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Group X hybrid histidine kinase Chk1 is dispensable for stress adaptation, host-pathogen interactions and virulence in the opportunistic yeast *Candida guilliermondii*

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29

30 **Abstract**

31

32 Hybrid histidine kinases (HHKs) progressively emerge as prominent sensing proteins in the
33 fungal kingdom and as ideal targets for future therapeutics. The group X HHK is of major interest,
34 since it was demonstrated to play an important role in stress adaptation, host-pathogen interactions
35 and virulence in some yeast and mold models, and particularly Chk1, that corresponds to the sole
36 group X HHK in *Candida albicans*. In the present work, we investigated the role of Chk1 in the
37 low-virulence species *Candida guilliermondii*, in order to gain insight into putative conservation of
38 the role of group X HHK in opportunistic yeasts. We demonstrated that disruption of the
39 corresponding gene *CHK1* does not influence growth, stress tolerance, drug susceptibility, protein
40 glycosylation or cell wall composition in *C. guilliermondii*. In addition, we showed that loss of
41 *CHK1* does not affect *C. guilliermondii* ability to interact with macrophages and to stimulate
42 cytokine production by human peripheral blood mononuclear cells. Finally, the *C. guilliermondii*
43 *chk1*null mutant was found to be as virulent as the wild-type strain in the experimental model
44 *Galleria mellonella*. Taken together, our results demonstrate that group X HHK function is not
45 conserved in *Candida* species.

46

47 *Key words:* Histidine kinases; stress adaptation; cell signaling; virulence; *Candida*

48

49

50 **1. Introduction**

51 In bacteria, archaea, slime molds, plants and fungi, hybrid histidine kinases (HHKs) sense
52 and transduce many intra- and extracellular signals, regulating a broad palette of physiological
53 processes. Although some HHKs appear to be present in humans, typical HHK-like sensor proteins
54 have not yet been reported in mammals, promoting these proteins as ideal targets for future therapies
55 [1].

56 Since their pioneering identification in fungal cells, a limited number of fungal HHKs have been
57 studied over the last fifteen years, and their functions in fundamental cellular processes are not yet
58 clearly identified [2,4]. HHKs are involved in cell signaling systems referred to as His-to-Asp
59 phosphorelays and several canonical schemes depicting transduction pathways involving HHKs in
60 fungi have emerged. To date, HHKs act as primary sensors for various environmental stimuli, and
61 upon activation, initiate phosphate transfer events between various proteins, leading to an adaptive
62 response [4]. The number of HHK-encoding genes differs sharply from one fungal clade to another,
63 and the N-terminus sensing domain is highly variable among HHK structures. Considering these
64 observations, several classifications of fungal HHKs were established, and currently, they are
65 categorized into sixteen groups [4-6].

66 Group X gathers some of the first described fungal HHKs; thus, it is one of the most thoroughly
67 studied groups in the fungal kingdom. In the dimorphic fungus *Penicillium marneffei*, the group X
68 HHK PmHhk1 was shown to regulate polarized growth, sporulation and cell wall composition [7].
69 In *Claviceps purpurea*, the agent of ergot of grasses, homologous HHK was involved in spore
70 germination, sensitivity to oxidative stress and fungicide resistance, and virulence of the mutant
71 strains was attenuated in both *C. purpurea* and *Magnaporthe oryzae* [8,9]. Most available data
72 concerning the role of fungal group X HHKs were gained from functional characterization of
73 *Candida albicans* Chk1, which was shown to play a crucial role in virulence, morphogenesis,
74 peroxide adaptation, cell wall composition, quorum sensing, biofilm formation and triazole

75 resistance [3]. Furthermore, during fungal infection, *C. albicans* Chk1 was required for survival
76 within human neutrophils and adherence to ex vivo human esophageal cells [3].

77 In the present work, we investigated the role of the *C. albicans* *CHK1* homolog in a low-
78 virulence-related yeast species, namely *Candida guilliermondii*, in order to gain insight into
79 putative conservation of the role of group X HHKs in the fungal CTG clade [10]. Indeed, although
80 *C. guilliermondii* is an infrequent agent of candidiasis, this species has been described as an
81 emerging pathogen intrinsically poorly susceptible to fluconazole and caspofungin, with a
82 propensity for causing treatment failure [11]. Recent studies specified that *C. guilliermondii*
83 accounts for 1-3% of all candidemia, but most cases of *C. guilliermondii* infection occur in
84 immunocompromised patients with hematological malignancies[12,13].

85

86 **2. Materials and methods**

87 *2.1. Strains and media*

88 Yeast strains (Table 1) were routinely cultivated in liquid YPD medium (1% yeast extract, 2%
89 peptone, 2% dextrose) at 30°C under agitation (150 rpm). YNB (0.67% yeast nitrogen base with
90 ammonium sulfate and without amino acids, 2% sucrose) agar (2%) plates were used for selection of
91 transformants following electroporation experiments. For heat inactivation, cells were resuspended
92 in phosphate-buffered saline (PBS) and incubated at 56°C for 1h [14]. Acid phosphatase expression
93 was induced by growing cells in YNB medium for 18 h at 28°C with constant shaking (200 rpm).

94 The murine macrophage cell line J774A.1 (ATCC TIB-67) was cultured in DMEM (Gibco)
95 containing 10% decompemented fetal bovine serum (FBS, Gibco) and 1 mM sodium pyruvate, or
96 in cRPMI (complete RPMI corresponding to RPMI-1640 (Sigma) without phenol red and
97 supplemented with 10% decompemented FBS, 1 mM sodium pyruvate and 2 g/l sodium
98 bicarbonate) for the infection experiments, at 37°C in 5% CO₂.

99

100 2.2. Nucleic acids purification and PCR amplification

101 Genomic DNA from *C. guilliermondii* strains was extracted using the Plant Nucleospin II kit
102 (Macherey-Nagel). DNA purification was performed using the Nucleospin Extract II kit
103 (Macherey-Nagel). PCR assays were performed with Phusion DNA polymerase (New England
104 Biolabs).

106 2.3. Plasmid construction

107 An 8,569bp PCR fragment was first amplified from the *C. guilliermondii*CHK1 locus
108 (Genbank accession numberXM_001483618) (Fig. 1A) with the primer pair CHK1S5'-
109 TGGTTTCCTGATTTCAGTTCGCTATACCC-3' and CHK1R 5'-
110 AGTACCCACGACCAGTACCAGGATAAGG-3'[15]. This amplicon was cloned into the pGEM-T
111 easy vector (Promega) to yield plasmid pG-CHK1(Fig. 1B). The pG-CHK1plasmid was digested
112 with *Cla*I (Fig. 1B) to delete a 4,041 bp central fragment in the cloned *CHK1* coding sequence, and
113 the resulting digested plasmid was ligated to the *REP-URA3-REP* fragment released after digestion
114 of the pG-*REP-URA3-REP* plasmid[16](Fig. 1C) with *Cla*Ito yield plasmid pG-5'*CHK1-REP-*
115 *URA3-REP-3'CHK1*(Fig. 1D). The 5'*CHK1-REP-URA3-REP-3'CHK1*disruption cassette was
116 released from pGEM-T vector after digestion of the pG-5'*CHK1-REP-URA3-REP-3'CHK1*with
117 *Not*I (Fig. 1E).

