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Candida albicans Cek1 MAPK signaling enhances fungicidal activity of salivary Histatin 5

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Running title: *C. albicans* Cek1 Enhances Susceptibility to Histatin 5

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1 **ABSTRACT**

2 *Candida albicans* is a major etiological organism for oropharyngeal candidiasis (OPC), while
3 salivary Histatin 5 (Hst 5) is a human fungicidal protein that protects the oral cavity from OPC.
4 *C. albicans* senses its environment by MAPKinase activation that can also modulate the
5 activity of some antifungal drugs including Hst 5. We found that phosphorylation of the MAPK
6 Cek1, induced either by N-acetyl-glucosamine (GlcNAc) or serum, or its constitutive
7 activation by deletion of its phosphatase Cpp1, elevated the susceptibility of *C. albicans* cells
8 to Hst 5. Cek1 phosphorylation but not hyphal formation was needed for increased Hst 5
9 sensitivity. Interference with the Cek1 pathway by deletion of its head sensor proteins, Msb2
10 and Sho1, or by addition of secreted aspartyl protease (SAP) cleavage inhibitors such as
11 pepstatin A, reduced Hst 5 susceptibility under Cek1 inducing conditions. Changes in fungal
12 cell surface glycostructures also modulated Hst 5 sensitivity, and Cek1 inducing conditions
13 resulted in a higher uptake rate of Hst 5. These results show that there is a consistent
14 relationship between activation of Cek1 MAPK and increased Hst 5 susceptibility in *C.*
15 *albicans*.

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17 **INTRODUCTION**

18 *Candida albicans* is the major etiological organism of oral candidiasis (thrush) in individuals
19 whose immune system is impaired. Naturally occurring antimicrobial peptides such as defensins
20 and histatins are promising candidates for treatment of fungal infections because of their distinct
21 mechanism of action from conventional azole and polyene-based antifungal drugs (1). Salivary
22 Histatin 5 (Hst 5) is a fungicidal histidine-rich protein constitutively produced by human salivary
23 gland cells, with physiological concentrations in saliva ranging from 10 to 30 μM (2). Hst 5
24 initially binds to the *C. albicans* cell wall followed by active translocation into the cytosol by Dur3
25 and Dur31 polyamine transporters (3). Although Hst 5 appears to have several intracellular
26 targets (4), it ultimately induces selective leakage of small intracellular ions and nucleotides,
27 causing gradual cell death (4).

28 The oral cavity is a challenging environment for fungal colonization due to wide fluctuations in
29 temperature, tonicity, and osmolarity. *C. albicans* senses environmental changes through its
30 membrane sensors that elicit responses through various signaling pathways, one of the most
31 important being MAP kinase (MAPK) signal transduction pathways (5). Four MAPK pathways
32 have been identified in *C. albicans*: the high-osmolarity glycerol (HOG) pathway, the cell wall
33 integrity Mkc1 pathway, and the Cek1 and Cek2 pathways. The HOG MAPK network is involved
34 in adaptation to both osmotic and oxidative stresses (6-11) as well as heavy metal stresses (11).
35 We found that Hst 5 treatment of *C. albicans* cells induced rapid activation of the Hog1 pathway
36 (12), related to Hst 5 induction of cellular osmotic stress. *C. albicans* cells that were first
37 subjected to osmotic stress, to induce Hog1 phosphorylation, became resistant to Hst 5.
38 Conversely, *C. albicans hog1Δ/Δ* knock-outs were more susceptible to Hst 5, illustrating the
39 protective role the Hog1 MAPK pathway plays in Hst 5 susceptibility (12). However, the Mkc1

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40 pathway that is involved in cell wall repair (13, 14), biofilm formation (15), response to some
41 stress conditions (13), and protection from echinocandin antifungal drugs (16); does not directly
42 modulate the antifungal activity of Hst 5 (12). Likewise, Cek2 participates in mating (17) but has
43 no known role in modulating antifungal drug activity (18).

44 The *C. albicans* Cek1 MAPK pathway is involved in cell wall biogenesis, hyphal development,
45 and virulence (5, 19). Although largely known for its role in hyphal formation, the Cek1 pathway
46 is not absolutely necessary for hyphae induction as illustrated by *C. albicans cek1Δ/Δ* cells that
47 form hyphae in medium containing serum (20). Also, cells lacking Cph1, Cek1 pathway's
48 dedicated transcription factor involved in hyphal formation, still show some virulence in a murine
49 model of systemic candidiasis (21). However *cek1Δ/Δ* cells are avirulent in the same
50 experimental model of infection, suggesting that Cek1 has an important function beyond hyphae
51 formation. This function has been illustrated in experiments that showed Cek1 activation in
52 response to damage to cell surface glycostructures (22). Interestingly, this activation did not
53 lead to hyphal formation, suggesting that Cek1 plays an important role in cell wall maintenance
54 and remodeling that is independent of its role in hyphal formation. It must be noted that the
55 Mkc1 pathway also has an important role in hyphal formation (23). Nevertheless, filamentation
56 induced by any signal involving Cek1 or Mkc1 represents a situation wherein the cell wall
57 undergoes extensive changes.

58 There is considerable cross-talk between the Hog1 and Cek1 pathways (24). The Hog1
59 pathway can repress the Cek1 MAP Kinase pathway under basal conditions (19), so that *C.*
60 *albicans hog1Δ/Δ* cells have constitutively higher levels of Cek1 phosphorylation. Since
61 *hog1Δ/Δ* cells are more susceptible to Hst 5, we considered the possibility that the

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62 accompanying Cek1 MAPK activation may also potentiate Hst 5 antifungal activity. *C. albicans*
63 cells grown in the presence of N-Acetyl-D-glucosamine (GlcNAc) at 37°C showed optimal
64 release of the extracellular inhibitory domain of Msb2 (head sensor of Cek1 pathway),
65 potentially by the action of secreted aspartic proteases (SAPs), along with corresponding levels
66 of Cek1 phosphorylation (25). A second sensor of the Cek1 pathway, the 4-pass or tetraspan
67 protein called Sho1 (26, 27), is needed for Cek1 activation as well, while the phosphatase Cpp1
68 removes the phosphate group from activated Cek1 to negatively regulate the pathway. We
69 subjected *C. albicans* cells to conditions that either induced optimal Cek1 phosphorylation or
70 had an inhibitory effect on the pathway, followed by evaluation of Hst 5 susceptibility. We also
71 examined various *C. albicans* mutants lacking proteins involved in the Cek1 pathway, with
72 regards to Hst 5 susceptibility. Our results provide compelling evidence that Cek1 activation
73 enhances Hst 5 mediated killing and thus plays an important role in Hst 5 susceptibility.

