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Candida albicans Iff11, a secreted protein required for cell wall structure and virulence

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1 **ABSTRACT**

2 The *Candida albicans* cell wall is the immediate point of contact with the host
3 and is implicated in the host-fungal interaction and virulence. To date a number of
4 cell wall proteins have been identified and associated with virulence. Analysis of the
5 *C. albicans* genome has identified the *IFF* gene family as encoding the largest family
6 of cell wall related proteins. This family is also conserved in a range of other
7 *Candida* species. Iff11 differs from other family members in lacking a GPI anchor,
8 and we have demonstrated it to be *O*-glycosylated and secreted in *C. albicans*. A null
9 mutant lacking *IFF11* was hypersensitive to cell wall damaging agents, suggesting a
10 role in cell wall organization. In a murine model of systemic infection the null mutant
11 was highly attenuated in virulence, and survival-standardized infections suggest it is
12 required to establish an infection. This work provides the first evidence of the
13 importance of this gene family in the host-fungal interaction and virulence.

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14 ## INTRODUCTION

15 *Candida albicans* is the most common opportunistic fungal pathogen of
16 humans. It is carried as a commensal in a significant proportion of individuals and
17 can cause superficial epithelial infections and, in the immunocompromised, life-
18 threatening systemic infections (5, 21, 24). A number of different virulence factors
19 are involved with *C. albicans* pathogenicity, of these the cell wall and secreted
20 proteins are proposed to play a key role (15, 26, 27). The cell surface of *C. albicans*
21 is the first point of contact with the host and plays a role in adhesion, colonization and
22 immunomodulation (6, 20, 25, 28). The cell wall is comprised of an inner layer
23 consisting of glucans and chitin which provide mechanical strength, and an outer layer
24 enriched in mannoproteins (7, 15, 20, 26) that determine cell surface properties and
25 play a vital role in the host-fungal interaction.

26 The major class of cell surface mannoproteins is the covalently attached
27 glycosylphosphatidylinositol (GPI)-linked cell wall proteins (GPI-CWPs) (15, 25).
28 These have a common structure consisting of an N-terminal signal sequence,
29 conserved domain, variable length Ser/Thr rich region and C-terminal GPI-anchor.
30 Genomic approaches have identified 104 open reading frames in the *C. albicans*
31 genome encoding potential GPI-CWPs (11). To date only 12 GPI-CWPs have been
32 shown to be expressed by proteomic analysis of cell walls derived from exponentially
33 growing yeast cells (10). These include 5 carbohydrate-active enzymes (Cht2, Crh11,
34 Pga4, Phr1 and Scw1), potential adhesion proteins of the *ALS* family (Als1 and Als4),
35 a superoxide dismutase (Sod4) and others (Ywp1, Ecm33, Rbt5 and Ssr1). It is
36 proposed that the majority of GPI-CWPs are differentially expressed under different
37 growth conditions.

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38 The *IFF* gene family was initially identified during the annotation of the

39 *C. albicans* genome (9) and includes the previously reported hypha-specific GPI-

40 CWP *HYR1* (1). In the current annotation of the genome there are 12 members of the

41 family (*IFF1-11*, *HYR1*): it is therefore the largest family of cell wall and related

42 proteins. A similar family of varying number is also present in a range of other

43 pathogenic *Candida* species. We chose to target *IFF11* from the family for

44 experimental investigation. Uniquely among *IFF* family members it lacked a

45 potential GPI-anchor or transmembrane domain, so its analysis was therefore

46 expected to avoid the problem of functional redundancy commonly associated with

47 gene families. Moreover, we were intrigued by the possibility that, without a GPI-

48 anchor, the protein may play a role both inside and outside the cell. Analysis of the

49 localization of Iff11 showed it to be secreted and not associated with the cell wall.

50 Deletion of *IFF11* resulted in a cell wall defect identified through hypersensitivity to

51 cell wall stress. In a murine model of infection the *iff11*Δ null mutant was highly

52 attenuated in virulence. This work provides the first example of this conserved gene

53 family's role in virulence.

54

55 **MATERIALS AND METHODS**

56 **Strains, media and culture conditions.** All strains constructed and used in

57 this study are detailed in Table 1. Strains were grown as yeasts at 30 ºC in YEPD

58 medium (1% yeast extract, 2% mycological peptone [Oxoid, Basingstoke, UK], 2%

59 glucose), SD medium (0.67% yeast nitrogen base, 2% glucose) or SC-U (0.67% yeast

60 nitrogen base, 2% glucose, 0.077% complete supplement mixture minus uracil

61 [Qbiogene, Cambridge, UK]). Uridine (50 µg/ml) was added to media as required.

62 Pseudohyphal cells were induced by subculture of stationary phase cells into fresh

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63 YEPD at 37 ºC. Hyphal cells were induced in YEPD + 20% (v:v) newborn calf
64 serum, RPMI 1640, Lee's medium pH 6.5 (16) at 37 ºC, or solid spider medium at
65 30 ºC (17). Prior to virulence testing strains were grown at 30 ºC in NGY medium
66 (0.1% neopeptone [Becton-Dickinson, Cowley, Oxford, UK], 0.1% yeast extract,
67 0.4% glucose) for 16-18 h.