119 2.4. Yeast transformation

120 Transformation of *C. guilliermondii* cells was performed by electroporation as
121 described[17].

123 2.5. mRNA detection

124 Total RNA was extracted from *C. guilliermondii* cultures (YPD) with NucleoSpin RNA
125 Plant (Macherey-Nagel). First-strand cDNAs were synthesized from 1 µg of total RNA using

126 random hexamer primers (0.5 μ M) with 15 units of Superscript III reverse transcriptase (Invitrogen).
127 The *C. guilliermondii* *CHK1* mRNAs were detected by PCR using primers CHK1S and CHK1R
128 under the following conditions: 95°C for 10 min and 30 cycles with denaturation at 95°C for 30 s
129 followed by annealing at 60°C for 30 s and extension at 72°C for 4 min. The *C. guilliermondii* Actin
130 1 gene (*ACT1*) was used as a housekeeping gene and amplified by using primers qACT1-F 5'-
131 CCGACTTGGGATTGGAAGCCGC-3' and qACT1-R 5'-
132 CTCAGGAGGAGCGATGATCTTAACC-3' with the following conditions: 95°C for 10 min and
133 30 cycles with denaturation at 95°C for 30 sec followed by annealing at 60°C for 30 s and extension
134 at 72°C for 1 min.

135

136 2.6. Sensitivity test for stress responses, drugs and antifungal compounds

137 EUCAST testing was performed according to EUCAST EDef 7.1 methodology[18]. All
138 strains were cultured on YNB before susceptibility testing. RPMI (Sigma Aldrich) was
139 supplemented with antifungal agents, including 0.03-16.0 μ g/mL amphotericin B or caspofungin,
140 0.125-64.0 μ g/mL flucytosine, 0.25-128.0 μ g/mL fluconazole, and 0.015-8.0 μ g/mL voriconazole;
141 or with other chemical compounds, including 0.125-1.0 mM sodium dodecyl sulfate (SDS, Thermo
142 Fisher), 0.25-2.0 mM caffeine (Sigma-Aldrich), 0.25-2.0 M NaCl (Thermo Fisher) or sorbitol
143 (AppliChem), 1.0-11.5mM H₂O₂, 0.003-0.375 mM menadione (Sigma Aldrich), 0.003-0.375mg/mL
144 Congo red (Sigma Aldrich). Inoculated plates were incubated at 32°C and read with a
145 spectrophotometer after 48 h. According to EUCAST EDef 7.1 recommendations, MIC 90 was
146 determined for amphotericin B and MIC 50 for other drugs and compounds. The EUCAST MICs
147 for the quality control strain *C. parapsilosis* ATCC 22019 were all within the recommended ranges
148 for the licensed compounds. Due to the low solubility of calcofluor white (CFW) and the high
149 natural resistance of *C. guilliermondii* towards this compound[19], comparison of susceptibility of
150 wild type (WT) and mutant strains to CFW was achieved using a standard drop plate assay protocol.
151 For this purpose, each strain was incubated overnight at 35°C in YPD broth, washed, serially diluted

152 (10² to 10⁵ dilutions) in distilled water and spotted (4 µL) onto solid YPD medium supplemented or
153 not with 100-600 µg/mL CFW. Plates were incubated for 3 days at 32°C.

154

155 2.7. Analysis of cell wall composition

156 Quantification of cell wall mannan, β-glucan and chitin was performed by high-performance
157 anion-exchange chromatography coupled with pulsed amperometric detection (HPAEC-PAD),
158 measuring the levels of the corresponding monosaccharides: mannose, glucose and N-
159 acetylglucosamine, respectively. Cells grown in YPD broth were washed twice with PBS and
160 broken with glass beads. Cell walls were pelleted by centrifuging and purified as described[14]. The
161 samples were then acid-hydrolyzed with trifluoro-acetic acid, and analyzed by HPAEC-PAD in
162 Dionex equipment as previously reported[14].

163 To determine the content of cell wall proteins, walls were alkali-hydrolyzed before quantifying
164 using a standard colorimetric method, while the levels of cell wall phosphomannan were quantified
165 by cell ability to bind the cationic dye Alcian blue, as described[20].

166

167 2.8. Zymogram analysis

168 Acid phosphatase was used as a marker of the status of the N-linked glycosylation pathway,
169 as previously reported[21]. Cells grown overnight in YNB broth were collected by centrifuging,
170 washed with 10 mM Tris-HCl, pH 6.8 and disrupted with glass beads in a Braun homogenizer. The
171 homogenate was centrifuged at 20,000 x g for 10 min, the soluble fraction saved and loaded onto
172 native 8% (w/v) polyacrylamide gels. Electrophoresis was run at 110 V for 1.5 h, the gel rinsed with
173 100 mM sodium acetate, pH 5.8 and enzyme activity was visualized by incubating with 0.4 mM 4-
174 methylumbellyferyl-phosphate (Sigma) in 100 mM sodium acetate, pH 5.8 for 30 min at 37 °C.

175

176 2.9. Cytokine production by human peripheral blood mononuclear cells

177 The Ethics Committee of the Universidad de Guanajuato approved the use of human cells in
178 this study (permission number 17082011). Healthy adult volunteers provided blood samples after a
179 written informed consent was signed. Peripheral blood mononuclear cells (PBMCs) were isolated
180 using a Ficoll gradient, as previously reported[22]. A 100 μ L aliquot of 5×10^6 PBMCs/ml was
181 added to U-bottom 96-well microplates along with 100 μ L containing 1×10^5 fungal cells. The
182 interaction was incubated for 24 h at 37 °C with 5% (v/v) CO₂. Supernatants were collected by
183 centrifuging and used to quantify levels of IL-1 α , TNF α , IL-6, IL-10 by ELISA (Preprotech). The
184 IL-1 β levels were measured using a commercial kit from R&D. For IL-1 α quantification, stimulated
185 PBMCs were disrupted by three sequential freezing cycles as reported[23].

186

187 *2.10. Macrophage infection with yeasts*

188 Yeast cells were labelled overnight in liquid YPD medium supplemented with 5 μ g/ml
189 CFW. Macrophages were infected as previously described in cRPMI medium[24]. We used a
190 multiplicity of infection (MOI) of 1 macrophage to 1 yeast (1M:1Y) for all experiments, except for
191 yeast viability, where we used 10M:1Y. Briefly, 2×10^5 macrophages per well were adhered
192 overnight in 96-well plates at 37°C in 5% CO₂ and infected with the required number of CFW-
193 labeled yeasts in cRPMI in the presence of 5 μ g/ml CFW to allow the continuous labeling of newly
194 replicated yeasts outside the macrophages.

195

196 *2.11. Fluorimetry and flow cytometry assays*

197 Fluorimetry and flow cytometry assays were conducted as previously described using a
198 FluoStar Optima fluorimeter (BMG Labtech) and a FACSCanto II (Becton Dickinson),
199 respectively[24]. Fluorimetry was used to determine multiplication of the fungal biomass and the
200 ratio of ingested fungal cells after 1 h, 5 h and 24 h of infection. Briefly, we measured the total
201 fluorescence of CFW-labeled yeasts in an infection well. Then, a final concentration of 250 μ g/ml of
202 trypan blue was used to quench the fluorescence of non-ingested CFW-labeled yeasts, and we

203 measured the fluorescence of internalized CFW-labeled yeasts. Flow cytometry was used to
204 measure macrophage and yeast mortality rates and the ratio of macrophages engaged in
205 phagocytosis. Briefly, macrophages were double-stained with anti-mouse CD16-APC (membrane
206 stain) and calcein-AM (a marker of active metabolism) after 24 h of incubation with CFW-labeled
207 yeasts. The percentage of macrophage viability was calculated as the number of macrophages
208 positive for both fluorescence (calcein and anti-CD16) in an infection assay versus the uninfected
209 macrophage control. Phagocytosing macrophages were quantified as the number of macrophages
210 positive for calcein, anti-CD16 and CFW fluorescence. To determine yeast mortality inside
211 macrophages, macrophages were infected with CFW-labeled yeasts at a MOI of 10 M:1Y in a 96-
212 well plate. After 24 h, wells were washed, macrophages were detached and lysed with 0.1% Triton
213 X-100 (Acros Organics) to release ingested yeast cells. We used 1 µg/ml propidium iodide (PI)
214 (Sigma) (λ_{ex} 535 nm, λ_{em} 617 nm) to stain collected yeasts for flow cytometry analysis. PI only
215 enters dead cells. Heat-killed yeasts were used as positive controls and live yeasts were used as
216 negative controls for PI staining. For heat inactivation, yeast cells were incubated for 30 min at
217 90°C. For analysis, the CFW population was selected and the ratio of the population positive for the
218 PI signal was then measured to determine yeast mortality.