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75 **MATERIALS and METHODS**

76 **Strains and chemicals.** The genotypes of strains used in this study are described in Table 1. *C.*
77 *albicans* strain CAI-4 (28) was used as the WT strain, *cek1Δ/Δ* (20) was kindly provided by Dr.
78 Malcolm Whiteway (Concordia University, Montreal, Canada), *hog1Δ/Δ* (9) was kindly provided
79 by Dr. Janet Quinn, (Newcastle University, Newcastle, United Kingdom), and *cpp1Δ/Δ* (29) was
80 kindly provided by Dr. Carol Kumamoto (Tufts University). Deletion mutants of Msb2, Sho1, and
81 Msb2/Sho1 were constructed in CAI-4 parental background as described (25). Cells were
82 cultured in yeast extract/peptone/dextrose (YPD; Difco) medium with uridine (50 µg/ml).
83 Pepstatin A (PA) (Sigma-Aldrich), a specific inhibitor of aspartic proteases, anti-β-(1,3)-glucan
84 monoclonal antibody (Biosupplies, Ltd, Australia), glycosylation inhibitor tunicamycin (Santa
85 Cruz Biotechnology, Inc.), and antifungal drug caspofungin (Sigma-Aldrich) were used in
86 candidacidal experiments, as indicated. Lee's media (pH=6.8) was freshly made as described
87 (30).

88 **Analysis of Cek1 phosphorylation.** For Cek1 phosphorylation studies, overnight cells were
89 diluted to an OD_{600}=0.3, grown to OD_{600} =1, followed by washing and re-suspension in pre-
90 warmed YNB media with 1.25% N-acetyl-D-glucosamine (GlcNAc) as carbon source and
91 growth at 37°C for 1 h. For all other experiments, cell were incubated at 30°C for 1 h in YNB
92 media with 1.25% glucose (Glc) as carbon source after reaching OD_{600} =1. Fetal bovine serum
93 (FBS, Life Technologies, Grand Island, NY) was also used to induce Cek1 phosphorylation.
94 After 1 h incubation, cells were harvested at 2,500 X *g* and 4°C for 2 min, and washed with 10
95 mM pH 7.4 sodium phosphate buffer (NaPB). For protein extraction, cell pellets were placed on
96 ice and re-suspended in 300 ml 10% TCA buffer (10 mM Tris HCl pH 8.0, 10% trichloroacetic
97 acid, 25 mM NH_4OAc, 1 mM Sodium EDTA). Total cellular lysate were isolated by disrupting

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98 cells with acid washed beads by vortexing for 1 min x 10 cycles using FastPrepH-24 Instrument
99 (MP Biomedicals LLC). Samples were placed on ice for 5 min between each cycle. The beads
100 were removed and the samples were centrifuged at 4°C for 10 min at 15,000 X *g*. The
101 supernatant was removed and resuspended in 150 μl of buffer (0.1 M Tris HCl pH 11.0, 3%
102 SDS). Samples were boiled for 5 min, and then centrifuged at 15,000 X *g* for 30 sec.
103 Normalized protein content (20 μg) was separated by SDS-PAGE on 12% gels and transferred
104 to nitrocellulose membranes. After transfer, membranes were incubated with primary antibodies
105 at 4°C for 16 h in 5% BSA buffer (0.5 g BSA, 10 ml (TBST)), followed by washing with TBST.
106 For Cek1 phosphorylation, anti-phospho p42/44 MAPK ERK1/2 Thr202/Tyr204 rabbit
107 monoclonal (Signaling Technology) antibody (P-Cek1) was used as the primary antibody. Cek1
108 protein was used as loading control and detected by a polyclonal Cek1 antibody (raised against
109 two fragments of Cek1 protein, from 86-101 and 111-125, by Genemed Synthesis, Inc). This
110 Cek1 antibody recognizes Cek1p as well as its close homologue Cek2p. Goat α-rabbit IgG-HRP
111 (Jackson ImmunoResearch Laboratories, Inc.) was used as the secondary antibody. The
112 membranes were then incubated with secondary antibodies at 25°C for 1 h in blocking buffer,
113 washed, and used for detection using SuperSignal West Pico detection kit (Thermo Scientific).

114 **Microscopic studies.** Morphology of cells used for candidacidal assays were examined
115 microscopically using an Axio Fluorescence Microscope and visualized at 40X magnification.
116 Cells were photographed and the degree of germination of at least 100 observed cells under
117 various conditions was calculated and documented as follows: cell groups without any
118 germination (-); cell groups with germination less than 50% and having small (less than 2X the
119 length of the mother cell) germ tubes (+/-); cell groups having 50% - 100% germination as well
120 as germ tubes 2-3X length of mother cells (+); and cell groups with 100% germination as well as

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121 germ tubes at least 2-3X length of mother cells (++).

122 **Candidacidal Assay of Hst 5**. Killing assays of *C. albicans* strains by Hst 5 was performed
123 using the microdilution plate assay as previously described (12). For pre-treatment, *C. albicans*
124 cells were first incubated for 1 h in either YNB containing 1.25% Glc at 30°C, YNB containing
125 1.25% GlcNAc at 37°C, 10 mM sodium phosphate buffer at pH 7.4 (NaPB) containing
126 tunicamycin (5 μg/ml), 10 mM NaPB containing caspofungin (20 ng/ml), or Lee's media. Cells
127 were washed twice in NaPB and re-suspended in NaPB at a concentration of 10^6 cells/ml. To
128 test the effect of pepstatin A (PA), a specific inhibitor of aspartic proteases, cells were
129 pretreated with GlcNAc at 37°C or Glc at 30°C for 1 h with or without 10 μM PA. To test the
130 involvement of β-1, 3-glucans, cells were first incubated with β-1,3-glucan monoclonal antibody
131 (10 μg/ml) at room temperature for 30 min, and washed twice in NaPB before addition of Hst 5
132 (31 μM). The cell suspensions (50 μl) were then mixed with Hst 5 (31 μM) for 30 min at 30°C
133 with shaking. Cell suspensions were diluted in 10 mM NaPB, and aliquots of 500 cells were
134 spread onto YPD plus uridine agar plates and incubated for 48 h at room temperature. Assays
135 were performed in triplicate for each strain. Cell death was calculated as [1 - (number of
136 colonies recovered from Hst 5-treated cells/ number of colonies from control cells)] X 100%, to
137 determine percent (%) killing. Differences between experimental groups were evaluated for
138 significance using unpaired Student's t-test, and analyzed by Prism 5.0 software.