68 **Construction of *iff11*Δ null mutant and re-integrant strains.** The *IFF11*
69 gene was disrupted by ura-blasting (12). The regions of homology were amplified by
70 PCR (upstream primer pair 5'-ATTTGTATGTTGCTCTAGGC-3' and 5'-
71 GTCGACTAGTGTTGAGCTGAAG-3', SalI restriction site underlined; and
72 downstream primer pair 5'-AGATCTATCTTCAAAATCACCAC-3' and 5'-
73 GAGCTCCTCGTCAGTGTTTGTCA-3', BglII and SacI restriction sites underlined
74 respectively) and cloned into pMB-7 (12). The disruption cassette was released by
75 digestion with HindIII and SacI and was flanked by 468 bp upstream and 500 bp
76 downstream to target *IFF11*. *IFF11* was disrupted in strain CAI-4 by sequential
77 rounds of gene replacement and recycling of the *URA3* marker by selection on SD
78 medium plus 5-fluoroorotic acid (1 mg/ml) and uridine (50 μg/ml). The *ura* null
79 mutant was transformed with StuI-digested CIp10 (19) to avoid potential problems
80 associated with the level of *URA3* expression (4). As a control a re-integrant strain
81 was constructed in which the wild type gene was introduced into the null mutant. The
82 *IFF11* open reading frame plus 491 bp promoter and 476 bp terminator were
83 amplified by PCR (primer pair 5'-ATTTGTATGTTGCTCTAGGC-3' and 5'-
84 GAGCTCCTCGTCAGTGTTTGTCA-3') and cloned into pGEM-T Easy (Promega
85 Ltd, Southampton, UK). The plasmid inserted was subcloned into the NotI site of
86 CIp10. The resulting plasmid was linearized with StuI and transformed into the
87 *iff11*Δ null mutant.

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88 **Construction of *IFF11* tagged and overexpressing strains.** For

89 overexpression studies the enolase (*ENO1*) promoter was utilized. The *ENO1*

90 promoter (-983 to -1) was amplified by PCR (primer pair, 5'-

91 GAGCTCCATTTGTATCTTTAGTAGACATG-3' and 5'-

92 GCGGCCGCTGTTGTAATATTCCTGAATTATC-3', SacI and NotI restriction sites

93 underlined respectively) and cloned into pGEM-T Easy. The plasmid insert was

94 subcloned into the relevant sites of CIp10 to create CIp10-ENO a convenient vector

95 for constructing overexpressing strains.

96 To internally tag *IFF11* with the V5 epitope the open reading frame plus 540

97 bp promoter and 496 bp terminator was amplified by PCR (primer pair, 5'-

98 GAAAATCAATATCGTTAGTAGTG-3' and 5'-

99 CAAAATACATAGACAATGACTC-3') and cloned into pGEM-T Easy. The

100 resulting vector was amplified by inverse PCR (primer pair, 5'-

101 GAAGGATCCGGTAAGCCTATCCCTAACCCTCTCCTCGGTCTCGATTCTACG

102 GCTATCACAAGCACCCAATC-3' and 5'-

103 GAAGGATCCGGTGGCACTTGATTAATAAC-3', BamHI sites underlined), the

104 product digested with BamHI and self ligated to introduce the V5 epitope tag in

105 frame. The internally tagged open reading frame plus promoter and terminator were

106 then subcloned on a NotI fragment into CIp10 to create CIp10-IFF11-V5. For

107 overexpression studies either the wild type or V5 tagged open reading frame plus 496

108 bp terminator were amplified by PCR (primer pair, 5'-

109 AAGATGTTATTGTCAAACCTTG-3' and 5'-CAAAATACATAGACAATGACTC-

110 3', start codon underlined) and cloned into pGEM-T Easy. The plasmid inserts were

111 then cloned on a NotI fragment into CIp10-ENO to create CIp10-ENO-IFF11 and

112 CIp10-ENO-IFF11-V5 respectively. Orientation of the open reading frame in relation

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113 to the *ENO1* promoter was confirmed by PCR. To construct *IFF11* overexpressing
114 and tagged strains CIp10-IFF11-V5, CIp10-ENO-IFF11 and CIp10-ENO-IFF11-V5
115 were linearized with StuI and independently transformed into both CAI-4 and
116 SBC101 (*iff11*Δ ura^- null mutant).

117 **Preparation of protein extracts and western blot analysis.** Secreted protein
118 fractions from cells grown in SC-U were filter sterilized (0.45 μm pore diameter) then
119 concentrated and buffer exchanged to phosphate-buffered saline (PBS) by
120 ultrafiltration (Amicon ultra-10 kDa cutoff; Millipore, Watford, UK). Samples were
121 then further concentrated by ethanol precipitation and reconstituted in PBS. Whole
122 cells were extracted with β-mercaptoethanol according to published protocols (29).
123 Briefly, washed cells were incubated in ammonium carbonate buffer (pH 8)
124 containing 1% β-mercaptoethanol for 30 min at 37 °C. The supernatant was then
125 collected by centrifugation, buffer exchanged and concentrated by ultrafiltration and
126 ethanol precipitation as before.

