi1 is a key player in the cellular response to caspofungin in C. albicans.

In S. cerevisiae, Knr4 is proposed to link the Ca2+/calcineurin/Crz1 signaling pathway with the Slt2/Mpk1 cell-wall integrity pathway. The localization of Smi1-GFP in signaling pathways, giving it a central role in cell-wall integrity signaling. The f了 fungal targets. An advantage of Smi1 as a drug target over Hsp90, the cell wall related phenotypes reported for GS-1 mutants of C. albicans in a similar manner to that observed for GS-1 in N. crassa. This protein family is conserved among fungi, including other fungal pathogens of animals (C. glabrata, A. nidulans, N. crassa), developing drugs that target Smi1 might lead to broader antifungal applications in domains such as agriculture.

Acknowledgements

Dr. Jean-Luc Parrou (LJSBP, Toulouse) for vector YEpplac195 PGK/CYC1 and Gregory Da Costa (INRA, Jouy-en-Josas) for technical help. SZ is an Institut Pasteur International Network Affiliation Program Fellow (Institut Pasteur de Tunis, Institut Pasteur, Paris) and has also been supported by grants from the European Commission (FinSysB PITN-GA-2008-214004), the Agence Nationale de la Recherche (KANJII, ANR-08-MIE-033-01) and the French Government’s Investissement d’Avenir Program (Institut de Recherche Technologique BIOASTER, ANR-10-AIRT-03) to Chd’E.

HMY acknowledges the FEBS society and the organizers of the Human Fungal Pathogen Schools 2015 and 2017 for a great introduction to C. albicans and other human fungal pathogens, as well as Formation permanente of the Centre INRA Toulouse Occitanie for financing her attendance to these researcher schools.

HMY is forever grateful to Frans M. Klis for his kindness throughout the years and his great advice to “Start working on C. albicans.”

Declarations of interest

None.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.tcs.2018.10.002.

References

Anxxer, O., Björnham, O., Castelain, M., Koul, E., Schled, S., Füllman, E., Andersson, M., 2010. Unraveling the Secrets of Bacterial Adhesion Organelles Using Single Molecule Force Spectroscopy. In: DIVA. Springer Verlag, pp. 337-362.

Basmajj, F., Martin-Yken, H., Durand, F., Dagkessamanskaia, A., Pichereaux, C., Rossignol, M., Francois, J., 2006. The “interactome” of the Knr4/Smi1, a protein implicated in coordinating cell wall synthesis with bud emergence in Saccharomyces cerevisiae. Mol. Genet. Genomics 275, 217–230.

Benoit, M., Gabriel, D., Gerich, G., Gaub, H.E., 2000. Discrete interactions in cell adhesion measured by single-molecule force spectroscopy. Nat. Cell Biol. 2, 313–317.

Birkaya, B., Madidi, A., Joshi, J., Fee, S.J., Cullen, P.J., 2009. Role of the cell wall integrity and filamentous growth mitogen-activated protein kinase pathways in cell wall remodeling during filamentous growth. Eukaryot. Cell 8, 1118–1133.

Brachmann, C.B., Davies, A., Cost, G.J., Caputo, E., Li, J., Hieter, P., Boeke, J.D., 1998. Designer deletion strains derived from Saccharomyces cerevisiae S288C a useful set of strains and plasmids for PCR-mediated gene disruption and other applications. Yeast (Chichester Engl.) 14, 115–132.

Brockerhoff, S.E., Davis, T.N., 1992. Calmodulin concentrates at regions of cell growth in Saccharomyces cerevisiae. J. Cell Biol. 118, 619–629.

Brown, A.J.P., Leach, M.D., Nicholls, S., 2010. The relevance of heat shock regulation in fungal pathogens of humans. Virulence 1, 330–332.

Brown, G.D., Denning, D.W., Gov, N.A.R., Leviitz, S.M., Netea, M.G., White, T.C., 2012. Hidden killers: human fungal infections. Sci. Transl. Med. 4 165rv13–165rv13.

Chauveau, M., Neuvez, A., Cabral, V., Zaussi, S., Goyard, S., Bachelier-Bas, S., Firon, A., Legrand, M., Diogo, D., Nauleau, C., et al., 2012. A versatile overexpression strategy in the pathogenic yeast candida albicans: identification of regulators of morphogenesis and fitness. PLoS One 7, e45912.

Chopinet, L., Formosa, C., Rols, M.P., Dufu, R.E., Dague, E., 2013. Imaging living cells surface and quantifying its properties at high resolution using AFM in Quanta mode. Micron. 50, 26–33.

Dagkessamanskaia, A., El Azzouzi, K., Kikuchi, Y., Ohyya, Y., Francois, J.M., Martin-Yken, H., 2013. Imaging living cells force microscopy analysis of yeast mutants defective in cell wall architecture. Yeast 27, 673–684.