219

220 2.12. Virulence assays in the insect species *Galleria mellonella*

221 Groups of 10 *G. mellonella* larvae of at least 1 cm length and uniform color were inoculated in the
222 last left proleg with the fungal suspension using a Hamilton syringe (701N, 26's gauge, 10 µL
223 capacity)[25]. Infection was achieved by injecting 10 µL inoculum into the hemocoel. Animal
224 groups were kept at 37°C and mortality and phenotypical changes were recorded for 15 days. As a
225 control, a group of 10 animals was injected with PBS.

226

227 2.13. Statistical analysis

228 Data are cumulative results of all experiments performed and are shown as mean \pm SD or
229 SE. The Mann-Whitney U test was used to establish statistical significance, which was set at $P <$
230 0.05. Mortality results were analyzed using the Log-rank test and shown in Kaplan-Meier charts,
231 with a significance level set at $P <$ 0.05

232

233 3. Results

234 3.1. Disruption of the *C. guilliermondii* CHK1 gene

235 We previously provided a complete bioinformatics characterization of both the *C. guilliermondii*
236 *CHK1* gene and Chk1 deduced protein in a global study deciphering the subcellular localization of
237 the series of HHKs found in this species[15]. Briefly, the *C. guilliermondii* Chk1 predicted protein
238 is composed of 2428 amino acids and shares 30% identity with Chk1 from *C. albicans*. *C.*
239 *guilliermondii* Chk1 displays all subdomains specifically found in *C. albicans* Chk1, thus belonging
240 to the group X HHK[4]. Importantly, no further homologous sequences were found in the genome
241 of *C. guilliermondii*, indicating that, as observed in *C. albicans*, a unique copy of the *CHK1* gene is
242 present and thus encodes the sole member of group X HHK in *C. guilliermondii*.

243 Among the series of selectable markers available for *C. guilliermondii* genetics[26-30], we chose
244 to use the recently developed *URA3* blaster system to disrupt *C. guilliermondii* *CHK1*[16]. This was
245 done to recreate the genetic modifications performed in *C. albicans*, where the *URA3* blaster system
246 was initially used to generate *C. albicans* *chk1* mutants[31,32]. For this purpose, a 5'*CHK1-REP-*
247 *URA3-REP-3'CHK1* fragment with 2,900 bp and 1,600 bp of the 5' and 3' *CHK1* homologous arms,
248 respectively, was generated. This disruption cassette was used to transform the *C. guilliermondii*
249 U312 (*ura3* $_{\Delta 290}$) recipient strain (auxotroph for uracil) to prototrophy. Ura⁺ transformants were
250 selected on minimal medium plates. The efficiency of transformation was roughly 5-6 transformants
251 per μ g of DNA. A series of 30 randomly selected Ura⁺ transformants were analyzed by colony PCR
252 as previously described[33]. Homologous integration of the 5'*CHK1-REP-URA3-REP-3'CHK1*
253 cassette at the *CHK1* locus (Fig. 2A) occurred in 3 of the analyzed transformants and was derived

254 from gene replacement, resulting in disruption of the target gene and in the genotype *ura3*,
255 *chk1Δ::REP-URA3-REP* (abbreviated *chk1Δ*, see a representative clone in Fig. 2B). For the
256 remaining Ura⁺ transformants, it is highly likely that they were derived from gene replacement at the
257 *ura3* locus (not studied). Loss of intact *C. guilliermondii* *CHK1* was confirmed by both PCR and
258 RT-PCR analyses (Fig. 2B). Lack of amplification of *CHK1* cDNA in the *chk1Δ* mutant was
259 unlikely to be related to poor quality of the RNA preparations, as these were successfully used to
260 synthesize and amplify the *ACT1* cDNA (Fig. 2B). Therefore, a *C. guilliermondii* *chk1Δ* null mutant
261 was generated.

262

263 3.2. Growth, stress tolerance, and drug susceptibility of the *C. guilliermondii* *chk1Δ* null mutant

264 Since the *chk1Δ* null mutant was Ura⁺, we first compared its growth kinetics with WT strain
265 ATCC 6260 (the parental strain of U312)[16]: both exhibited similar doubling times in liquid
266 YPD or YNB media (data not shown). Furthermore, no differences were observed in colony growth
267 (diameter and aspect) of either the WT or mutant strains when cultured on solid YNB or YPD media
268 (data not shown). These results suggest that *CHK1* deletion does not affect *C. guilliermondii* overall
269 development.

270 Since previous works demonstrated the involvement of HHK in the capacity of adaptation to
271 hyperosmotic conditions in several fungal species[3], we next examined the osmotolerance of the
272 *chk1Δ* null mutant. Both WT and null mutant strains displayed similar growth rates in high-
273 osmolarity media containing NaCl or sorbitol (Table 2). These results demonstrate that the *CHK1*
274 deletion has no effect on the growth and the capacity of adaptation of *C. guilliermondii* yeast cells to
275 hyperosmotic conditions.

276 It is now well described that Chk1 plays a major role in the perception of oxidant conditions in *C.*
277 *albicans*[34]. Thus, the impact of *C. guilliermondii* *CHK1* in resistance to oxidant stresses was
278 evaluated in media supplemented either with H₂O₂ or with menadione. Neither hypersensitivity nor
279 resistance of the *chk1Δ* mutant towards these oxidant conditions was observed (Table 2).

280 Finally, because it was previously shown that the *C. albicans chk1*Δ null mutant is hypersensitive
281 to fluconazole and voriconazole[35], we monitored the effect of a series of clinical antifungals
282 (amphotericin B, flucytosine, fluconazole, voriconazole, and caspofungin) against both *C.*
283 *guilliermondii* reference strain ATCC 6260 and the *chk1*Δ null mutant. Neither hypersensitivity nor
284 resistance to these antifungals of the *chk1*Δ mutant was observed. Indeed, the MICs of both strains
285 were similar to those previously described for *C. guilliermondii* (Table 2)[11].

286

287 3.3. Protein glycosylation and cell wall composition in the *C. guilliermondii chk1*Δ null mutant

288 It was previously reported that disruption of *CHK1* in *C. albicans* led to shortening in cell
289 wall mannans[36], and this allowed exposure of β1,3-glucan at the cell surface, resulting in
290 increased phagocytosis by phagocytic cells[37]. Thus, we assessed whether loss of *CHK1* affected
291 the glycosylation pathways in *C. guilliermondii*. Using zymogram analysis of secreted acid
292 phosphatase, a protein reporter of the status of the *N*-linked glycosylation pathway[20], we found no
293 differences in the mobility of the enzyme extracted from WT control cells and the *chk1*Δ null
294 mutant, indicating no obvious defects in this biosynthetic pathway (Fig. 3). In order to confirm this
295 result, we quantified cell wall phosphomannan content, an indirect indicator of the status of
296 protein glycosylation pathways[14,20,38,39], and found no significant differences in the content of
297 this moiety attached to both *N*-linked and *O*-linked mannans (Table 3)[14]. We next determined the
298 cell wall composition of the null mutant by HPAEC-PAD and we could not find significant
299 differences in the content of chitin, glucan and mannan (Table 3). Moreover, protein content within
300 the cell wall was similar in both the WT control strain and the *chk1*Δ null mutant. These results are
301 in line with the fact that drugs interfering with the biosynthesis and structure of the fungal cell wall,
302 such as caffeine, Congo red (Table 2) and calcofluor white (not shown) displayed similar effects on
303 WT and mutant strains. Furthermore, neither flocculation at the macroscopic level nor aggregation
304 of cells observed by microscopy, as described in the *C. albicanschk1*Δ mutant[31], were observed in

305 liquid cultures of the corresponding *C. guilliermondii* mutant. Therefore, loss of *CHK1* has no
306 obvious impact on *C. guilliermondii* cell wall composition or protein glycosylation.