139 **Time Lapse Confocal Microscopy.** Overnight grown cells were diluted with fresh YPD media
140 to OD_{600} of 0.4 and grown till OD_{600} of 0.8–1.0. *C. albicans* cells, pretreated with Glc at 30°C or
141 GlcNAc at 37°C for 1 h, were washed by NaPB twice and diluted to obtain 10^6 cells/ml in NaPB.
142 For analyzing rate of cell death following Hst 5 exposure, chambered cover glass slides (Lab-

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143 TekII) were coated with concanavalin A (100 mg/ml) for 60 min and washed twice with water.
144 Cells (10^6 cells/ml) were fixed on concanavalin A-coated slides for 30 min at room temperature.
145 The plates were then washed twice with NaPB, followed by addition of Hst 5 (31 μM) and
146 propidium iodide (PI) (5 μg/ml). Images were captured each minute using a Zeiss LSM 510
147 Meta Confocal Microscope and Plan Apochromat 63/1.4 (oil) objective. ImageJ software was
148 used for image acquisition and total mean fluorescent intensity of total PI uptake of cells per
149 minute was calculated.

150 **Detection of cytoplasmic Hst 5.** For cytosolic levels of Hst 5, *C. albicans* cells were pretreated
151 with Glc at 30°C or GlcNAc at 37°C for 1 h, then cells (10^7) were collected and re-suspended in
152 1 ml of NaPB with Biotin labeled Hst 5 (BHst 5) (final concentration of 31 μM). The cell mixtures
153 were incubated with constant shaking for 30 min, and the cell pellet was subjected to
154 cytoplasmic extraction as described before (31). Cytoplasmic BHst 5 was detected by Western
155 blots using streptavidin conjugated with horseradish peroxidase (Pierce) normalized by total
156 cytosolic protein.

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158 **RESULTS**

159 **Hst 5 treatment does not induce Cek1 phosphorylation.** In order to assess whether
160 addition of Hst 5 to *C. albicans* may activate Cek1 MAPK, phosphorylation of Cek1 following
161 Hst 5 exposure was measured. Cells were pretreated with either GlcNAc at 37°C for 1 h (a
162 Cek1 inducing condition), or with Glc at 30°C that does not induce Cek1 phosphorylation,
163 followed by exposure to 31 μM Hst 5 for 0, 10, 20, and 30 min (Fig. 1). Hst 5 treatment alone
164 did not cause Cek1 phosphorylation in *C. albicans* cells pretreated with Glc (Fig. 1, left). Cells
165 pretreated with GlcNAc showed substantial Cek1 phosphorylation at 0 min that slowly
166 diminished by 20 mins in Hst treated cells (Fig. 1, middle), and was very similar to that of
167 control cells without Hst 5 (Fig. 1, right panel). The reduction in Cek1 phosphorylation at 30
168 min compared to control cells is most likely because of Hst 5 mediated cell death, although
169 Cek1 phosphorylation was also diminished in control cells by 30 mins. Thus, Hst 5 itself does
170 not induce Cek1 phosphorylation.

171 **Cells exhibiting Cek1 phosphorylation have higher Hst 5 susceptibility.** Next we wanted
172 to determine whether *C. albicans* cells treated with Cek1 phosphorylation-inducing conditions
173 would alter cell susceptibility to subsequent exposure with Hst 5 (Fig. 2). Previously, we found
174 that combining a temperature shift from 30°C to 37°C with GlcNAc as a carbon source
175 induced high levels of Cek1 phosphorylation; while *C. albicans* exposed to either 37°C in Glc,
176 or GlcNAc alone, exhibited lesser levels of phosphorylation (25). Hence, we pretreated cells to
177 stimulate different levels of Cek1 phosphorylation (Glc at 30°C as negative control, Glc at
178 37°C, GlcNAc at 30°C, and GlcNAc at 37°C as positive control), prior to exposure of cells to
179 Hst 5 (31 μM). We also monitored germination under these conditions; and as expected, cells

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180 treated with GlcNAc at 37°C showed robust germination (+), while less than 50% of cells
181 pretreated with Glc at 37°C were germinated (+/-). Cells pre-incubated with GlcNAc at 37°C
182 had maximal Cek1 phosphorylation and showed significantly ($P<0.001$) increased
183 susceptibility to Hst 5 (from 50% to 77% killing) compared with control cells (Fig. 2). However,
184 *C. albicans* cells pretreated with conditions that only weakly induce Cek1 phosphorylation (Glc
185 at 37°C, GlcNAc at 30°C) did not have a statistically significant change in Hst 5 susceptibility
186 (Fig. 2).

187 **Conditions affecting Cek1 activation alter Hst 5 susceptibility.** To further show the
188 dependence of Hst 5 killing on prior strong Cek1 activation, we examined Hst 5 antifungal
189 activity in mutants lacking components of Cek1 MAPK pathway (*cek1Δ/Δ*, Fig. 3 or *msb2Δ/Δ*,
190 *sho1Δ/Δ*, and *msb2/sho1Δ/Δ*, Fig. 4). We also examined *C. albicans hog1Δ/Δ* cells (Fig. 3),
191 which show constitutively high levels of Cek1 phosphorylation (19). *C. albicans cek1Δ/Δ*
192 mutants did not respond to GlcNAc at 37°C and did not have increased susceptibility to Hst 5
193 after pretreatment with GlcNAc at 37°C as compared to WT control cells (Fig. 3A). In contrast,
194 *C. albicans hog1Δ/Δ* mutants showed elevated levels of Cek1 activation and increased Hst 5
195 killing irrespective of pretreatment (GlcNAc at 37°C or Glc at 30°C) (Fig. 3A). To determine
196 whether there is further cross-talk between Hog1 and Cek1 MAPK with respect to Hst 5
197 susceptibility, *C. albicans* cells were osmotically stressed with 1M NaCl to induce strong Hog1
198 phosphorylation. As we found previously (12), cells pretreated with conditions that induce P-
199 Hog1 were half as susceptible to Hst 5 killing as untreated cells (Fig. 3B), most likely because
200 of the negative effect Hog1 activation has on the Cek1 pathway (19); and these Hog1 inducing
201 conditions did not activate Cek1.

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202 Furthermore, *C. albicans* cells lacking Cek1 MAPK head sensor proteins (*msb2Δ/Δ*, *sho1Δ/Δ*
203 and *msb2/sho1Δ/Δ*) showed negligible Cek1 phosphorylation in response to GlcNAc at 37°C
204 and proportionally reduced subsequent *C. albicans* Hst 5 susceptibility (Fig. 4A, grey bars);
205 that was also not evident in the control cells pretreated with Glc at 30°C (Fig. 4A, white bars).
206 However, all three mutant strains (*msb2Δ/Δ*, *sho1Δ/Δ,* and *msb2/sho1Δ/Δ*) exhibited hyphal
207 formation (Fig. 4A) even in the absence of Cek1 phosphorylation, showing that changes in Hst
208 5 sensitivity are independent of hyphal induction. Presence of 10 μM Pepstatin A (PA), a
209 specific inhibitor of aspartic proteases that mediate Cek1 signaling through Msb2 cleavage
210 (25), inhibited Cek1 phosphorylation in cells pretreated with GlcNAc at 37°C and
211 correspondingly lowered Hst 5 susceptibility to levels similar to that of control cells pretreated
212 with Glc at 30°C and not subjected to protease treatment (Fig. 4B). However, in this instance it
213 was not possible to correlate killing with hyphal induction since PA treatment at this
214 concentration inhibited hyphal formation. Thus, these data further supported the link between
215 Cek1 activation and Hst 5 susceptibility.