127 Cell walls were prepared from β-mercaptoethanol-extracted cells by a method
128 modified from De Groot *et al*. (10). Cells were resuspended in 10 mM Tris-HCl
129 pH 7.5 plus protease inhibitor (Roche Applied Science) and disrupted by glass beads
130 in a Fast Prep machine (Qbiogene). Cell walls were washed extensively with 1 M
131 NaCl and water and then extracted twice for 5 min at 100 °C in 2% (w/v) sodium
132 dodecyl sulfate (SDS), 100 mM EDTA, 40 mM β-mercaptoethanol, 50 mM Tris-HCl
133 pH 7.5 to remove nonconvalently attached proteins and cytoplasmic contaminants.
134 Cell walls were then washed in water and freeze dried. Mild alkali extracts were
135 prepared by treating cell walls with 30 mM NaOH for 16 h at 4 °C. The reaction was
136 stopped by neutralization with 30 mM acetic acid, followed by the addition of 0.4%
137 SDS and incubation at 100 °C for 5 min. GPI-linked cell wall proteins were released

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138 by resuspending cell walls in HF-pyridine (Sigma-Aldrich, Gillingham, UK) at 4 ºC

139 for 3 h. The reaction was quenched by adding an equal volume of water prior to

140 dialysis to remove HF-pyridine. Mild alkali and HF-pyridine extracts were freeze

141 dried and resuspended in PBS. All secreted and cell wall extracts were prepared

142 sequentially from the same set of cells and extracts were resuspended at a volume to

143 represent the proportion of original culture volume. Soluble protein extracts were

144 prepared according to standard protocols (3), and 50 µg of protein was used in

145 subsequent analysis.

146 Protein extracts were separated on a 4-12% NuPAGE bis-Tris gel (Invitrogen,

147 Paisley, UK) and blotted onto a polyvinylidene difluoride membrane. The membrane

148 was blocked with 5% bovine serum albumin (BSA) in TBS-T (Tris buffered saline

149 containing 0.1% Tween-20) for 2 h at room temperature and subsequently incubated

150 with the primary antibody, mouse anti-V5 (Invitrogen), at 1:5000 dilution in TST-T

151 for 1 h. The membrane was then washed twice for 5 min and twice for 15 min in

152 TST-T before incubation with the horseradish peroxidase-conjugated secondary

153 antibody, anti-mouse IgG-HRP (Invitrogen), at 1:10000 dilution in TBS-T for 1 h.

154 After the final washes, twice for 5 min and twice for 15 min in TST-T, the membrane

155 was processed and exposed to film according to manufacturer's instructions

156 (Lumiglo; New England Biolabs, Hitchin, UK).

157 **Phenotypic analysis.** Strains were screened for hypersensitivity to cell wall

158 perturbing agents by the microdilution method and for phosphomannan content by

159 Alcian Blue binding as described previously (2, 3, 13). Briefly, for cell wall

160 sensitivity, standardised inocula were prepared from 24-h YEPD cultures by washing

161 cells with water and resuspending at A_{600} = 1. YEPD medium was inoculated with the

162 strains at an A_{600} = 0.01 and 95 µl volumes dispensed into 96-well microtiter plates.

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163 Cell wall stressing agents were added in a 5 µl volume across a range of doubling
164 dilutions. Plates were incubated for 16 h at 30 ºC and the A_{600} determined. The
165 agents tested were: Calcofluor White (500 µg/ml), Congo Red (500 µg/ml), SDS
166 (0.1%), hygromycin B (500 µg/ml), NaCl (1 M), KCl (1 M), caffeine (50 mM),
167 vanadate (80 mM) and tunicamycin (100 µg/ml). The concentrations listed are the
168 maximum concentration tested for each agent.

169 Cell surface hydrophobicity was screened in an octane partitioning assay.
170 Washed stationary phase cells (2 ml) were thoroughly mixed with octane (1 ml) and
171 allowed to separate for 5 min. Once separated the change in A_{600} of the aqueous
172 phase was calculated, and the proportion of cells that were hydrophobic and
173 partitioned with the octane layer determined. Adherence of yeast cells to buccal
174 epithelial cells (BEC) was assessed as described previously (3, 23); at least 150 BEC
175 were scored for adherence. A range of enzyme activities was screened for in
176 concentrated secreted protein extracts (10 kDa ultrafiltration residues of membrane-
177 sterilized culture filtrates) and intact cells. For exoglucanase activity a range of
178 glucans (cellulose, glycogen, laminarin and pustulan) was incubated with
179 concentrated secreted protein extracts and glucose released measured enzymically
180 (Glucose (GO) assay kit; Sigma-Aldrich). Protease activity was measured as
181 previously described (8). API ZYM kits (BioMérieux Industry, Missouri, USA) were
182 used according to the manufacturer's instructions to detect activities of acid and
183 alkaline phosphatases, C4 esterase, C8 esterase-lipase, C14 lipase, leucine, valine and
184 cysteine aminopeptidases, trypsin, chymotrypsin, phosphoamidase, α- and β-
185 glucosidases, α- and β-galactosidases, β-glucuronidase, *N*-acetyl-β-D-
186 glucosaminidase, α-mannosidase and α-fucosidase. API ZYM tests were done with

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187 wild type, *iffΔ* null mutant and the overexpressing strain, as well as with concentrated
188 secreted protein extract.