307

308 3.4. Multi-parametric monitoring of interactions between *C. guilliermondii* cells and macrophages

309 Since it was recently demonstrated that deletion of *C. albicans* *CHK1* improves recognition
310 by phagocytes[37,40], we were thus interested in evaluation of cellular interactions of the *C.*
311 *guilliermondii* reference strain or the *chk1*Δnull mutant with macrophages. For this purpose, we
312 used a recently developed in vitro model allowing multiparametric monitoring[24]. The J774
313 macrophages were infected with the *C. guilliermondii* reference strain, and the behavior of both cell
314 types was monitored over time. Multiplication of the *C. guilliermondii* fungal biomass was inhibited
315 in the presence of J774 macrophages, as shown by total CFW fluorescence which did not increase
316 between 1 h and 24 h of infection (**Fig. 4A**). In the case of both strains, the rate of internalized yeast
317 cells was 40% after 1 h of infection, and reached a plateau of 80% at 5 h (Fig. 4B). *C.*
318 *guilliermondii* cells remained intramacrophagic at 24 h post-infection, as confirmed by
319 observation under the microscope (data not shown), with a mortality rate of roughly 20%(Fig. 4C).
320 The vast majority of the macrophages (nearly 90%) survived after 24 h of infection, and 63% were
321 engaged in phagocytosis (Fig. 4D). Finally, we compared the cellular interactions of *C.*
322 *guilliermondii* with those of other *Candida* species, including *C. albicans*, *C. glabrata* and *C.*
323 *lusitaniae* (Table 4). Globally, *C. guilliermondii* behaved similarly to *C. glabrata* and appeared to
324 be the least aggressive species, as indicated by the lowest macrophage killing and the highest
325 proportion of fungal cells internalized.

326

327 3.5. Loss of *CHK1* does not affect *C. guilliermondii* ability to stimulate cytokine production by 328 human PBMCs

329 Since we did not find obvious differences in the interaction of the *C. guilliermondii* *chk1*Δ
330 null mutant with macrophages, we next evaluated the ability of this mutant to stimulate cytokines by

331 human PBMCs. Our results showed that the WT control strain and the *chk1*Δ null mutant had a
332 similar ability to stimulate production of TNFα, IL-6, IL-1α, IL-1β, and IL-10 (**Fig. 5**). Artificial
333 exposure, by heat-killing, of inner wall components at the wall surface of *C. albicans* stimulated
334 differential cytokine production by human PBMCs[41]. Therefore, we performed experiments using
335 heat-killed *C. guilliermondii* cells. Despite observing a significant increment in cytokine production
336 upon inactivation of cells by heating (Fig. 5), no significant differences between WT control cells
337 and the *chk1*Δ null mutant were detected. Therefore, *CHK1* does not affect the *C. guilliermondii*-
338 human PBMC interaction.

339

340 3.6. The *C. guilliermondii* *chk1*Δ null mutant is as virulent as the wild type strain

341 Finally, we aimed to assess the impact of *CHK1* disruption on the virulence of
342 *C. guilliermondii*. Since no apparent defect in growth, wall composition or interaction with immune
343 cells was observed, we hypothesized that the virulence of the null mutant was not affected. Thus, we
344 did not perform experiments in mice, but in the alternative host *G. mellonella*, which has been
345 successfully used to assess the virulence of other *Candida* species, providing results similar to those
346 generated in the gold standard model, i.e. the mouse[25,42,43]. Again, we did not observe any
347 significant differences in mortality associated with the WT strain and the *chk1*Δ null mutant; both
348 strains killed the entire insect population in less than a week (**Fig. 6**). Therefore, the
349 *C. guilliermondii* *chk1*Δ null mutant does not display virulence attenuation.

350

351 4. Discussion

352 *C. albicans* group X HHK Chk1 plays a crucial role in morphogenesis (especially the yeast
353 to hyphae switch), virulence, peroxide adaptation, cell wall composition, quorum sensing, biofilm
354 formation and triazole resistance [31,32,34,35,44-48]. Furthermore, during fungal infection, *C.*
355 *albicans* Chk1 is required for survival in human neutrophils and adherence to ex vivo human
356 esophageal cells [37-40]. Therefore, all these studies suggest that group X HHK represents an

357 interesting fungal target for the discovery of new antifungal drugs. Via this perspective, it remains
358 essential to explore the conservation of the role of such potential targets in related species. In the
359 present work, we thus investigated the role of the *C. albicans*CHK1 homolog in the low-virulence
360 related yeast species *C. guilliermondii* in order to gain insight into putative conservation of the role
361 of these proteins in other members of the *Candida* CTG clade. For this purpose, we generated a *C.*
362 *guilliermondii* *chk1*Δ mutant strain and carried out a series of phenotypic analyses that were
363 previously investigated in the *C. albicans* *chk1*Δ mutant (above mentioned). We demonstrated that
364 disruption of the *CHK1* ortholog does not influence growth, stress tolerance, drug susceptibility,
365 protein glycosylation or cell wall composition in *C. guilliermondii*. In addition, it was found that
366 loss of *CHK1* does not affect *C. guilliermondii* ability to interact with macrophages and to
367 stimulate cytokine production by human PBMCs. Finally, the *C. guilliermondii* *chk1*Δ null mutant
368 was shown to be as virulent as the WT strain in the experimental model *G. mellonella*. Importantly,
369 it was previously demonstrated that the *CHK1* homolog plays no obvious role in the low-virulence
370 related yeast species *C. lusitanae*[49]. Therefore, although experimental conditions used in the
371 present study were not strictly identical to those reported for *C. albicans* *chk1*Δ mutant phenotypic
372 analysis, taken together, and in line with what was previously suggested in *C. lusitanae*[49], our
373 results suggest that group X HHK function is not conserved in *C. albicans*, *C. lusitanae* or *C.*
374 *guilliermondii*. This divergence in the function of several HHK groups was already reported in
375 molds [3,4]. For instance, group III HHK could play an opposing role in virulence in the two
376 closely related species of plant pathogens *Alternaria brassicicola* and *Alternaria longipes*[50,51].
377 While *C. albicans*, *C. lusitanae* and *C. guilliermondii* commonly belong to the particular fungal
378 CTG clade, it is now accepted that the genetic distance between any *Candida* species is considerable
379 [52]. Thus, in agreement with low conservation of the amino acid sequence of *C. albicans*, *C.*
380 *lusitanae* and *C. guilliermondii* Chk1, it is likely that differences observed in the function of these
381 crucial sensing proteins could underlie, at their own scale, prominent traits leading to specific
382 strategies of these yeast species to adapt to their specific environmental niches and hosts. Finally, it

383 is important to remember that, as observed in other closely related *Candida* species [49], *C.*
384 *guilliermondii* harbors two additional HHK genes, namely *SLN1* and *NIK1*, which encode HHKs
385 belonging to groups VI and III, respectively [15]. Although the structures of the sensing domains in
386 Chk1, Sln1 and Nik1 deduced proteins are highly divergent, it is not excluded that functional
387 redundancy could occur between these three HHKs, and further efforts will be needed to address
388 this pending hypothesis.