216 Since *C. albicans* phosphatase Cpp1 negatively regulates the Cek1 MAPK pathway (20), we
217 further hypothesized that a *C. albicans* strain lacking Cpp1 and having constitutive levels of
218 Cek1 phosphorylation would be hypersensitive to Hst 5, even under non-inducing conditions.
219 As expected, *cpp1Δ/Δ* cells showed constitutive levels of Cek1 phosphorylation similar to that
220 of GlcNAc-induced WT cells; and these cells were also more sensitive to Hst 5 under either
221 non-inducing (Glc at 30°C) or inducing (GlcNAc at 37°C) conditions (Fig. 5A). By comparison,
222 the sensitivity of wild-type cells to Hst 5 was elevated only after pretreatment with GlcNAc at
223 37°C. The elevated sensitivity of *cpp1Δ/Δ* cells to Hst 5 was not due to the extent of hyphal
224 formation, since there was no difference in Hst 5 susceptibility between cells pretreated with

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225 GlcNAc at 37°C (100% cells showed germination, ++) and cells pretreated with Glc (50-100%
226 germination, +); and these cells were less sensitive to Hst 5 than germinated WT cells.

227 To determine whether subjecting *C. albicans* cells to other conditions that stimulate Cek1
228 would also create Hst 5 hypersensitivity similar to GlcNAc at 37°C, we pretreated cells with
229 the cell wall damaging agent caspofungin (20 ng/ml) as this was reported to induce robust
230 Cek1 phosphorylation (32). Unexpectedly, cells pretreated with caspofungin showed only a
231 minor increase in Cek1 phosphorylation after pretreatment from 30 - 120 min when compared
232 with cells pretreated at GlcNAc at 37°C (Fig. 5B). Increasing doses of caspofungin beyond
233 those previously reported (32) did not raise Cek1 phosphorylation levels more than those
234 induced by GlcNAc at 37°C (data not shown). Correspondingly, caspofungin pretreated cells
235 had no difference in their susceptibility to Hst 5, in agreement with our observed lack of Cek1
236 phosphorylation (Fig. 5B). Thus, unlike previous reports, our data shows that caspofungin
237 treatment of *C. albicans* cells is not a robust inducer of the Cek1 MAPK pathway, at least
238 under our assay conditions.

239 **Hst 5 susceptibility increased upon induction of Cek1 phosphorylation independently**
240 **from hyphal formation.** To further assess the relationship between Hst 5 susceptibility, Cek1
241 phosphorylation and hyphal induction, we examined other hyphal-inducing conditions (Fig. 6A).
242 Cells pretreated with 10% FBS showed even higher levels of germination (100% cells showed
243 germination, ++) than cells pretreated at GlcNAc at 37°C (+), but sensitivity to Hst 5 killing was
244 not significantly ($P>0.05$) increased (Fig. 6A). Next, we tested hyphal formation in *C. albicans*
245 *cek1Δ/Δ* cells in response to serum and found strong germination (+) (Fig. 6A), (as shown
246 previously (20)). However, germinated *cek1Δ/Δ* cells had the same sensitivity to Hst 5 as WT

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247 cells pretreated with Glu having no hyphal formation (Fig. 6A), thus suggesting that increased
248 Hst 5 sensitivity is mediated through Cek1 phosphorylation specifically and is not merely a
249 result of serum-induced hyphae formation. To further address this, we induced hyphae using
250 Lee's Media at 37°C temperature for 1 h, a condition that by-passes the Cek1 pathway as we
251 do not observe Cek1 phosphorylation under these conditions (Fig. 6B). After growth in Lee's
252 media, cells exhibited hyphae formation comparable to that induced by GlcNAc at 37°C (Fig.
253 2) however, these cells did not show any increase in Hst 5 sensitivity in the absence of Cek1
254 phosphorylation (Fig. 6B). Thus we conclude that Cek1 phosphorylation and not hyphal
255 formation is the reason for elevation of Hst 5 sensitivity in cells pretreated with Cek1 activating
256 conditions.

257 **Cek1 activation leads to higher Hst 5 uptake rates in *C. albicans*.** Since Candida cell
258 surface β-1,3-glucans play an important role in Hst 5 binding (33), and the Cek1 pathway
259 modulates cell surface exposure of β-1,3-glucans (22), we examined whether changes in Hst
260 5 susceptibility are a result of altered surface β-1,3-glucan exposure in response to Cek1
261 inducing conditions (GlcNAc at 37°C). For cells pretreated with Glc at 30°C, addition of an
262 antibody specific to β-1,3-glucan reduced Hst 5 killing (31 μM) from 51% to 40% (Fig. 7A),
263 similar to what we have previously reported (33). However, addition of β-1,3-glucan-specific
264 antibody to cells pretreated with GlcNAc at 37°C reduced Hst 5 killing to the level of cells
265 treated with Glc at 30°C and Ab, from 72% to 40% (Fig. 7A). Thus, elevated Hst 5 killing of
266 cells pretreated with GlcNAc at 37°C may be due, in part, to increased Hst 5 binding to
267 exposed *C. albicans* surface β-1,3-glucans as a result of Cek1-mediated cell wall remodeling.

268 To further test address the role of changes in cell surface glycostructures, we pre-treated cells

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269 with tunicamycin (5 µg/ml), a classic glycosylation inhibitor, before addition of Hst 5 to
270 determine whether this might alter subsequent Hst 5 susceptibility. However, tunicamycin
271 itself reduced *C. albicans* viability similarly to that of Hst 5 alone (Fig. 7B). Pre-treatment of
272 cells with tunicamycin followed by Hst 5 further increased cell killing from 51% to 75% (Fig.
273 7B). Although treatment with tunicamycin did enhance cell susceptibility to Hst 5, this result
274 might also be due to a combinatorial/synergistic drug effect in addition to cellular defects in
275 glycosylation.