Dague, E., Bittar, R., Ranchon, H., Durand, F., Yken, H.M., Francois, J.M., 2010. An atomic force microscopy analysis of yeast mutants defective in cell wall architecture. Yeast 27, 673–684.

Dague, E., Javert, E., Laplatine, V., Viallet, B., Thibault, C., Resler, L., 2011. Assembly of live microorganisms on microstructured PDMS stamps by convective/capillary deformation. Nanotechnology 22, 395102.

Davis, D., Edwards, J.E., Mitchell, A.P., Ibrahim, A.S., 2000. Candida albicans RIM101 hormone response pathway is required for host-pathogen interactions. Infect. Immun. 68, 5953–5959.

Delgado, M.L., Gil, M.L., Gobaldu, Z., 2003. Starvation and temperature upshift cause an increase in the enzymatically active cell-wall-associated glyceraldehyde-3-phosphate dehydrogenase protein in yeast. FEMS Yeast Res. 4, 297–303.

Desai, J.V., Mitchell, A.P., Andes, D.R., 2014. Fungal biofilms, drug resistance, and recurrent infection. Cold Spring Harb. Perspect. Med. 4 a019729–a019729.

Davison, F., van Drogen, F., Peters, M., 2002. Spa2p functions as a scaffold-like protein to recruit the Mpk1 MAP kinase module to sites of polarized growth. Curr. Biol. 12, 1698–1703.

Enderlin, C.S., Selitrennikoff, C.P., 1994. Cloning and characterization of a Neurospora crassa gene required for (1,3) beta-glucan-synthetic activity and cell wall formation. Proc. Natl. Acad. Sci. U.S.A. 91, 9500–9504.

Ene, I.V., Walker, M., Schiavone, M., Lee, K.K., Martin-Yken, H., Dague, E., Gov, N.A.R., Munro, C.A., Brown, A.J.P., 2015. Cell wall remodeling enzymes modulate fungal cell wall elasticity and osmotic stress resistance. mBio 6, e00886–15.

Ficaro, S.B., McCleland, M.L., Stakenberg, P.T., Burke, D.J., Ross, M.M., Shabanowitz, J., Hunt, D.F., White, F.M., 2002. Proteoproteome analysis by mass spectrometry and its application to Saccharomyces cerevisiae. Nat. Biotechnol. 20, 301–305.

Firon, A., Amgd, S., Iraqui, I., Gouda, S., Goyard, S., Prévost, M.C., Gabon, J., d’Enfert, C., 2007. The SUN41 and SUN42 genes are essential for cell separation in Candida albicans. Mol. Microbiol. 66, 1256–1275.

Füehl, R.R., Sperry, A.O., Garrard, W.T., 1993. Yeast calmodulin and a conserved nuclear protein in yeast. FEMS Yeast Res. 4, 297–303.

Formosa, C., Schiavone, M., Martin-Yken, H., Francois, J.M., Dufu, R.E., Dague, E., 2013. Nano-scale effects of caspofungin against two yeast species, Saccharomyces cerevisiae and Candida albicans. Antimicrob. Agents Chemother. 57, 3488–3506.

Formosa, C., Pillet, F., Schiavone, M., Dufu, R.E., Resler, I., Dague, E., 2014a.
Generation of living cell arrays for atomic force microscopy studies. Nat. Protoc. 10, 199–204.

Formosa, C., Schiavone, M., Boisrame, A., Richard, M.L., Duval, R.E., Dague, E. 2014b. Multimodal imaging of adhesive nanodomains at the surface of Candida albicans by atomic force microscopy spectroscopies. Nanomed. Nanotechnol. Biomed. Imprint.

Gietz, R.D., Woods, R.A., 2006. Yeast transformation by the LiAEC/SS carrier DNA/PEG method. In: Yeast Protocols. Humana Press, Totowa, NJ, pp. 107–120.

Girard, M., 1957. Der Spitzentropfen von Polyvinzucis viscosiz (L.). Planta 50, 47–59.

Gola, S., Martin, K., Walshe, A., Dunkler, A., Wendland, J., 2003. New modules for PCR-based gene targeting in Candida albicans: rapid and efficient gene targeting using 100 bp of flanking homology region. Yeast 20, 1339–1347.

Harris, S.D., Read, N.D., Roberson, R.W., Shaw, B., Seiler, S., Plamann, M., Momany, M., 2005. Polarimere meets Spitzentropfens: microcopy, genetics, and genomics converge. Eukaryot. Cell 4, 225–229.

Hertz, H., 1881. Ueber die Berührung fester elastischer Körper. J Für Reine Angew Math 156–171.

Hinterdorfer, P., Baumgartner, W., Gruber, H.J., Schlicker, K., Schindler, H., 1996. Detection and localization of individual antibody-antigenic reaction events by atomic force microscopy. Proc. Natl. Acad. Sci. U.S.A. 93, 3477–3481.

Hutter, J.J., Bichhoefer, J., 1993. Calibration of atomic-force microscope tips. Rev. Sci. Instrum. 64, 1866–1873.