389

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399

400 **References**

- 401 1. Bem AE, Velikova N, Pellicer MT, BaarlenPv, Marina A, Wells JM. Bacterial histidine kinases as novel
402 antibacterial drug targets. *ACS ChemBiol*2015;10:213-224.
- 403 2. Chauhan N, Latge JP, Calderone R. Signalling and oxidant adaptation in *Candida albicans* and
404 *Aspergillus fumigatus*. *Nat Rev Microbiol* 2006;4:435-444.
- 405 3. Li D, Agrellos OA, Calderone R. Histidine kinases keep fungi safe and vigorous.
406 *CurrOpinMicrobiol*2010;13:424-430.
- 407 4. Defosse TA, Sharma A, Mondal AK, Dugé de Bernonville T, Latgé JP, Calderone R, *et al.*Hybrid
408 histidine kinases in pathogenic fungi. *MolMicrobiol*2015;95:914-924.

- 409 5. Catlett NL, Yoder OC, Turgeon BG. Whole-genome analysis of two-component signal transduction
410 genes in fungal pathogens. *Eukaryot Cell* 2003;2:1151-1161.
- 411 6. Lavín JL, Ramírez L, Ussery DW, Pisabarro AG, Oguiza JA. Genomic analysis of two-component
412 signal transduction proteins in basidiomycetes. *J MolMicrobiolBiotechnol* 2010;18:63-73.
- 413 7. Wang F, Tao J, Qian Z, You S, Dong H, Shen H, *et al.* A histidine kinase PmHHK1 regulates polar
414 growth, sporulation and cell wall composition in the dimorphic fungus *Penicillium marneffe*. *Mycol Res*
415 2009;113:915-923.
- 416 8. Nathues E, Jörgens C, Lorenz N, Tudzynski P. The histidine kinase CpHK2 has impact on spore
417 germination, oxidative stress and fungicide resistance, and virulence of the ergot fungus
418 *Clavicepspurpurea*. *Mol Plant Pathol*2007;8:653-665.
- 419 9. Jacob S, Foster AJ, Yemelin A, Thines E. Histidine kinases mediate differentiation, stress response, and
420 pathogenicity in *Magnaportheoryzae*. *Microbiologyopen* 2014;3:668-687.
- 421 10. Papon N, Savini V, Lanoue A, Simkin AJ, Crèche J, Giglioli-Guivarc'h N, *et al.* *Candida guilliermondii*:
422 biotechnological applications, perspectives for biological control, emerging clinical importance and
423 recent advances in genetics. *Curr Genet*2013;59:73-90.
- 424 11. Pfaller MA, Diekema DJ, Mendez M, Kibbler C, Erzsebet P, Chang SC, *et al.* *Candida guilliermondii*, an
425 opportunistic fungal pathogen with decreased susceptibility to fluconazole: geographic and temporal
426 trends from the ARTEMIS DISK antifungal surveillance program. *J ClinMicrobiol* 2006;44:3551-3556.
- 427 12. Girmenia C, Pizzarelli G, Cristini F, Barchiesi F, Spreghini E, Scalise G, *et al.* *Candida*
428 *guilliermondii*fungemia in patients with hematologic malignancies. *J ClinMicrobiol.* 2006;44:2458-
429 2464.
- 430 13. Savini V, Catavittello C, Onofrillo D, Masciarelli G, Astolfi D, Balbinot A, *et al.* What do we know
431 about *Candida guilliermondii*? A voyage throughout past and current literature about this emerging
432 yeast. *Mycoses* 2011;54:434-441.
- 433 14. Mora-Montes HM, Bates S, Netea MG, Díaz-Jiménez DF, López-Romero E, Zinker S, *et al.*
434 Endoplasmic reticulum alpha-glycosidases of *Candida albicans* are required for N glycosylation, cell
435 wall integrity, and normal host-fungus interaction. *EukaryotCell* 2007;6:2184-2193.

- 436 15. Foureau E, Clastre M, Montoya EJ, Besseau S, Oudin A, Glévarec G, *et al.* Subcellular localization of the
437 histidine kinase receptors Sln1p, Nik1p and Chk1p in the yeast CTG clade species *Candida*
438 *guilliermondii*. Fungal Genet Biol 2014;65: 25-36.
- 439 16. Foureau E, Courdavault V, Simkin AJ, Pichon O, Crèche J, Giglioli-Guivarc'h N, *et al.* Optimization of
440 the URA-blaster disruption system in *Candida guilliermondii*: efficient gene targeting using the URA3
441 marker. J Microbiol Methods 2012;91:117-120.
- 442 17. Millerioux Y, Clastre M, Simkin AJ, Marais E, Sibirny AA, Noël T, *et al.* Development of a URA5
443 integrative cassette for gene disruption in the *Candida guilliermondii* ATCC 6260 strain. J Microbiol
444 Methods 2011;84:355-358.
- 445 18. Rodriguez-Tudela JL, Arendrup MC, Barchiesi F, Bille J, Chryssanthou E, Cuenca-Estrella M, *et al.*
446 EUCAST Definitive Document EDef 7.1: method for the determination of broth dilution MICs of
447 antifungal agents for fermentative yeasts: Subcommittee on Antifungal Susceptibility Testing (AFST) of
448 the ESCMID European Committee for Antimicrobial Susceptibility Testing (EUCAST). Clin Microbiol
449 Infect 2008;14:398-405.
- 450 19. Walker LA, Gow NA, Munro CA. Elevated chitin content reduces the susceptibility of *Candida* species
451 to caspofungin. Antimicrob Agents Chemother 2013;57:146-154.
- 452 20. Bates S, MacCallum DM, Bertram G, Munro CA, Hughes HB, Buurman ET, *et al.* *Candida albicans*
453 Pmr1p, a secretory pathway P-type Ca²⁺/Mn²⁺-ATPase, is required for glycosylation and virulence. J
454 Biol Chem 2005;280:23408-23415.
- 455 21. Hobson RP, Munro CA, Bates S, MacCallum DM, Cutler JE, Heinsbroek SE, *et al.* Loss of cell wall
456 mannosylphosphate in *Candida albicans* does not influence macrophage recognition. J
457 Biol Chem 2004;279:39628-39635.
- 458 22. Endres S, Ghorbani R, Lonnemann G, van der Meer JW, Dinarello CA. Measurement of
459 immunoreactive interleukin-1 beta from human mononuclear cells: optimization of recovery,
460 intrasubject consistency, and comparison with interleukin-1 alpha and tumor necrosis factor.
461 Clin Immunol Immunopathol 1988;49:424-438.
- 462 23. Mora-Montes HM, Netea MG, Ferwerda G, Lenardon MD, Brown GD, Mistry AR, *et al.* Recognition
463 and blocking of innate immunity cells by *Candida albicans* chitin. Infect Immun. 2011;79:1961-1970.