189 To study the possible effects *in trans* of Iff11 on cell wall hypersensitivity, a
190 concentrated culture filtrate was used in rescue experiments both by addition to the
191 Calcofluor White sensitivity assay and by addition to the pre-culture used to grow the
192 inocula of wild type and *iff11*Δ cells.

193 **Virulence studies.** For virulence tests female immunocompetent BALB/c
194 mice were challenged intravenously. Challenge strains were grown in NGY medium
195 at 30 ºC for 16-18 h and cells were then washed in water and resuspended in
196 physiological saline. Groups of 6 mice were challenged with $2x10^4$ colony forming
197 units (cfu)/g body weight and monitored over 28 days. Mice showing signs of illness
198 or distress were humanely terminated and recorded as dying on the following day.
199 Mice surviving the course of the experiment were humanely terminated on day 28.
200 Kidneys and brain were removed post mortem, homogenized and tissue burdens
201 calculated by viable counting. Doses for a survival-standardized infection were
202 determined by challenging pairs of mice intravenously with varying doses of the test
203 strains and monitoring outcome. For survival-standardized infection studies (22)
204 groups of 3 mice were infected with challenge strains, humanely terminated on days
205 2, 4 and 7 post infection and spleen, kidney and brain tissue burdens determined. All
206 animal experimentation was done in accordance with UK Home Office regulations
207 and was approved by both the Home Office and an institutional ethical review
208 committee. Data from virulence studies were analyzed statistically by Kaplan-
209 Meier/LogRank tests for survival and by Mann-Whitney *U* tests for burden
210 differences.

211 **RESULTS AND DISCUSSION**

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212 **The *IFF* gene familiy.** The *IFF* (IPF Family F) gene family was initially
213 identified in the annotation of the *C. albicans* genome, CandidaDB (9). In the release
214 of the genome called assembly 19 there are 12 members of the gene family, *IFF1* to
215 *IFF11* and the previously reported hypha-specific gene *HYR1* (1) (Table 2; assembly
216 20 was released after this work was completed). The majority of the family displays a
217 structure characteristic of GPI-anchored cell wall proteins, consisting of signal
218 sequence, conserved domain, variable length Ser/Thr rich region and potential GPI-
219 anchor (Table 2). The homology between family members is mainly found in the
220 conserved N-terminal domain of 350 amino acids (Table 3). Multiple sequence
221 alignments of the conserved domain demonstrate clustering of some family members
222 suggesting recent duplication. The conserved domain does not display significant
223 homology to proteins of known function. In addition to the Ser/Thr rich region which
224 is potentially *O*-glycosylated, all family members except Iff11 also contain potential
225 *N*-glycosylation sites. Iff7 and Iff11 also deviate from the general structure displayed
226 by family members as both lack a potential GPI-anchor site. In the case of Iff7 the
227 protein contains a potential C-terminal transmembrane domain suggesting it is
228 membrane bound. As Iff11 lacks both a potential GPI-anchor signal and a potential
229 transmembrane domain it is likely either to be secreted or attached to the cell wall by
230 other means. Iff11 also displays the lowest level of homology to the majority of other
231 members (30-39% identity), except Iff10 (56.6% identity). *IFF11* is directly
232 downstream of *IFF10* on chromosome 3, suggesting they resulted from tandem
233 duplication.

234 A similar gene family of varying number can be identified in a range of related
235 fungi including the *Candida* species *C. dubliniensis*, *C. guilliermondii*, *C. lusitaniae*,
236 *C. tropicalis*, *C. glabrata*, *Kluyveromyces lactis* and *Debaryomyces hansenii*.

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237 However, the family is not present in *Saccharomyces cerevisiae* or in the genome
238 sequences currently available for a range of fungi pathogenic to humans including
239 *Aspergillus fumigatus, A. terreus, Coccidioides immitis, Cryptococcus neoformans,*
240 and *Histoplasma capsulatum*: our search extended to these and other fungal genomes
241 currently available (http://www.broad.mit.edu/annotation/fgi/). The *C. albicans*
242 genome also carries the *ALS* gene family of cell wall proteins (14). The *IFF* family is
243 of comparable size and these are the largest families of cell wall related proteins in the
244 genome as presently annotated.
245 As *IFF11* is a family member that differs in lacking a GPI-anchor, and hence
246 may be localized differently, it was chosen for further analysis as it may not display
247 functional redundancy — a problem commonly associated with the analysis of gene
248 families. The previously identified member of the family, *HYR1* is known to display
249 hypha-specific expression but its inactivation generated no obvious phenotype (1).
250 We therefore analyzed the expression of *IFF11*. *IFF11* mRNA was found to be
251 constitutively expressed in yeast, pseudohyphal and hyphal growth forms, and
252 compared to positive control *EFB1* was expressed at a low level (data not shown).
253 **Localization of Iff11**. The bioinformatics analysis of Iff11 suggested that it
254 lacks a potential GPI-anchor signal. We therefore tested whether it is secreted or cell
255 wall-associated. In order to localize Iff11 an internal epitope tag approach was
256 chosen to avoid potential problems with alteration of the N- or C- terminal signals.
257 An internal V5 epitope tag was inserted into a *IFF11* cassette through inverse PCR
258 and self ligation. This was targeted to insert the V5 tag in frame at position 349 aa
259 between the conserved domain and the Ser/Thr rich region. To determine the cellular
260 localization of Iff11 secreted and cell wall fractions were analyzed by western
261 blotting. When the V5-tagged version of *IFF11* was expressed under its native