Kathiravan, M.K., Salake, A.B., Choue, A.S., Dushe, P.B., Watode, R.P., Mukta, M.S., Gadhsw, S., 2012. The biology and chemistry of antifungal agents: a review. Bioorg. Med. Chem. 20, 5678–5698.

LaFayette, S.L., Collins, C., Zas, A.K., Schell, W.A., Betancourt-Quinzo, M., Gunatilaka, A.A.L., Perfect, J.R., Cowen, L.E., 2010. PKC signaling regulates drug resistance of the fungal pathogen Candida albicans via circuitry comprised of Mcp1, calcineurin, and Hap90. PloS Pathog. 6, e1001069.

Leage, G., Silic, A.M., Menard, P., Shapiro, J., Hussein, S., Bussey, H., 2004. Analysis of beta-1,3-glucan assembly in Candida albicans. Curr. Genet. 47, 117–126.

Liu, R., Formosa, C., Dagkessamanskaia, Dague, E., Francois, J.M., Martin-Yken, 2015. Combining Atomic Force Microscopy and genetics to investigate the role of Kmr4 in Saccharomyces cerevisiae sensitivity to K9 Killer toxin. Lett. Appl. NanoBiosci. 4, 306–315.

Liu, T.T., Lee, R.E.B., Barker, K.S., Lee, R.E., Wei, I., Homayouni, R., Rogers, P.D., 2005. Genome-wide expression profiling of the response to azole, polyene, echinocandin, and pyrimidine antifungal agents in C. albicans. Antimicrob. Agents Chemother. 49, 2226–2236.

Markovich, S., Yekutiel, A., Shalit, I., Shadkchan, Y., Osherov, N., 2004. Genomic approach to identification of mutations affecting caspofungin susceptibility in Saccharomyces cerevisiae. Antimicrob. Agents Chemother. 48, 3871–3876.

Martin-Herraez, K., Dagkessamanskaia, A., Satchanska, G., Dallies, N., François, J., 1999. KNR4, a suppressor of Saccharomyces cerevisiae cwh mutants, is involved in the transcriptional control of chitin synthase genes. Microbiol. Biotechnol. Read. Engl. 145 (Pt 1), 249–258.

Martin-Yken, H., Dagkessamanskaia, A., Basmaji, F., Lagorce, A., Francois, J., 1999. KNR4, a member of the MFS transporter family drug resistance in Candida albicans. Mol. Microbiol. 34, 1347–1358.

Martino-Yken, H., Dagkessamanskaia, A., Basmaji, F., Lagorce, A., Francois, J., 2003. The beta-1,3-glucan assembly in Candida albicans: rapid and efficient gene targeting using 100 bp of flanking homology region. Yeast 20, 1339–1347.

Martin-Yken, H., Dagkessamanskaia, A., Basmaji, F., Lagorce, A., Francois, J., 2003. The beta-1,3-glucan assembly in Candida albicans: rapid and efficient gene targeting using 100 bp of flanking homology region. Yeast 20, 1339–1347.

Nevsky, A.A., Perfect, J.R., Cowen, L.E., 2010. PKC signaling regulates drug resistance of the fungal pathogen Candida albicans via circuitry comprised of Mcp1, calcineurin, and Hap90. PloS Pathog. 6, e1001069.

Oliveira, H.C., Costa-Orlandi, C.B., Mendes-Giannini, M.J.S., Fusco-Almeida, A.M., 2017. Antifungal therapy: new advances in the understanding and treatment of mycoses. Front. Microbiol. 8.

Walker, L.A., Gow, N.A.R., Munro, C.A., 2013. Elevated chitin content reduces the susceptibility of Candida albicans to antifungal agents. Future Microbiol. 8.

Walker, L.A., Gow, N.A.R., Munro, C.A., 2010. Fungal echinocandin resistance. Fungal Genet. Biol. 47, 109–126.

Walther, L.A., Gow, N.A.R., Munro, C.A., 2013. Mechanisms of resistance to asazole antifungal agents in Candida albicans isolates from AIDS patients involve specific multidrug transporters. Antimicrob. Agents Chemother. 39, 2376–2386.

Yang, F., Zhang, L., Wakabayashi, H., Sakiyama, A., Watanabe, A., 2009. Rapid hypothesis testing with 100 bp of flanking homology region. Yeast 20, 1339–1347.

Yekutiel, A., Shalit, I., Shadkchan, Y., Osherov, N., 2004. Genomic approach to identification of mutations affecting caspofungin susceptibility in Saccharomyces cerevisiae. Antimicrob. Agents Chemother. 48, 3871–3876.

Zhang, L., Wakabayashi, H., Myers, J., Jiang, Y., Cao, Y., Jimenez-Ortigosa, C., Perlin, D.S., 2015. Mechanisms of echinocandin antifungal drug resistance. Ann. N. Y. Acad. Sci. 1354, 1–11.