- 464 24. Dementhon K, El-Kirat-Chatel S, Noël T. Development of an in vitro model for the multi-parametric
465 quantification of the cellular interactions between *Candida* yeasts and phagocytes. PLoS One
466 2012;7:e32621.
- 467 25. Gago S, García-Rodas R, Cuesta I, Mellado E, Alastruey-Izquierdo A. *Candida parapsilosis*, *Candida*
468 *orthopsilosis*, and *Candida metapsilosis* virulence in the non-conventional host *Galleria mellonella*.
469 Virulence 2014;5:278-285.
- 470 26. Obando Montoya EJ, Mélin C, Blanc N, Lanoue A, Foureau E, Boudesocque L, *et al.* Disrupting the
471 methionine biosynthetic pathway in *Candida guilliermondii*: characterization of the *MET2* gene as
472 counter-selectable marker. Yeast 2014;31:243-251.
- 473 27. Foureau E, Courdavault V, Rojas LF, Dutilleul C, Simkin AJ, Crèche J, *et al.* Efficient gene targeting in
474 a *Candida guilliermondii* non-homologous end-joining pathway-deficient strain. Biotechnol Lett
475 2013;35:1035-1043.
- 476 28. Foureau E, Courdavault V, Simkin AJ, Sibirny AA, Crèche J, Giglioli-Guivarc'h N, *et al.* Transformation
477 of *Candida guilliermondii* wild-type strains using the *Staphylococcus aureus* MRSA 252 ble gene as a
478 phleomycin-resistant marker. FEMS Yeast Res 2013;13:354-358.
- 479 29. Foureau E, Clastre M, Millerioux Y, Simkin AJ, Cornet L, Dutilleul C, *et al.* *TRP5*/5-fluoroanthranilic
480 acid counter-selection system for gene disruption in *Candida guilliermondii*. Curr Genet 2012;58:245-
481 254.
- 482 30. Millerioux Y, Clastre M, Simkin AJ, Courdavault V, Marais E, Sibirny AA, *et al.* Drug-resistant cassettes
483 for the efficient transformation of *Candida guilliermondii* wild-type strains. FEMS Yeast Res
484 2011;11:457-463.
- 485 31. Calera JA, Calderone R. Flocculation of hyphae is associated with a deletion in the putative CaHK1 two-
486 component histidine kinase gene from *Candida albicans*. Microbiology 1999;145:1431-1442.
- 487 32. Yamada-Okabe T, Mio T, Ono N. Roles of three histidine kinase genes in hyphal development and
488 virulence of the pathogenic fungus *Candida albicans*. J Bacteriol 1999;181:7243-7247.
- 489 33. Akada R, Murakane T, Nishizawa Y. DNA extraction method for screening yeast clones by PCR.
490 Biotechniques 2000;28:668-670,672,674.

- 491 34. Li D, Gurkovska V, Sheridan M, Calderone R, Chauhan N. Studies on the regulation of the two-
492 component histidine kinase gene CHK1 in *Candida albicans* using the heterologous lacZ reporter gene.
493 *Microbiology* 2004;150:3305-3313.
- 494 35. Chauhan N, Kruppa M, Calderone R. The Ssk1p response regulator and Chk1p histidine kinase mutants
495 of *Candida albicans* are hypersensitive to fluconazole and voriconazole. *Antimicrob Agents*
496 *Chemother*2007;51:3747-3751.
- 497 36. Li D, Williams D, Lowman D, Monteiro MA, Tan X, Kruppa M, *et al.* The *Candida albicans* histidine
498 kinase Chk1p: signaling and cell wall mannan. *Fungal Genet Biol.* 2009;46:731-741.
- 499 37. Klippel N, Cui S, Groebe L, Bilitewski U. Deletion of the *Candida albicans* histidine kinase gene CHK1
500 improves recognition by phagocytes through an increased exposure of cell wall beta-1,3-glucans.
501 *Microbiology* 2010;156:3432-3444.
- 502 38. Bates S, Hughes HB, Munro CA, Thomas WP, MacCallum DM, Bertram G, *et al.* Outer chain N-
503 glycans are required for cell wall integrity and virulence of *Candida albicans*. *J BiolChem.* 2006;281:90-
504 98.
- 505 39. Lopes-Bezerra LM, Lozoya-Perez NE, Lopez-Ramirez LA, Martinez-Alvarez JA, Teixeira MM, Felipe
506 MS, *et al.* Functional characterization of *Sporothrixschenckii*glycosidases involved in the N-linked
507 glycosylation pathway. *Med Mycol* 2015;53:60-68.
- 508 40. Torosantucci A, Chiani P, De Bernardis F, Cassone A, Calera JA, Calderone R. Deletion of the two-
509 component histidine kinase gene (CHK1) of *Candida albicans* contributes to enhanced growth inhibition
510 and killing by human neutrophils in vitro. *Infect Immun*2002;70:985-987.
- 511 41. Gow NA, Netea MG, Munro CA, Ferwerda G, Bates S, Mora-Montes HM, *et al.* Immune recognition of
512 *Candida albicans* beta-glucan by dectin-1. *J Infect Dis* 2007;196:1565-1571.
- 513 42. Arendrup M, Horn T, Frimodt-Møller N. In vivo pathogenicity of eight medically relevant *Candida*
514 species in an animal model. *Infection* 2002;30:286-291.
- 515 43. Cook SM, McArthur JD. Developing *Galleria mellonella* as a model host for human pathogens.
516 *Virulence* 2013;4:350-353.

- 517 44. Calera JA, Zhao XJ, De Bernardis F, Sheridan M, Calderone R. Avirulence of *Candida albicans* CaHK1
518 mutants in a murine model of hematogenously disseminated candidiasis. *Infect Immun* 1999;67:4280-
519 4284.
- 520 45. Kruppa M, Goins T, Cutler JE, *et al.* The role of the *Candida albicans* histidine kinase CHK1 gene in
521 the regulation of cell wall mannan and glucan biosynthesis. *FEMS Yeast Res.* 2003;3:289-299.
- 522 46. Kruppa M, Jabra-Rizk MA, Meiller TF, Calderone R. The histidine kinases of *Candida albicans*:
523 regulation of cell wall mannan biosynthesis. *FEMS Yeast Res* 2004;4:409-416.
- 524 47. Kruppa M. Quorum sensing and *Candida albicans*. *Mycoses*2009;52:1-10.
- 525 48. Li D, Bernhardt J, Calderone R. Temporal expression of the *Candida albicans* genes CHK1 and CSSK1,
526 adherence, and morphogenesis in a model of reconstituted human esophageal epithelial candidiasis. *Curr*
527 *Top Med Chem* 2002;2:939-971.
- 528 49. Chapeland-Leclerc F, Paccallet P, Ruprich-Robert G, Rebutier D, Chastin C, Papon N. Differential
529 involvement of histidine kinase receptors in pseudohyphal development, stress adaptation, and drug
530 sensitivity of the opportunistic yeast *Candida lusitanae*. *Eukaryot Cell* 2007;6:1782-1794.
- 531 50. Cho Y, Kim KH, La Rota M, Scott D, Santopietro G, Callihan M, *et al.* Identification of novel virulence
532 factors associated with signal transduction pathways in *Alternariabrassicicola*. *Mol Microbiol*
533 2009;72:1316-1333.
- 534 51. Luo YY, Yang JK, Zhu ML, Liu CJ, Li HY, Lu ZB, *et al.* The group III two-component histidine kinase
535 AlHK1 is involved in fungicides resistance, osmosensitivity, spore production and impacts negatively
536 pathogenicity in *Alternarialongipes*. *CurrMicrobiol*2012;64:449-456.
- 537 52. Papon N, Courdavault V, Clastre M, Bennett RJ. Emerging and emerged pathogenic *Candida* species:
538 beyond the *Candida albicans* paradigm. *PLoSPathog* 2013;9:e1003550.