276 Since a reason for higher Hst 5 susceptibility is its increased uptake by *C. albicans* cells, we
277 compared cellular uptake of Hst 5 under Cek1 inducing and non-inducing conditions.
278 Intracellular translocation of biotin labeled Hst 5 (BHst 5) as a function of pretreatment of cells
279 with either Cek1 inducing or non-inducing conditions was measured. Cells exposed to GlcNAc
280 at 37°C had higher cytosolic levels of BHst 5 after 30 min compared with cells treated with Glc
281 at 30°C (Fig 7C). To examine the dynamics of early uptake of Hst 5 in cells exposed to P-
282 Cek1 conditions, we measured propidium iodide (PI) uptake visualized by time-lapse confocal
283 microscopy since intracellular PI is a measure of cell death and directly relates to levels of
284 intracellular Hst 5 (34). *C. albicans* pretreated with GlcNAc at 37°C showed 21% PI positive
285 cells after 6 min, compared with only 7% PI positive cells for those cells pretreated with Glc at
286 30°C (Fig. 7D); showing that the rate of cell death is higher in cells pretreated with GlcNAc at
287 37°C. These data show that conditions inducing Cek1 phosphorylation ultimately lead to
288 higher Hst 5 uptake and increased *C. albicans* susceptibility.

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290 **DISCUSSION**

291 Although many factors (environmental high salt, fungal energy depletion) are known to
292 decrease the candidacidal activity of Hst 5, this is the first report of a means to render *C.*
293 *albicans* cells more sensitive to this fungicidal protein. We show here that phosphorylation of
294 Cek1, induced either by GlcNAc (Fig. 2) or serum (Fig. 6A), or its constitutive activation by
295 deletion of its phosphatase Cpp1 (Fig. 5A), makes cells more susceptible to Hst 5. Conversely,
296 interference with the Cek1 pathway by deletion of its head sensor proteins, Msb2 and Sho1
297 (Fig. 4A), or by addition of cleavage inhibitors such as Pepstatin A (Fig. 4B), blocked elevation
298 in Hst 5 susceptibility following Cek1 inducing conditions. We found that optimal Cek1
299 phosphorylation resulted in a 50% increase in Hst 5 killing activity *in vitro*. However, our *in vitro*
300 assays only measure a short time span of interaction of Hst 5 with cells, compared with
301 conditions likely found *in vivo* in which fungal cells are continuously exposed to Hst 5
302 replenished by salivary secretions. Thus, *C. albicans* cells in the oral environment that better
303 transport and accumulate Hst 5 (due to activation of Cek1 MAPKinase) are likely to have even
304 higher sensitivity to Hst 5 when compared to *in vitro* conditions. In this regard, we found that
305 increasing *in vitro* killing of Hst 5 conjugates by 50% resulted in more than four log-fold
306 reduction in fungal tongue burden in murine OPC (34). Similar animal experiments are needed
307 to test the effects of Cek1 activation *in vivo*.

308 The *C. albicans* cell wall is a complex and dynamic structure consisting of a core of β-1,3-
309 glucans covalently linked to β-1,6-glucans and chitin, with an outer layer or matrix composed
310 mainly of mannose-glycosylated proteins (35). Cek1 plays a role in maintenance of cell wall
311 structure through control of β-glucan exposure (22) and mannosylation status (35) of cell wall
312 glycoproteins; and the pathway responds to cell surface damage caused by agents such as

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313 caspofungin and tunicamycin (32). Previously we have shown that Hst 5 binds to *C. albicans*
314 surface β-1,3-glucans and this binding facilitates its antifungal activity (33). Our discovery that
315 conditions inducing P-Cek1 caused greater cellular uptake of Hst 5 and that treatment with
316 antibody that blocks *C. albicans* cells surface β-1,3-glucans led to reduction of Hst 5 killing (Fig.
317 7A), suggested that the Cek 1 pathway influences Hst 5 susceptibility in part through exposure
318 of cell wall β-1,3-glucans. We also tested the expression levels of Dur3 and Dur31 transporters
319 (data not shown) that are necessary for uptake of Hst 5 (3), both under Cek1 inducing and non-
320 inducing conditions. We did not observe any difference in expression levels of either *DUR3* or
321 *DUR31* genes (data not shown), showing that the probable cause of enhanced Hst 5 uptake
322 under inducing conditions is not a change in expression levels of transporters genes. Instead, it
323 is possible that other Cek1-mediated factors might increase Dur transporter accessibility or its
324 substrate affinities.

325 It is important to note that elevated Hst 5 sensitivity mediated by Cek1 was not coupled to
326 hyphae formation following Cek1 activation. This is not surprising since other pathways such
327 as Mkc1 (23) and PKA (36) are also involved in hyphae formation, while Cek1 activation is not
328 required for the process of germination under all growth conditions (20). Nevertheless, cell wall
329 changes during germination may still have some role in increasing *C. albicans* susceptibility to
330 Hst 5 since cell wall remodeling is an important part of the process of hyphae formation. Even
331 subtle increases in Cek1 phosphorylation, may translate into corresponding changes in Hst 5
332 susceptibility (see Fig. 5A), underscoring the role of Cek1 pathway in Hst 5 susceptibility. We
333 also observed greater Hst 5 susceptibility for cell lacking Hog1, even under conditions that do
334 not induce Cek1 phosphorylation (Fig. 3). This is likely a result of higher levels of constitutive
335 Cek1 phosphorylation in the absence of Hog1; since Hog1 is known to repress the Cek1

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336 pathway (19). It is quite possible that Cek1 inducing conditions may also repress Hog1
337 activation that has a protective role in Hst 5 mediated insult to *C. albicans* cells, thereby
338 providing an alternative explanation for our results, independent of Cek1 mediated cell wall
339 remodeling.

340 This study highlights the involvement of MAPK pathways in modulating *C. albicans*
341 susceptibility to Hst 5, and further underscores the importance of environmental conditions that
342 activate such pathways. New modalities in treatment of oral candidiasis might be combinatorial
343 therapies using biopeptides such as Hst 5 along with oral treatments that "prime" fungal cells
344 for enhanced susceptibility. Thus, exposing fungal cells *in vivo* to conditions that induce Cek1
345 might further improve Hst 5 killing both by native secreted peptides or therapeutically applied
346 Hsts.

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349 Institutes of Health, USA.

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FIGURE LEGENDS

FIG 1. Hst 5 itself does not initiate Cek1 phosphorylation. *C. albicans* cells were pretreated with Cek1-inducing or non-inducing conditions, GlcNAc at 37°C or Glc 30°C, respectively, for 1 h, followed by washing with 10 mM NaPB before exposure to 31 μM Hst 5 for 0, 10, 20, and 30 min. Non-inducing conditions (left panel) were used as negative control for P-Cek, while inducing conditions without treatment with Hst 5 (right panel) were used as positive control. Phosphorylation of Cek1 was detected by α–phospho p42/44 MAPK ERK1/2 Thr202/Tyr204 rabbit monoclonal (P-Cek1) as the primary antibody, while Cek1/2 protein was used as loading control.