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262 promoter no signal could be detected, either in secreted, cell wall or total protein
263 extracts. This is consistent with the finding that *IFF11* was constitutively expressed
264 at a low level. Therefore, for localization studies Iff11-V5 was overexpressed under
265 the control of the *ENO1* (enolase) promoter. Western blot analysis of total soluble
266 protein extracts identified three specific bands associated with Iff11-V5
267 overexpression (Fig. 1A, lane 9, 10) present at 53 kDa, 64 kDa and 90 kDa. The 53
268 kDa protein band matched the predicted size of Iff11 after processing of the N-
269 terminal signal sequence. The presence of the specific 64 kDa band on western blot
270 analysis is suggestive of an intermediate in post translational modification. The 90
271 kDa band is presumably fully modified (see later), mature Iff11 present in the
272 secretory pathway. To determine the localization of Iff11, secreted and cell wall
273 fractions were analyzed (Fig. 1A). No specific bands could be detected in a range of
274 cell wall fractions, including non-convalently attached proteins (whole cell β-
275 mercaptoethanol- and SDS-treated cell wall extracts) and covalently attached proteins
276 (mild alkali, HF-pyridine and β-1,3-glucanase extracts). In contrast, the secreted
277 protein fraction contained a specific band at ~90 kDa. Therefore, Iff11 appears to be
278 a secreted protein and is not found associated with the cell wall. However, it is not
279 possible to discount that a small, non-detectable, level of Iff11 may be associated with
280 the cell wall.

281 Modification by glycosylation is common for cell wall and secreted proteins.
282 To access the nature of Iff11 modification the Iff11-V5 secreted protein extracts were
283 treated with endoglycosidase H (endo H) and jack bean mannosidase (Fig. 1B).
284 Consistent with bioinformatics analysis Iff11 was found not to be modified by *N*-
285 glycosylation as there was no apparent shift in mobility after treatment with endo H.
286 However, after treatment with jack bean mannosidase there was a clear increase in the

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287 mobility of Iff11-V5. This, in combination with the absence of any effect of endo H,

288 implies that Iff11 is modified by *O*-glycosylation. The increase in molecular mass

289 from the predicted (53 kDa) to that observed (~90 kDa) is suggestive of extensive *O*-

290 glycosylation. *C. albicans* *O*-mannan comprises one to five α-1,2-linked mannose

291 residues (18), the first added in the endoplasmic reticulum (ER) and subsequent

292 residues in the Golgi. Jack bean mannosidase treatment would not release the first

293 residue of *O*-mannan, hence the mass of Iff11-V5 did not return to that predicted for

294 the unmodified form. The mobility of Iff11-V5 after jack bean mannosidase

295 treatment was close to that of the modification intermediate present in the soluble

296 protein extract. This is suggestive of a potential block in the secretory pathway at the

297 point of trafficking between the ER and Golgi in cells overexpressing Iff11-V5. No

298 difference in the localization or modification of Iff11-V5 was apparent in yeast or

299 hyphal cells (data not shown). In summary, localization studies suggest that Iff11 is

300 an extensively *O*-glycosylated secreted protein.

301 **Deletion of *IFF11*.** Both alleles of *IFF11* were disrupted in strain CAI-4 by

302 sequential gene deletion with the ura-blaster protocol (12), resulting in the deletion of

303 the entire open reading frame. *URA3* was introduced into the *iff11*Δ null mutant at the

304 *RPS1* locus to avoid potential problems with the level of *URA3* expression (4). *IFF11*

305 was also reintroduced into the *iff11*Δ null mutant to act as a re-integrant control. In

306 addition *IFF11*-V5 was introduced into the null mutant to confirm function of V5

307 tagged Iff11, and *IFF11* was also overexpressed under the control of the *ENO1*

308 (enolase) promoter.

309 Deletion of *IFF11* had no effect on growth rate in complete (YEPD) or

310 minimal (SD) medium. There was also no obvious effect on hyphal morphogenesis in

311 response to serum, Lee's medium at pH 6.5 or RPMI 1640 medium. However, there

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312 was a delay in developing the classic colony morphology displayed on spider
313 medium, and this was restored to wild type in the re-integrant strain. There was also
314 no change in antifungal susceptibility or the carbon assimilation profile. As secreted
315 proteins normally express an enzymic function we screened the *iff11*Δ null mutant and
316 *IFF11* overexpressing strains and secreted fractions for a range of activities. No
317 change was identified in protease activity, exoglucanase activity, or hydrolytic
318 enzyme activities screened by API-ZYM.