540 **FIGURE LEGENDS**

541 **Fig.1. Different steps in construction of the *CHK1* disruption cassette.** A.Schematic
542 representation of the *C. Guilliermondii* *CHK1* locus and position of primers CHK1S and CHK1R.
543 **B.** Representation of plasmid pG-*CHK1*. **C.** Structure of the pG-*RU3R* plasmid. **D.** Representation

544 of plasmid pG-5'*CHK1-REP-URA3-REP-3'CHK1*. **E.** Structure of the 5'*CHK1-REP-URA3-REP-*
545 3'*CHK1* disruption cassette.

546

547 **Fig. 2. Disruption of *C. guilliermondii*CHK1.** **A.** Schematic representation of the wild-type and
548 disrupted *CHK1* locus. **B.** PCR analysis of the *CHK1* locus in the ATCC 6260 wild-type strain and a
549 representative *chk1*Δ mutant using CHK1S and CHK1R primers (upper panel). The presence of
550 *CHK1* mRNAs in the ATCC 6260 wild-type strain and the representative *chk1*Δ mutant was detected
551 with reverse transcriptase-PCR (middle panel). The *C. Guilliermondii* actin 1 gene (*ACT1*) mRNAs
552 (lower panel) were detected as a control of RNA integrity.

553

554 **Fig. 3. Loss of *CHK1* does not affect protein N-linked glycosylation in *C. guilliermondii*.** Cell
555 homogenates were obtained from either the wild type or *chk1*Δ null mutant and used to perform a
556 zymogram to reveal acid phosphatase activity, a reporter of the N-linked mannosylation pathway, as
557 described in Materials and methods. Similar electrophoretic mobility of the enzyme was observed
558 in both samples.

559

560 **Fig. 4. Multi-parametric analysis of the interaction between J774 macrophages and yeast cells**
561 **over 24 h time course experiments.** Macrophages were infected with yeast as described in the
562 Materials and methods. **A.** Fluorimetry analysis of the multiplication of fungal biomass in the
563 presence of macrophages. CFW was added to the well at the beginning of the infection, and total
564 fluorescence of CFW-labeled yeast was measured over time. **B.** Fluorimetry analysis of the uptake
565 of yeast cells by macrophages. Trypan blue was used to quench the CFW fluorescence of non-
566 ingested yeast cells. Residual CFW fluorescence reflected internalized yeasts. **C.** Flow cytometry
567 analysis of the mortality of intramacrophagic yeast cells after 24 h of phagocytosis. Ingested CFW-
568 labeled yeast cells were released from macrophages using Triton X-100 and were stained with
569 propidium iodide. Dead cells showed both CFW and PI fluorescence. **D.** Flow cytometry analysis of

570 the survival of infected macrophages after 24 h of infection. The number of surviving macrophages,
571 positive for both calcein and anti-CD16 fluorescence, was determined in the presence and in the
572 absence of yeast cells to calculate percent survival. Macrophages associated with yeast, i.e.,
573 phagocytosing macrophages, were identified as macrophages positive for CFW fluorescence. Non-
574 phagocytosing macrophages were negative for CFW fluorescence. Results are shown as mean \pm S.E.
575 of three independent experiments performed in triplicate or quintuplet.

576

577 **Fig. 5. Loss of *CHK1* does not affect the ability of *C. guilliermondii* to stimulate cytokine**
578 **production by human PBMCs.** Live or heat-killed (HK) cells either from strain ATCC 6260
579 (closed bars) or the *chk1* Δ null mutant (open bars) were co-incubated with human PBMCs at 37°C
580 and 5% CO₂. Upon 24 h incubation, supernatants were collected and used to measure cytokine
581 levels by ELISA. Results are shown as mean \pm SD of three independent experiments performed in
582 duplicate, with a total of six donors. **P* < 0.05.

583

584 **Fig. 6. Virulence of *C. guilliermondii chk1* Δ in the insect model *G. mellonella*.** Groups containing
585 10 larvae were infected with 10 μ L of 1 x 10⁵ yeast/ μ L of strains ATCC 6260 (WT) or the *chk1* Δ
586 null mutant, and mortality was recorded daily for 15 days. As a control, one animal group was
587 injected with 10 μ L of PBS. Kaplan-Meier plots were generated with cumulative data of three
588 independent experiments, and each survival curve contained 30 animals.

Table 1. Strains used in this study

Species	Strain	Genotype
<i>Candida guilliermondii</i>	ATCC 6260 ^a	wild type
	U312 ^b	<i>ura3</i>
	<i>chk1Δ</i>	<i>ura3, chk1::REP-URA3-REP</i>
<i>Candida parapsilosis</i>	ATCC 22019 ^a	wild type
<i>Candida albicans</i>	SC5314 ^a	wild type
<i>Candida lusitanae</i>	ATCC 38533 ^a	wild type
<i>Candida glabrata</i>	ATCC 90030 ^a	wild type

^a Reference strain from American Type Culture Collection, ATCC Manassas, USA.

^b From [16].

Table 2. MIC determination of *C. guilliermondii* wild type strain and *chk1Δ* null mutant towards various antifungal drugs and other chemicals.

	WT	<i>chk1Δ</i>
Antifungals		
Amphotericin B (μg/mL)	0.5 (0.25-0.5)	0.25 (0.25-0.5)
Flucytosine (μg/mL)	0.25 (0.12-0.5)	0.25 (0.12-0.5)
Fluconazole (μg/mL)	4	2 (2-4)
Voriconazole (μg/mL)	0.06	0.06 (0.03-0.06)
Caspofungin (μg/mL)	2 (1-2)	2
Other chemicals		
Congo Red (mM)	0.03	0.03 (0.03-0.06)
SDS (mM)	0.375 (0.375-0.5)	0.5 (0.5-0.625)
Caffeine (mM)	0.75	0.75 (0.5-0.75)
NaCl (M)	1.25 (1-1.25)	1.25 (1-1.25)
Sorbitol (M)	1.25 (1-1.25)	1 (0.75-1.25)
H ₂ O ₂ (mM)	2.5 (2.5-4)	4 (2.5-4)
Menadione (mM)	2 (1-2)	2 (1-2)

Table 3. Cell wall composition of *C. guilliermondii* *chk1*Δ null mutant

Strain	Cell wall abundance				
	Chitin (%)	Mannan (%)	Glucan (%)	Phosphomannan content (μg) ^a	Protein (μg) ^b
ATCC6260	1.8 ± 1.2	43.7 ± 3.2	54.5 ± 4.3	58.8 ± 9.0	165.7 ± 14.6
<i>chk1</i> Δ	1.6 ± 1.3	42.7 ± 3.5	55.7 ± 3.7	59.8 ± 10.9	157.7 ± 15.6

