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

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3 A conserved fungal hub protein involved in adhesion and drug
4 resistance in the human pathogen *Candida albicans*.
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ABSTRACT

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54 Drug resistance and cellular adhesion are two key elements of the worldwide dissemination
55 and prevalence of the human fungal pathogen *Candida albicans*. Smi1 belongs to a family of
56 hub proteins conserved among the fungal kingdom, whose functions in cellular signaling
57 affect morphogenesis, cell wall synthesis and stress resistance. The data presented here
58 indicate that *C. albicans SMII* is a functional homolog of *Saccharomyces cerevisiae KNR4*
59 and is involved in the regulation of the fungal cell wall synthesis. Expression of *SMII* in the
60 *S. cerevisiae knr4Δ* null mutant rescued its sensitivity to Caspofungin and to heat stress.
61 Deletion of *SMII* in *C. albicans* resulted in sensitivity to the cell-wall-perturbing compounds
62 Calcofluor White and Caspofungin. Analysis of wild-type and mutant cells by Atomic Force
63 Microscopy showed that the Young's Modulus (stiffness) of the cell wall was significantly
64 reduced upon deletion of *SMII*, while cell surface adhesion measured by Single Force
65 Spectroscopy showed that the surface expression of adhesive molecules was also reduced in
66 the mutant. Over-expression of *SMII gene*, on the contrary, drastically increased the cell
67 surface adhesion phenotype *vs* the control strain. Finally, Smi1 cellular localization as
68 cytoplasmic patches and concentrated spots at the sites of new cell wall synthesis is consistent
69 with its role in the control of cell wall synthesis and appears conserved with the ones of its
70 counterparts in *S. cerevisiae* and *Neurospora crassa* respectively for yeast cells and hyphae of
71 *C. albicans*.
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INTRODUCTION

77 Fungal infections are responsible of the death of about 1.5 million people per year
78 worldwide. Hemiascomycetous yeasts of the genus *Candida* are the second most numerous
79 agents of fungal infections worldwide, with a prominent contribution by *Candida albicans*,
80 which causes over 400,000 cases of life-threatening systemic infections and 200,000 deaths
81 per year (Brown et al., 2012). Only four classes of antifungal (AF) drugs are available for
82 patient treatment and the emergence of resistance is becoming a serious concern. The
83 fungistatic group of Azoles and the more recently-developed fungicidal group of
84 Echinocandins constitute the two major classes of AF used to treat patients. Azoles block the
85 biosynthesis of ergosterol - an essential sterol for fungal cell membranes - by targeting the
86 cytochrome P450 14- α demethylase enzyme, Erg11, which catalyzes the conversion of
87 lanosterol to ergosterol, thereby affecting membrane integrity and inhibiting fungal growth
88 (Kathiravan et al., 2012; Vanden Bossche et al., 1995). On the other hand, Echinocandins, a
89 class of compounds developed between 2001 and 2006, target the catalytic sub-unit of the β -
90 1,3 glucan synthase protein complex, Fks1 (Odds et al., 2003). Studies of the molecular
91 mechanisms of resistance to these two classes of AF compounds (recently reviewed in
92 (Scorzoni et al., 2017)) have revealed that there are three major mechanisms leading to
93 resistance: overexpression of multidrug efflux pump-encoding genes, notably CDR1, CDR2
94 and MDR1 (Sanglard and Odds, 2002; Sanglard et al., 1995), point mutations in the target
95 proteins (ex: Erg11, Fks1) and alteration in the expression of proteins involved in sensitivity
96 to the drug (ex: Erg3, Erg11). In addition, the formation of fungal biofilms can also be
97 considered as a form of antifungal resistance due to the ability of the biofilm extracellular
98 matrix (ECM) to bind and entrap antifungal compounds, particularly Azoles and
99 Amphotericin B (Desai et al., 2014; Taff et al., 2013; Vedyappan et al., 2010; Zarnowski et
100 al., 2014).