319 **Cell wall sensitivity.** To determine the effect of deleting *IFF11* on cell wall
320 integrity, sensitivity to a range of compounds associated with cell wall defects was
321 determined. The *iff11*Δ null mutant was hypersensitive to the cell wall perturbing
322 agents Calcofluor White, Congo Red and SDS (Fig. 2). Sensitivity was returned to
323 wild type levels in the *iff11*Δ+*IFF11* re-integrant. There was no change in sensitivity
324 to high salt conditions, indicating that the mutant was not osmotically fragile. There
325 was also no change in sensitivity to hygromycin B, vanadate or tunicamycin, which
326 would have been indicative of glycosylation defects, or to caffeine, which is usually
327 associated with cell signalling defects. Overexpression of *IFF11* in the null mutant
328 background rescued cell wall sensitivity to wild type levels and did not affect the
329 overall level of resistance. The overexpression of IFF11-V5 in the null mutant
330 background also rescued cell wall sensitivity although it was not fully restored to wild
331 type levels, indicating that insertion of the V5 tag affected Iff11 function. However,
332 the rescue of the null mutant was sufficient to provide confidence in the localization
333 studies.

334 As Iff11 is secreted we wished to test if it could function *in trans* on the *iff11*Δ
335 null mutant strain. The null mutant was grown in the presence of wild type Iff11 from
336 concentrated spent medium and tested for cell wall sensitivity. No rescue of the

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337 *iff11*Δ null mutant was apparent after providing Iff11 in this form (data not shown).

338 The role of Iff11 in cell wall structure is therefore likely to be effected as the protein

339 passes through the secretory pathway.

340 The *iff11*Δ null mutant had no defect in phosphomannan content,

341 hydrophobicity or adhesion to BEC. However when *IFF11* was overexpressed a

342 decrease in phosphomannan content coupled with an increase in both hydrophobicity

343 and adhesion to BEC was observed. This could suggest that Iff11 has a potential role

344 in altering cell surface properties. However, as Iff11, an *O*-glycosylated protein, was

345 being overexpressed this could also be an artefact of depleting the GDP-mannose pool

346 required for glycosylation. This would lead to a phosphomannan defect which would

347 be directly linked to an increase in hydrophobicity. Therefore, studies of cell wall and

348 secreted proteins utilizing overexpression systems must be interpreted with care.

349 **Attenuated virulence of *iff11*Δ cells.** The virulence of the *iff11*Δ null mutant

350 compared to wild type and re-integrant controls was assessed in a murine model of

351 systemic infection. The *iff11*Δ null mutant was clearly attenuated in virulence (Fig. 3

352 and Table 4, LogRank test; $p < 0.001$) with a mean survival time of 27.7 days

353 compared to 9 days for the wild type control. The re-integrant control was restored

354 for virulence in terms of both mean survival time and organ burdens. In mice infected

355 with the *iff11*Δ null mutant there was a significant number of *Candida*-negative

356 organs (58% kidneys, 83% brains). However, where infection was detectable the

357 organ burdens were comparable to those seen in mice infected with the wild type

358 control strain (Mann-Whitney *U* test, p=0.052). When survival-standardized

359 infections were carried out, based on a challenge dose that results in similar mean

360 survival times for all strains tested (22), tissue burdens of mice infected with the

361 *iff11*Δ null mutant were comparable to those infected with the wild type control strain.

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362 This suggests that once an infection is established Iff11 is not required; suggesting
363 that it acts in the early stages of the infection process.

364 **Concluding remarks.** The *IFF* gene family is the largest family of cell wall
365 related proteins encoded by *C. albicans*. Here we have demonstrated one member of
366 the family, Iff11, is an *O*-glycosylated secreted protein. The other family members
367 are presumably attached to the cell wall or membrane as they contain potential GPI-
368 anchor signals or transmembrane domains. Deletion of *IFF11* resulted in a defective
369 cell wall, highlighted by hypersensitivity to cell wall perturbing agents. As Iff11 is
370 secreted and not maintained in the cell wall, and the null mutant has a defective wall,
371 it suggests that the protein carries out an enzymic rather than a structural role,
372 although we were unable to determine the nature of the enzyme activity despite tests
373 with an extensive range of substrates. Additionally as Iff11 cannot be provided *in*
374 *trans* its function is exerted as it passes through the secretory pathway or cell wall
375 upon release. Understanding the function and role of cell wall modifying enzymes is
376 critical to the future understanding of cell wall assembly.

377 The *iff11*Δ null mutant was clearly highly attenuated in virulence in a mouse
378 model of systemic infection. Further studies, using a survival-standardized infection
379 model, suggest that the defect in the null mutant is in initially establishing an
380 infection. Iff11 therefore does not display functional redundancy with the other
381 family members and was the ideal choice to characterize the role of the gene family.
382 The importance of Iff11 for virulence, coupled with the presence of the gene family in
383 *C. albicans* and other pathogenic *Candida* species also raises the possibility of this
384 family as a potential antifungal or vaccine target.