^aμg of Alcian Blue bound/OD₆₀₀=1^bμg of protein/mg of cell wall

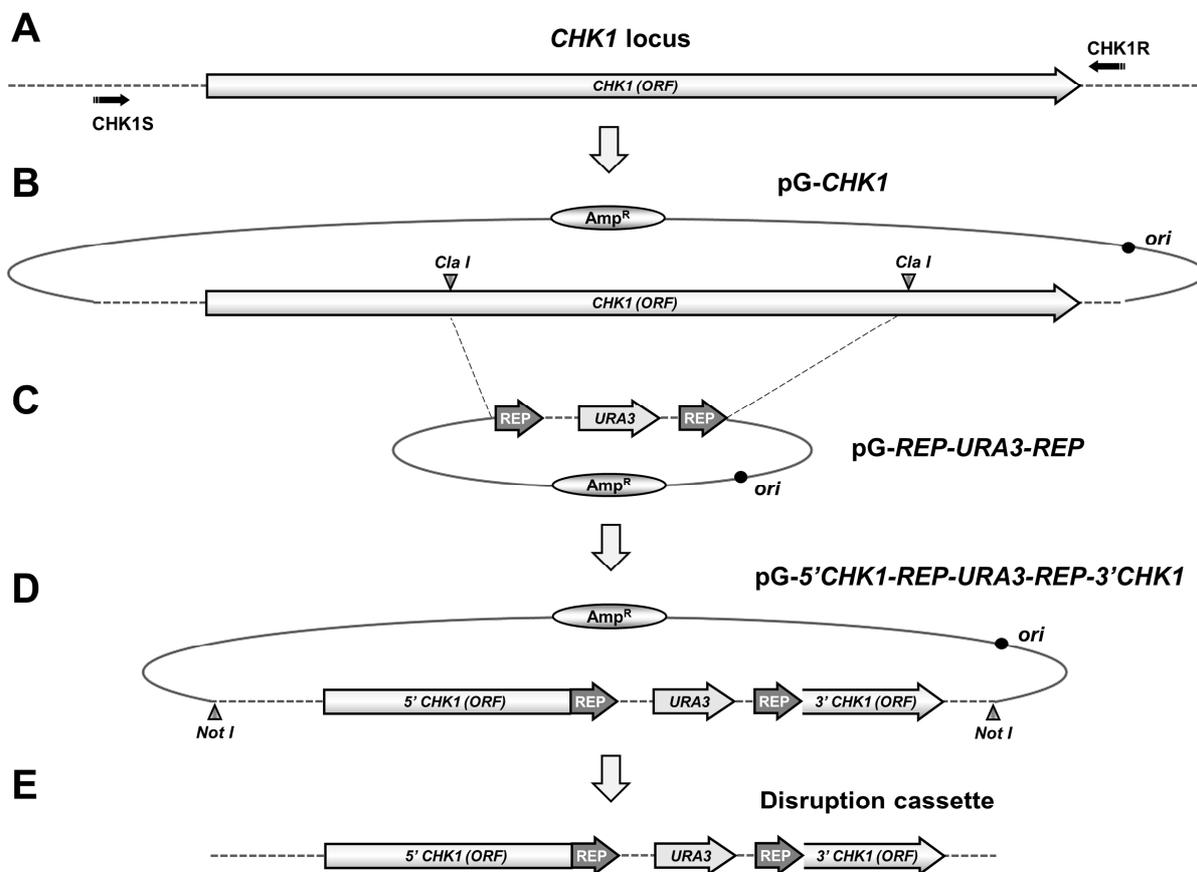
Table 4. Comparative analysis of the interactions involving J774 macrophages and different *Candida* species

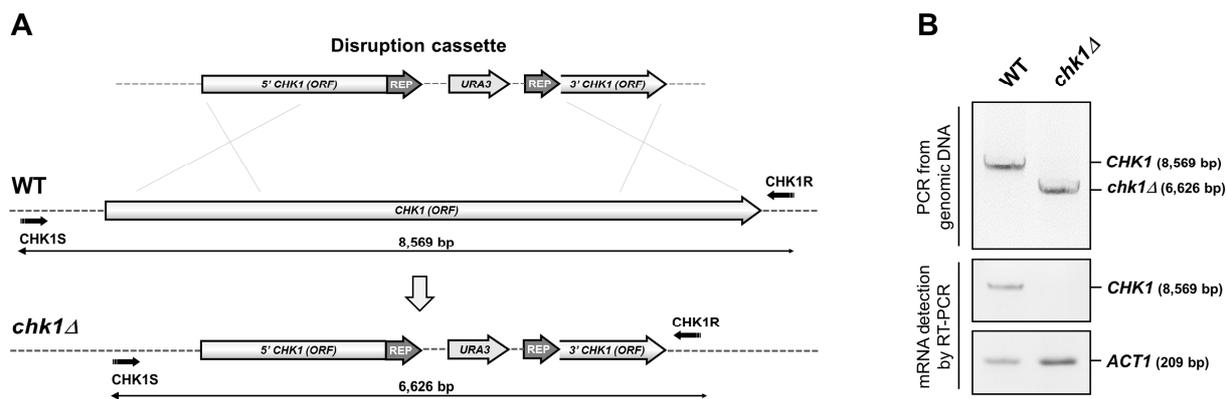
Species	Fungal biomass multiplication at 24 h ^a	Internalized yeast cells (%)			Macrophage (%) survival at 24 h (phagocytosing, %)	Yeast (%) mortality at 24 h

		1h	5 h	24 h		
<i>C. albicans</i> SC5314	6.8 ± 0.1	18 ± 2	10 ± 1	0	5 ± 3 (67 ± 17)	0
<i>C. glabrata</i> ATCC 90030	1.4 ± 0.1	55 ± 5	76 ± 2	67 ± 9	79 ± 12 (47 ± 11)	15 ± 5
<i>C. lusitaniae</i> ATCC 38533	3.6 ± 1.0	54 ± 5	64 ± 2	37 ± 4	73 ± 7 (57 ± 6)	26 ± 1
<i>C. guilliermondii</i> ATCC 6260	1.1 ± 0.1	41 ± 4	77 ± 10	81 ± 21	90 ± 24 (63 ± 1)	18 ± 1

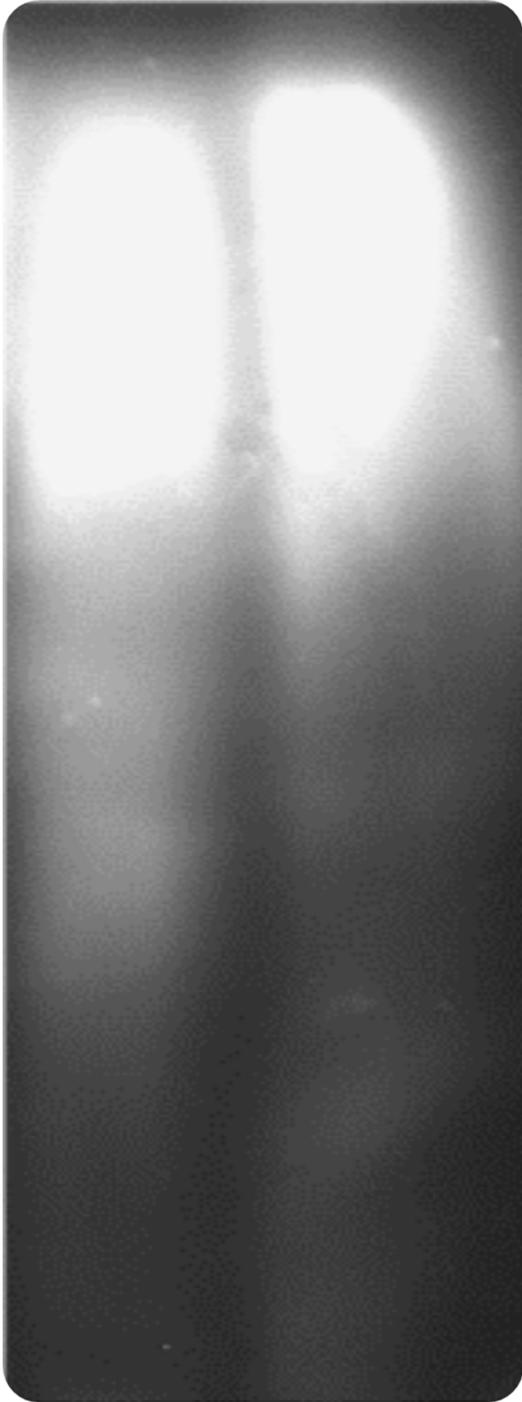
^acompared to initial fungal biomass at time = 1h.

Results are shown as mean ± S.E. of three to six independent experiments performed by triplicates or quintuplets.





WT **chk1 Δ**



ACCEPTED MANUSCRIPT