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

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

113 Here we describe the role of Smi1, a *C. albicans* protein homologous to the
114 *Saccharomyces cerevisiae* hub protein Knr4 which interacts physically with both the Slr2
115 MAP kinase and calcineurin, thus connecting the two primary signaling pathways involved in
116 cell wall maintenance during stress: the cell wall integrity pathway (CWI) and the calcineurin
117 pathway (Dagkessamanskaia et al., 2010; Martin-Yken et al., 2016). Knr4 is required for
118 resistance to cell wall stress induced by elevated temperature or the presence of antifungal
119 compounds, including Caspofungin (Lesage et al., 2004; Markovich et al., 2004). Knr4 also
120 plays a role in filamentous and pseudohyphal growth, mucin secretion and agar invasion
121 (Birkaya et al., 2009). Similarly, GS1 protein, the homolog of Knr4 and Smi1 in the model
122 filamentous fungi *Neurospora crassa*, is also involved in the control of morphogenesis,
123 Caspofungin sensitivity and new cell wall synthesis notably β -glucans (Enderlin and
124 Selitrennikoff, 1994; Resheat-Eini et al., 2008; Seiler and Plamann, 2003).

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

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

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140

141 **Material and Methods**

142 **Strains and growth media**

143

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

145 Depending on experimental conditions, yeast strains were grown in YPD (1 % (W/V) yeast
146 extract, 2 % (W/V) peptone, and 1 % (W/V) dextrose), YP (1 % (W/V) yeast extract, 2 %
147 (W/V) peptone) supplemented with 10 % Fetal Bovine Serum (FBS), or SD (synthetic
148 dextrose, 0.67 % (W/V) yeast nitrogen base (YNB; Difco) with 2 % glucose) supplemented as
149 necessary with arginine, histidine or uridine (20 mg/l). Agar (2 %) was used for growth on
150 solid medium. *Escherichia coli* strains TOP10 (Invitrogen) or DH5 α were used for DNA
151 cloning and maintenance of the plasmid constructs.

152

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

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

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

166 Construction of *C. albicans* *smi1* Δ/Δ and *smi1B* Δ/Δ knock-out mutants (Table 1) used PCR-
167 generated *ARG4* and *HIS1* disruption cassettes flanked by 120 base pairs of target homology
168 region (primer sequences are provided in supplementary material S1) as described by (Gola et
169 al., 2003) and (Schaub et al., 2006). Independent transformants were produced and the gene
170 replacements were verified by PCR on whole yeast cells as described previously (Gola et al.,
171 2003; Schaub et al., 2006).

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

175 Invitrogen). Construction of the Smi1 over-expressing strain was performed by recombination
176 of pEntry-SMI1 plasmid and pCip10-P_{TDH3}-GTW plasmid (Chauvel et al., 2012) also using
177 the Gateway systemTM (Invitrogen), followed by genome integration through targeted
178 homologous recombination at the genomic *RPS10* locus in the host strain BWP17.

179

180 ***GFP tagging of Smi1***

181 Smi1 was C-terminally-tagged with GFP by amplifying GFP-NAT cassette from the pGFP-
182 NAT1 plasmid (Milne et al., 2011) using primers gSmi1_F^a and gSmi1_R^a containing 100 bp
183 of flanking homology to the *SMI1* terminator and C-terminal of the *SMI1* ORF
184 (C1_07870C_A), respectively (Figure X –primer table). Transformants were selected on YPD
185 agar containing 300 µg/ml nourseothricin (Sigma). Integration of the GFP-NAT cassette was
186 confirmed by PCR using primers sSmi1_F and sFP_R, which anneal to the chromosome
187 outside the targeted region and within the cassette, respectively (primer sequences are
188 provided in Supplementary material Table S1).

189

190 ***Microscopy of Smi1-GFP***

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

198

199 ***Phenotypic Sensitivity tests:***

200 Drop tests to evaluate the sensitivity of different strains and mutants to cell wall affecting
201 drugs were performed as previously described (Ram et al., 1998) with minor modifications
202 (Martin et al., 1999). Briefly, yeast cells were grown in liquid YPD to OD₆₀₀ of 1+/- 0.1, then
203 concentrated by centrifugation and resuspended in sterile water to an OD₆₀₀ of 8. Serial
204 dilutions of 1/1, 1/10, 1/100 and 1/1000 were then spotted on solid media containing either
205 calcofluor white or Caspofungin at the indicated concentrations. Growth was scored and
206 photographs taken after 48h of incubation at 30 °C, or at 37 °C for testing the sensitivity to
207 elevated temperature.

208

209 ***Atomic Force Microscopy:***

210

211 ***Sample preparation for AFM experiments.***

212 Yeast cells were concentrated by centrifugation, washed two times in acetate buffer (18 mM
213 CH₃COONa, 1mM CaCl₂, 1mM MnCl₂, pH5.2), resuspended in the same buffer, and
214 immobilized on polydimethylsiloxane (PDMS) stamps prepared as described by Dague et al.
215 (Dague et al., 2011; Formosa et al., 2014a). Briefly, freshly oxygen-activated microstructured
216 PDMS stamps were covered with a total of 100 µl of cell suspension and allowed to stand for
217 15 min at room temperature. Yeast cells were then deposited into the stamps microstructures
218 by convective (capillary) assembly.