385 **ACKNOWLEDGEMENTS**

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FIGURE LEGENDS

FIG. 1. Localization and modification of Iff11. (A) Secreted, cell wall and cytoplasmic protein extracts were analyzed by western blotting with an anti-V5 antibody. Fractions were secreted proteins (*lanes 1, 2*), cell wall extracts from treatments with β-mercaptoethanol (*lanes 3, 4*), mild alkali (*lanes 5, 6*), or HF-pyridine (*lanes 7, 8*), and soluble proteins (*lanes 9, 10*). The extracts were prepared from wild type NGY152 (*lanes 1, 3, 5, 7, 9*) and SBC121 (overexpressing V5 tagged Iff11; *lanes 2, 4, 6, 8, 10*). All secreted and cell wall extracts were prepared sequentially from the same set of cells and extracts were resuspended at a volume to represent the proportion of original culture volume. For soluble protein extracts 50 µg of protein was analysed. The faint low molecular weight bands in lanes 9 and 10 are non-specific. (B) Glycosylation status of Iff11. The secreted protein extracts from SBC121 (overexpressing Iff11-V5) were untreated (*lanes 1, 3*) or treated with Endo H (*lane 2*) or jack bean mannosidase (*lane 4*), soluble cytoplasmic extracts were included as a control (*lane 5*). Note the low molecular weight band in each lane is non-specific.

FIG. 2. Sensitivity of *iff11*Δ null mutant to cell wall perturbing agents. Sensitivity was tested quantitatively by serial dilution of agents in multiwell plates. Agents to which *iff11*Δ displayed hypersensitivity (Calcofluor White, Congo Ted and SDS) are shown. Strains tested were wild type (*closed squares*), *iff11*Δ (*open squares*), re-integrant (*open triangles*), *iff11*Δ overexpressing Iff11 (closed diamonds) and *iff11*Δ over expressing Iff11-V5 (open diamonds). Error bars are means ± SD.

FIG. 3. Attenuation of virulence in the *iff11*Δ null mutant. The wild type (*closed squares*), *iff11*Δ (*open squares*) and re-integrant (*open triangles*) strains were tested

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for virulence in a mouse model of systemic infection. Six mice per strain were intravenously infected with $2x10^4$ cfu/g body weight.

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TABLE 1. *C. albicans* strains

Strain	Parent Strain	Genotype	Source
CAI-4	-	*ura3*Δ*::imm434/ura3*Δ*::imm434*	(11)
NGY152	CAI-4	As CAI-4 but *RPS1/rps1*Δ::CIp10	(4)
F3	CAI-4	As CAI-4 but *IFF11/iff11*Δ*::hisG-URA3-hisG*	This study
F3A	F3	As CAI-4 but *IFF11/iff11*Δ*::hisG*	This study
JMCA6	F3A	As CAI-4 but *iff11*Δ*::hisG/iff11*Δ*::hisG-URA3-hisG*	This study
SBC101	JMCA6	As CAI-4 but *iff11*Δ*::hisG/iff11*Δ*::hisG*	This study
SBC102	SBC101	As CAI-4 but *iff11*Δ*::hisG/iff11*Δ*::hisG*, *RPS1/rps1*Δ::CIp10	This study
SBC104	SBC101	As CAI-4 but *iff11*Δ*::hisG/iff11*Δ*::hisG*, *RPS1/rps1*Δ::CIp10-*IFF11*	This study
SCB119	CAI-4	As CAI-4 but *RPS1/rps1*Δ::CIp10-*IFF11-V5*	This study
SCB121	CAI-4	As CAI-4 but *RPS1/rps1*Δ::CIp10-*ENO1-IFF11-V5*	This study
SBC134	CAI-4	As CAI-4 but *RPS1/rps1*Δ::CIp10-*ENO1-IFF11*	This study
SBC136	SCB101	As CAI-4 but *iff11*Δ*::hisG/iff11*Δ*::hisG*, *RPS1/rps1*Δ::CIp10-*ENO-IFF11*	This study
SBC137	SBC101	As CAI-4 but *iff11*Δ*::hisG/iff11*Δ*::hisG*, *RPS1/rps1*Δ::CIp10-*ENO-IFF11-V5*	This study

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TABLE 2. Properties of *IFF* family

	alias	orf19 [a]	length (aa)	signal seq [b]	GPI anchor [c]	TMD [d]	N-Glycos [e]	Ser/Thr (%)
IFF1	*RBR3*	orf19.5124	1632	+ (20-21)	+ (1609)	-	5	42
IFF2	*HYR3*	orf19.575	1249	+ (20-21)	+ (1231)	-	39	36
IFF3		orf19.4361	942	+ (20-21)	+ (918)	-	3	33
IFF4		orf19.7472	1526	+ (20-21)	+ (1502)	-	3	35
IFF5		orf19.2879	1308	+ (20-21)	+ (1284)	-	10	36
IFF6		orf19.4072	1086	+ (19-20)	+ (1053)	-	31	38
IFF7	*HYR4*	orf19.3279	1225	+ (20-21)	-	+ (1205-1224)	11	27
IFF8		orf19.570	714	+ (19-20)	+ (690)	-	6	35
IFF9		orf19.465	941	+(20-21)	+ (917)	-	4	32
IFF10 [f]	*FLO9*	orf19.5404	1244	+ (19-20)	+ (1219)	-	7	33
IFF11		orf19.5399	511	+ (19-20)	-	-	0	25
HYR1		orf19.4975	919	+(20-21)	+ (985)	-	13	32