FIG 2. Cek1 activation makes *C. albicans* more susceptible to subsequent Hst 5 killing. To determine whether *C. albicans* cells pretreated with inducers of Cek1 phosphorylation (P-Cek1) would have altered susceptibility to subsequent exposure to Hst 5, cells were pretreated with Glc at 30°C or 37°C; and GlcNAc 30°C or 37°C, followed by treatment with 31 μM Hst 5. Cells pretreated with GlcNAc at 37°C had the highest levels of Cek1 phosphorylation and significantly ($P<0.001$) increased susceptibility to Hst 5 (by 30%) compared with conditions that only weakly induced Cek1 phosphorylation. Cells pretreated with GlcNAc at 37°C had 50-100% germination with germ tubes 2-3X length of mother cells (+), while cells incubated with Glc at 37°C had less than 50% germination with small germ tube (+/-); cells grown under Glc at 30˚C or GlcNAc at 30˚C had no germination (-).

FIG 3. Higher Hst 5 killing activity is dependent upon prior Cek1 phosphorylation. (A) Hst 5 antifungal activity was tested in *C. albicans* WT, *cek1Δ/Δ,* and *hog1Δ/Δ* after pretreatment of cells with either Cek1 inducing (GlcNAc at 37°C) or non-inducing conditions (Glc at 30°C). Mkc1 protein was used as a loading control since Cek1p and Hog1p were absent in respective

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samples. *C. albicans hog1Δ/Δ* cells, which had higher Cek1 phosphorylation compared to WT under non-inducing conditions, showed significantly more susceptibility to Hst 5 ($P<0.05$); while *cek1Δ/Δ* cells had no hypersensitivity to Hst 5, even after pretreatment with GlcNAc at 37°C. (B**)** Cells pretreated with 1 M NaCl exhibited strong Hog1 phosphorylation, but no Cek1 phosphorylation, and significantly less ($P<0.05$) cell death after 30 min incubation with 31 μM Hst 5.

FIG 4. Lack of Cek1 phosphorylation protects cells from Hst 5 killing. (A) *C. albicans msb2Δ/Δ, sho1Δ/Δ, and msb2/sho1Δ/Δ* cells pretreated with GlcNAc at 37°C had no Cek1 phosphorylation (P-Cek1) and showed reduced sensitivity to Hst 5, compared to the WT cells. All Msb2 mutants showed germination levels similar to WT despite differences in Hst 5 susceptibility. (B) Cells pretreated with pepstatin A (PA), lacked Cek1 phosphorylation and showed reduced sensitivity to Hst 5 ($P<0.05$) even under inducing conditions (GlcNAc at 37°C), compared to cells without pretreatment with PA.

FIG 5. Cells treated to induce Cek1 have higher Hst 5 susceptibility. (A) *C. albicans cpp1Δ/Δ* cells showed strong Cek1 phosphorylation (P-Cek1) irrespective of pretreatment with Glc at 30°C or GlcNAc at 37°C; and *cpp1Δ/Δ* cells had elevated sensitivity to Hst 5 under both pre-treatments, unlike WT cells that showed higher sensitivity only after pretreatment with GlcNAc at 37°C; Hst 5 sensitivity of *cpp1Δ/Δ* cells was significantly higher than the WT cells under non-inducing conditions. Although *cpp1Δ/Δ* cells had very high germination levels (100% cells showed germination, ++) after pretreatment with GlcNAc at 37°C, these cells were less sensitive to Hst 5 than less germinated WT cells (50-100% germination, +) (B) Cells pretreated with caspofungin (20 ng/mL) had no increase in Cek1 phosphorylation even after 120 min pretreatment and had no difference in susceptibility to Hst 5.

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FIG 6. Cek1 phosphorylation, but not germination, increases susceptibility to Hst 5. (A) *C. albicans* wild type cells pretreated with 10% fetal bovine serum (FBS) had very strong Cek1 phosphorylation (P-Cek1) accompanied by 100% germination (++), and were significantly (P<0.05) more sensitive to subsequent Hst 5 killing. However, there was no difference in Hst 5 sensitivity after 10% FBS pretreatment in *C. albicans cek1Δ/Δ* mutants despite having high germination (50-100% germination, +). (B) WT cells pretreated with Lee's media were 50-100% germinated (+), but did not show Cek1 phosphorylation, and had similar sensitivity to Hst 5 as cells pretreated with Glc at 30°C, both of which were significantly less sensitivity to Hst 5 compared to cells pretreated with GlcNAc at 37°C.

FIG 7. Cek1 activation results in higher Hst 5 binding, uptake, and cell death. (A) Cells pretreated with Cek1-inducing and non-inducing conditions were incubated with antibody to β-1,3-glucan, prior to Hst 5 susceptibility testing. Cells pretreated with GlcNAc at 37°C and incubated with the antibody showed reduced Hst 5 killing, similar to that of cells treated with Glc at 30°C and the antibody. (B) Cells pretreated with tunicamycin (5 µg/ml for 1 h at 37°C) had significantly (P<0.05) increased Hst 5 killing compared with cells treated with tunicamycin or Hst 5 alone. (C) Cells exposed to GlcNAc at 37°C had higher cytosolic levels of Biotin-labelled Hst 5 (BHst 5) after 30 min compared with cells treated with Glc at 30°C. (D) Propidium iodide (PI) uptake was visualized by time-lapse confocal microscopy after addition of Hst 5 to cells pretreated with GlcNAc at 37°C or Glc at 30°C. *C. albicans* pretreated with GlcNAc at 37°C showed a higher rate of PI uptake and higher cell death at 6 min, compared with cells pretreated with Glc at 30°C.