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

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

233

234

235 ***Fluorescence Microscopy:***

236

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

244

245 **Results:**

246

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

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

255 The *SMI1* open reading frame was amplified and cloned into a *S. cerevisiae* expression
256 vector, under the control of the strong and constitutive *ADHI* promoter. The *SMI1* coding
257 sequence is 1,863 bp and contains two GTC codons at positions 1717 and 1762, which have a
258 97 % chance of translation as a Serine in *C. albicans* through non-canonical codon usage in
259 this fungus, compared to a Leucine in *S. cerevisiae* (White et al., 1995), although these usage
260 frequencies appear to be sensitive to environmental stressors (Simões et al., 2016). These two
261 codons are located within the C-terminal of *Smi1*, which is less conserved than the central
262 domains among members of the fungal *Knr4/Smi1* super-family of proteins. The complete
263 sequence alignment of several fungal protein members of this super-family is shown in Figure
264 S2. The C-terminal part of *Knr4* is largely unstructured and not directly necessary for protein
265 function in *S. cerevisiae* (Dagkessamanskaia et al., 2010; Martin-Yken et al., 2016). Hence,
266 we tested the ability of the *C. albicans* *SMI1* gene, retaining its two ambiguous codons, to
267 complement the cell wall-related phenotypes of *S. cerevisiae* *knr4* null mutants. Our
268 phenotypic screen included sensitivity to Calcofluor White (CFW), SDS, caspofungin and
269 elevated temperature in haploid and diploid *S. cerevisiae* genetic backgrounds. CFW is a
270 compound that binds to nascent chitin fibrils and has been used since 1994 to identify fungal
271 cell wall mutants (Ram et al., 1994). Expression of *SMI1* gene in *S. cerevisiae* was able to
272 complement these phenotypes (Figure 1A) and also complemented the *smi1* Δ growth defect at
273 elevated temperature (Fig.1B), a specific defect in this organism that is linked to cell cycle
274 progression through START (Fishel et al., 1993, Martin-Yken et al., 2003). Hence, the
275 function of the *Smi1/Knr4* proteins appears to be conserved between the two species despite
276 their phylogenetic distance and the differences between the two protein sequences, which
277 share only 34% identity and 49% similarity (Figure S1).

278

279

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

281

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).

292

293

294 **3. Cell Wall Biophysics**

295

296 **3.1 Cell Wall Strength / Elasticity**

297

298 Atomic Force Microscopy under liquid conditions can be used to investigate the
299 nanomechanical 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 $YM = 1 / \text{Elasticity}$. 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

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

320

321

322 **3.2 Adhesion:**

323 Another cell surface feature that can be easily and very precisely measured by AFM is
324 the ability to adhere to surfaces, using Single Molecule Force Spectroscopy (Axner et al.,
325 2010; Benoit et al., 2000; Formosa et al., 2014a; Hinterdorfer et al., 1996; Neuman and Nagy,
326 2008). Here, adhesion between the cell surface and the AFM bare tips, constituted of Si_3N_4
327 was measured by scanning square areas on the top of yeast cells. We recorded force curves
328 whose retract parts were then used to generate adhesion maps where the intensity of each
329 pixel corresponds to the force required to dissociate the AFM tip from the sample, *i.e.* the
330 adhesion force, expressed in picoNewtons pN (see Figure 4). It can be seen immediately from
331 these heat maps that the *smi1 Δ/Δ* mutant cell surface is almost not adhesive compared to the
332 control strain, while the *SMII* overexpressing cells on the other hand display a marked
333 increase in their surface adhesion. In addition to the visual representation by these adhesion
334 maps, the cellular adhesion was also evaluated by three quantitative parameters: the mean
335 adhesion force and specific energy of each adhesion event observed and the overall
336 proportion of these adhesive events among all the recorded force curves. In order to quantify
337 these values we defined as an adhesive event any force curve showing an adhesion force
338 above 50pN. With this threshold level we calculated the percentage of adhesive events for
339 each cell type and measured the area situated below the retract curves, which represent the
340 adhesion energy of the event (Table 2). These values indicated that adhesive events were
341 encountered more frequently at the surface of the over-expressing strain SMII-OE (63% of
342 the recorded 21,500 force curves) than on the control strain (46% of over 13,300 force
343 curves), and were rare on the deletion mutant surface (19% of over 12,300 force curves). The
344 average adhesion force measured on the control strain was of 127 ± 52 pN, calculated on
345 6256 adhesive force curves. This has to be compared with an average force of 70 ± 16 pN