[a] orf19 numbers refer to the *C. albicans* database assembly 19 (http://www.candidagenome.org)

[b] signal sequences were predicted using SignalP (http://www.cbs.dtu.dk/services/SignalP/)

[c] GPI anchor attachment was predicted using DGPI (http://129.194.185.165/dgpi)

[d] Potential transmembrane domains were predicted using TMHHH (http://www.cbs.dtu.dk/services/TMHMM-2.0/)

[e] Potential *N*-glycosylation sites were predicted using NetNGlyc (http://www.cbs.dtu.dk/services/NetNGlyc/)

[f] Sequence for *IFF10* contains a potential frameshift mutation joining orf19.5404 to orf19.5401

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TABLE 3. Homology of *IFF* gene family products (% identity)[a]

	Iff1	Iff2	Iff3	Iff4	Iff5	Iff6	Ifff7	Iff8	Iff9	Iff10	Iff11	Hyr1
Iff1	100											
Iff2	59.4	100										
Iff3	55.6	63.7	100									
Iff4	55.1	62.3	76.3	100								
Iff5	53.5	60.9	85.4	74	100							
Iff6	45.6	50.1	46.5	48.3	46.5	100						
Iff7	48	53.1	55.1	53.2	51.4	39.1	100					
Iff8	45.6	54.1	50.1	49.4	47.5	46.8	41.8	100				
Iff9	55.1	62.9	98	74.9	83.7	45.6	54.3	49	100			
Iff10	30.2	35.8	35.4	35.5	34.2	33.8	34.2	31.9	34.6	100		
Iff11	30	38.5	34.2	34.1	33.6	34.6	34.7	35.1	33.3	56.6	100	
Hyr1	35.4	45.5	42.2	41.6	41.9	34.4	37.5	40.8	41.9	29.3	30.9	100

[a] Protein homology was calculated over the first 350 amino acids incorporating the conserved domain

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TABLE 4. Mean survival times and organ burdens for groups of 6 BALB/c mice infected with *iff11*Δ null mutant and control strains

Strain	Mean survival (days ± SD)	Kidney burden (log_{10}cfu/g ± SD)	Brain burden (log_{10}cfu/g ± SD)
IFF11 (NGY152)	9.0 ± 4.5	6.3 ± 0.6	2.1 ± 0.7
*iff11*Δ (SBC102)	27.7 ± 0.8	3.2 ± 2.1	1.5 ± 0.4
*iff11*Δ + *IFF11* (SBC104)	10.7 ± 8.5	6.0 ± 0.3	2.2 ± 0.8

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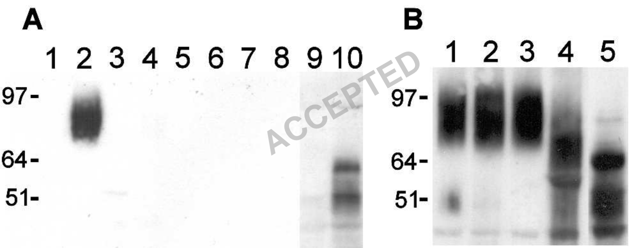

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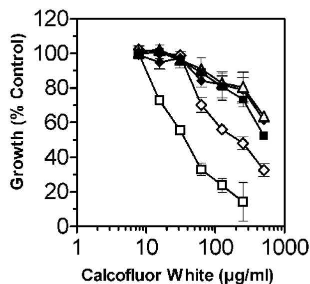
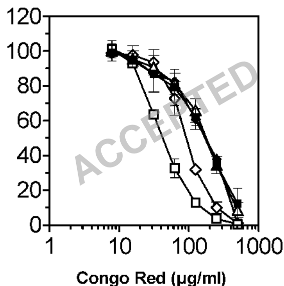

Growth (% Control)
0
20
40
60
80
100
120
1
10
100
1000
Calcofluor White (µg/ml)


0
20
40
60
80
100
120
1
10
100
1000
Congo Red (µg/ml)
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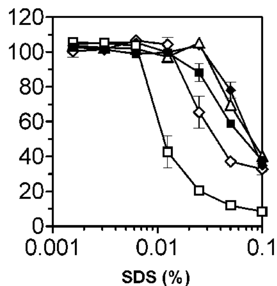
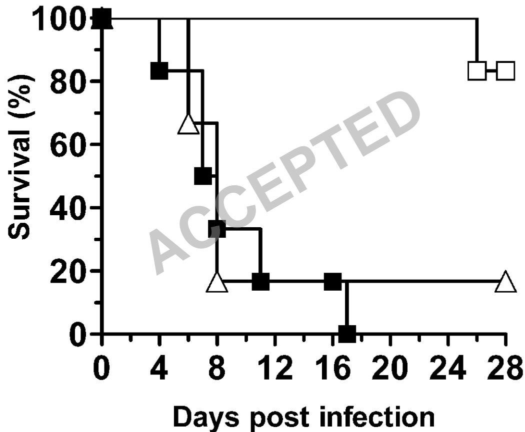

0
20
40
60
80
100
120
0.001
0.01
0.1
SDS (%)



Survival (%)
100
80
60
40
20
0
0
4
8
12
16
20
24
28
Days post infection
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