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TABLE 1

CAI-4	*ura3Δ::imm434/URA3*	(28)
cek1Δ/Δ	*ura3Δ::imm434/ura3Δ::imm434 cek1Δ ::hisG-URA-hisG/cek1Δ ::hisG*	(20)
hog1Δ/Δ	*Δura3::imm434/Δura3::imm434 his1::hisG/his1::hisG hog1::loxP-ura3-loxP/hog1::loxP-HIS1-loxP CIp20 (URA3 HIS1)*	(9)
cpp1Δ/Δ	*cpp1Δ::hisG/cpp1Δ::hisG-URA3-hisG*	(29)
msb2Δ/Δ	*ura3Δ::imm434/ura3Δ::imm434, his1Δ::hisG/his1Δ::hisG-URA3-hisG msb2Δ::FRT/msb2Δ::FRT*	(25)
sho1Δ/Δ	*ura3Δ::imm434/ura3Δ::imm434 his1Δ::hisG/his1Δ::hisG sho1::hisG/sho1:hisG-URA3-hisG*	(25)
msb2Δ/Δ /sho1Δ/Δ	*ura3Δ::imm434/ura3Δ::imm434 his1Δ::hisG/his1Δ::hisG msb2Δ::FRT sho1::hisG/sho1::hisG-URA-hisG*	(25)

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FIGURES

FIGURE 1

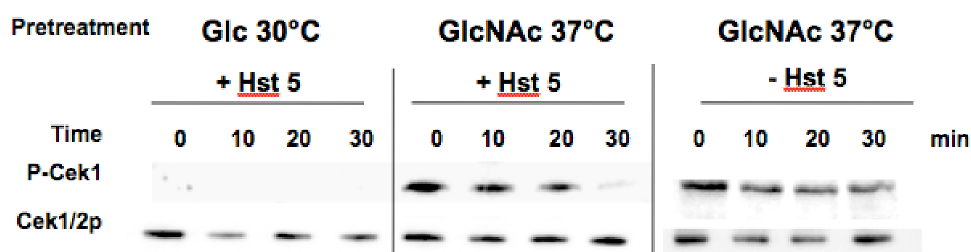

FIG 1. Hst 5 itself does not initiate Cek1 phosphorylation. *C. albicans* cells were pretreated with Cek1-inducing or non-inducing conditions, GlcNAc at 37°C or Glc 30°C, respectively, for 1 h, followed by washing with 10 mM NaPB before exposure to 31 μM Hst 5 for 0, 10, 20, and 30 min. Non-inducing conditions (left panel) were used as negative control for P-Cek, while inducing conditions without treatment with Hst 5 (right panel) were used as positive control. Phosphorylation of Cek1 was detected by α–phospho p42/44 MAPK ERK1/2 Thr202/Tyr204 rabbit monoclonal (P-Cek1) as the primary antibody, while Cek1/2 protein was used as loading control.

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FIGURE 2

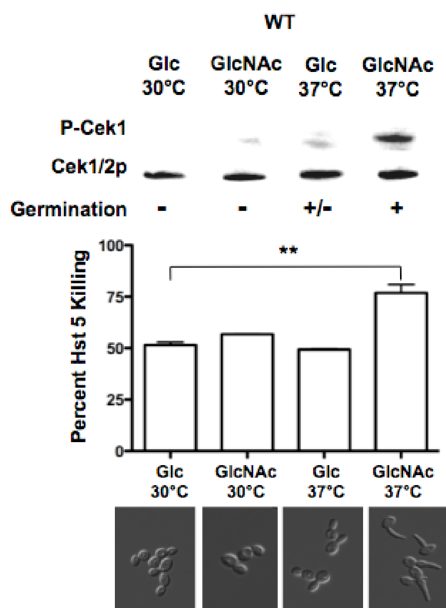

FIG 2. Cek1 activation makes *C. albicans* more susceptible to subsequent Hst 5 killing. To determine whether *C. albicans* cells pretreated with inducers of Cek1 phosphorylation (P-Cek1) would have altered susceptibility to subsequent exposure to Hst 5, cells were pretreated with Glc at 30°C or 37°C; and GlcNAc 30°C or 37°C, followed by treatment with 31 μM Hst 5. Cells pretreated with GlcNAc at 37°C had the highest levels of Cek1 phosphorylation and significantly ($P<0.001$) increased susceptibility to Hst 5 (by 30%) compared with conditions that only weakly induced Cek1 phosphorylation. Cells pretreated with GlcNAc at 37°C had 50-100% germination with germ tubes 2-3X length of mother cells (+), while cells incubated with Glc at 37°C had less than 50% germination with small germ tube (+/-); cells grown under Glc at 30˚C or GlcNAc at 30˚C had no germination (-).