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

356

357

358

359 **4. Cellular Localization:**

360

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

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

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

383

384

385

386 **DISCUSSION**

387

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

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

419

420 Our results show that homozygous deletion of *SMII* gene leads to a marked increase in
421 the sensitivity of *C. albicans* to both CFW and Caspofungin at 30 °C. Since Caspofungin
422 tolerance has been reported in *C. albicans* mutants with elevated cell-wall chitin (Perlin,
423 2015; Plaine et al., 2008; Walker et al., 2013; Yang et al., 2017) while elevated chitin is
424 known to induce hypersensitivity to CFW (Elorza et al., 1983; Roncero and Durán, 1985), this
425 finding may appear puzzling at first glance. However, this is consistent with the phenotypes
426 observed for the *S. cerevisiae knr4* Δ mutant (Lesage et al., 2004; Martin et al., 1999).
427 Moreover, as our cell wall stiffness measurements, these phenotypes indicate a severely
428 modified cell wall organization of the deletion mutant *smi1* Δ/Δ compared to the control strain,
429 coherent with a possible upstream role of Smi1 in cellular signaling pathways allowing this
430 protein to influence simultaneously the expression level of different cell wall synthesis genes.
431 Consistent with this, early results obtained in the bakers' yeast established a role for Knr4 in
432 the transcriptional control of all *S. cerevisiae* chitin synthase genes (Martin et al., 1999). The
433 complex interplay of cellular signaling pathways controlling *C. albicans* susceptibility to
434 echinocandins has been described by Munro and colleagues (Walker et al., 2010), and it now
435 seems possible that Smi1 is one piece of this puzzle.

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

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

447 localization has been reported for the MAP kinase Slr2 and for Calmodulin, the phosphatase
448 which activates Calcineurin (Brockerhoff and Davis, 1992; van Drogen and Peter, 2002). In
449 the model filamentous fungus, *N. crassa*, GS1, the homolog of Smi1, localizes at the
450 Spitzenkörper within the hyphal tip (Verdin et al., 2009; Sánchez-León et al., 2011). This
451 localization is conserved in another ascomycete filamentous fungus, *A. nidulans* (also called
452 *Emericella nidulans*), (Schultzhaus et al., 2015). The results presented here indicate that Smi1
453 also localizes in or around the Spitzenkörper of *C. albicans* hyphae in a similar manner to that
454 observed for GS-1 in *N. crassa*. Finally, given the cell wall related phenotypes reported for
455 GS-1 mutants of *N. crassa* (Enderlin and Selitrennikoff, 1994; Resheat-Eini et al., 2008;
456 Seiler and Plamann, 2003), it is tempting to speculate that the function of these proteins is not
457 only conserved between *C. albicans* and *S. cerevisiae*, but also, to some extent at least, in
458 filamentous fungi. The role of Smi1 in the control of cell wall synthesis, cellular adhesion and
459 drug resistance is of great interest with regard to finding new antifungal targets. The decisive
460 advantage of Smi1 as a drug target over Hsp90, calcineurin, Pkc1 or other MAP kinases is the
461 fact that the Knr4/Smi1 superfamily of proteins is specific of the Fungal Kingdom and absent
462 from host cells (Martin-Yken et al., 2016). In addition, since this protein family is conserved
463 among fungi, including other fungal pathogens of mammals (*C. glabrata* and *Aspergillus*
464 *species* notably) and also plants (ex: *Magnaporthe grisea*), developing drugs that target Smi1
465 might lead to broader antifungal applications in domains such as agriculture.

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478 advice to "*Start working on C. albicans*".

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Strain name	Genotype	References
<i>Candida albicans</i> strains :		
BWP17	<i>ura3Δ::λimm434/ura3Δ::λimm434, arg4Δ::hisG/arg4Δ::hisG, his1Δ::hisG/his1Δ::hisG</i>	(Wilson et al., 1999)
CEC161	Isogenic to BWP17 but <i>arg4Δ::hisG/ARG4, his1Δ::hisG/HIS1</i>	(Firon et al., 2007)
BWP17 AHU	Isogenic to CEC161 but <i>RPS1/rps1::Clp10-URA3</i>	(Chauvel et al., 2012)
<i>smi1Δ/Δ</i>	Isogenic to BWP17 but <i>smi1Δ::HIS1/smi1Δ::ARG4</i>	This study
<i>smi1Δ/Δ - AHU</i>	Isogenic to <i>smi1Δ/Δ</i> but <i>RPS1/rps1::Clp10-URA3</i>	This study
<i>smi1Δ/Δ + P_{TDH3} SMI1</i>	Isogenic to <i>smi1Δ/Δ</i> but <i>RPS1/rps1::Clp10-P_{TDH3}-SMI1</i>	This study
<i>smi1BΔ/Δ</i>	Isogenic to BWP17 but <i>smi1BΔ::HIS1/smi1BΔ::ARG4</i>	This study
SMI1-OE	Isogenic to CEC2908 but <i>RPS1/rps1::Clp10-P_{TDH3}-SMI1</i>	This study
DAY185	<i>ura3::imm434/ura3::imm434 his1::hisG::HIS1/his1::hisG arg4::hisG::ARG4-URA3/arg4::hisG</i>	Davis et al., 2000
SMI1-GFP	DAY185 <i>SMI1::SMI1-GFP-NAT</i>	This study
<i>Saccharomyces cerevisiae</i> strains :		
BY4741a	MAT a; <i>his3Δ1 leu2Δ0; met15Δ0; ura3Δ0</i>	(Brachmann et al., 1998)
<i>knr4Δ</i>	BY4741a <i>YGR229c::KanMX4</i>	YKO Collection (Open Biosystems)
W303-2N	MAT a/α <i>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</i>	
HM1315	W303-2N <i>YGR229c::KanMX4/ YGR229c::KanMX4</i>	This study.

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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)
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BWP17 AHU	782 (+/- 158)	46.1	127 (+/- 52)	1.77 x10⁻¹⁷
<i>smi1</i> 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

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491 **Table 2: Summary of Atomic Force Microscopy measurements for BWP17 AHU,**492 ***smi1*Δ/Δ AHU, and SMI1-OE strains.**

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494 Column 1: Mean values with standard deviation of Young's Moduli calculated from force
495 curves obtained as described above (3.1).496 Column 2: Percentage of adhesive events measured by AFM, calculated from at least 12,000
497 force curves for each cell type, with a threshold level for the definition of an adhesive event as
498 50pN on the retraction curve.499 Column 3: Mean values of Adhesion forces for each cell type, calculated from adhesive force
500 curves obtained as described above.501 Column 4: Mean values of the Adhesion Energy for each strain, calculated from the area
502 below the force curves presenting an adhesion event.

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LEGENDS TO FIGURES

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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*SMI* bearing *C. albicans* *SMI* gene or p*KNR4* bearing *S. cerevisiae* *KNR4* gene were grown in liquid SD medium lacking uracile 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 control strain W3032N and mutant strain HM1315 *knr4* Δ/Δ with either empty YEplac195 *PGK/CYC1* plasmid or p*SMI* (Yeplac195 bearing *C. albicans* *SMI* 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 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.

Figure 2. Calcofluor White and caspofungin sensitivity of the *C. albicans* *smi1* Δ/Δ mutant.

Control strain BWP17 and mutant strain *smi1* Δ/Δ 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 40mg of CFW or 150 ng caspofungin ml⁻¹. Growth was scored after 2 days at 30°C.

Figure 3. Young Moduli of *smi1* Δ/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.

539

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

542 Adhesion force measurements performed on single *C. albicans* cells by Atomic Force
543 Microscopy. The adhesion maps presented have been recorded on three independent and
544 representative *C. albicans* cells for each cell type. Each analyzed area covers $1 \times 1 \mu\text{m}^2$.

545 Adhesion scales are shown and read as follows: bright yellow = maximum adhesion force at 2
546 nN; dark red = minimum at 0.0 nN.

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548 **Figure 5. Smi1-GFP localizes to apical growth sites in yeast and hyphae.** Cells were
549 grown on Ibidi μ -slides in YNB medium at 30°C for 2h (yeast) and 20 % FCS, 2 % glucose at
550 37°C for 3h (hyphae). Smi1-GFP localised transiently to emerging bud tips in yeast (arrows)
551 and to septa in hyphae (asterisks) but was maintained consistently at hyphal/branch tips
552 (arrows). Punctate fluorescence patches were also observed throughout yeast and hyphal cells.
553 Images are maximum projections of individual z-stacks.

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