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FIGURE 3

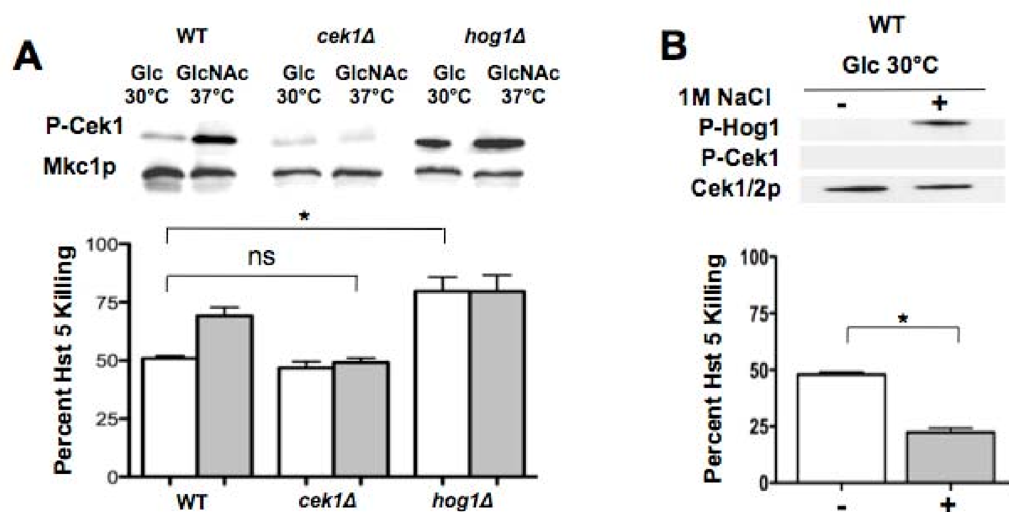

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FIGURE 4

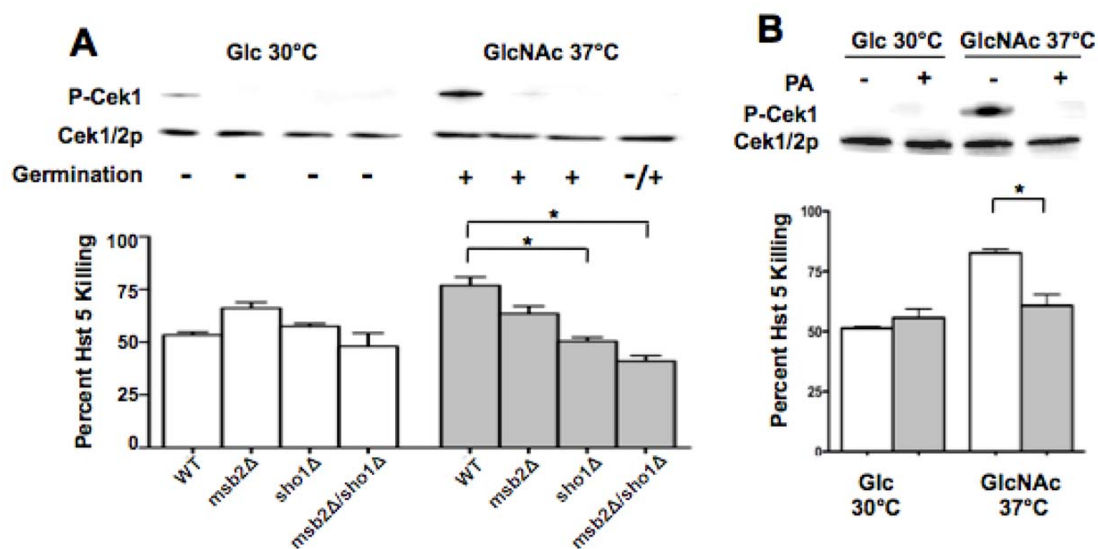

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FIGURE 5

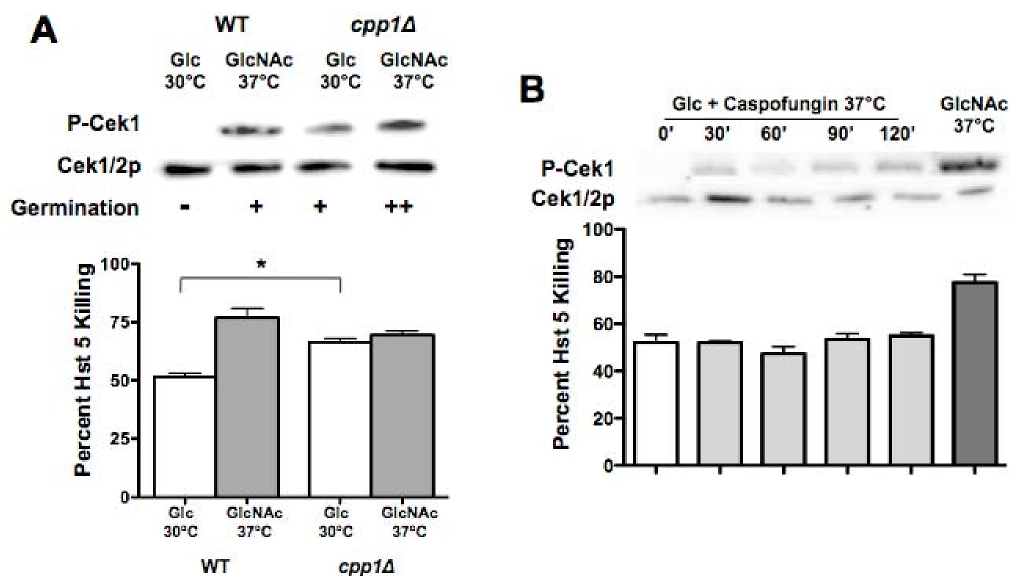

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FIGURE 6

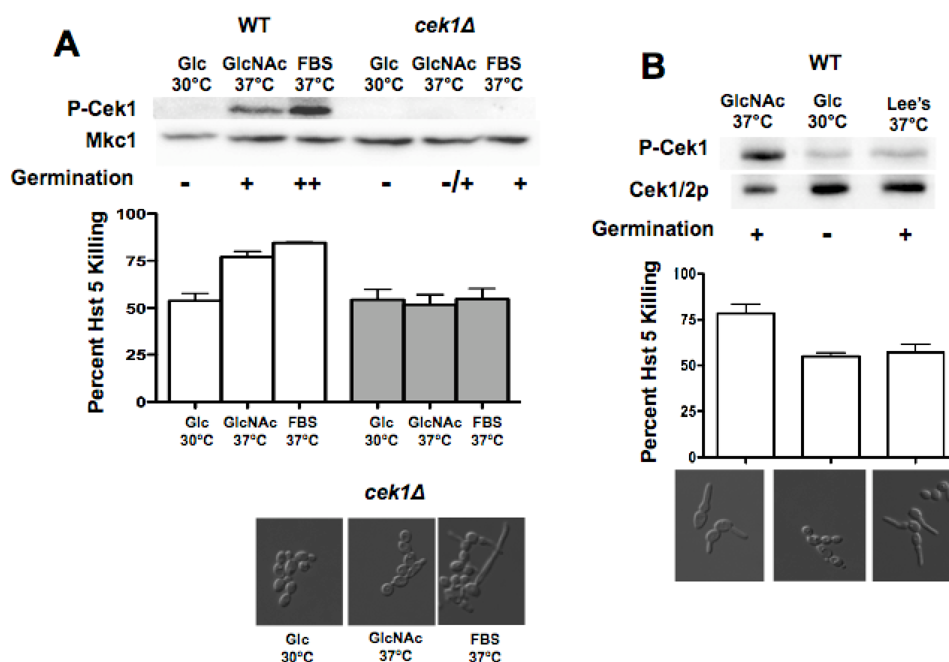

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FIGURE 7

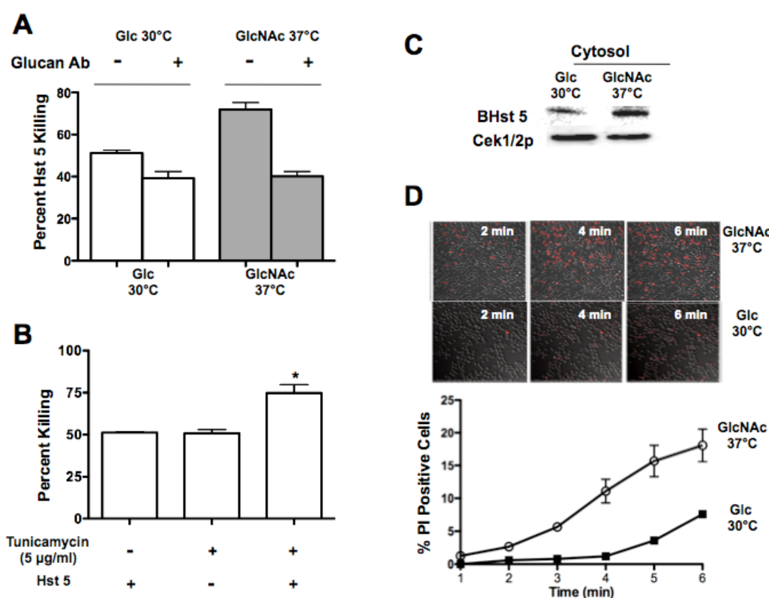

FIG 7. Cek1 activation results in higher Hst 5 binding, uptake, and cell death. (A) Cells pretreated with Cek1-inducing and non-inducing conditions were incubated with antibody to β-1,3-glucan, prior to Hst 5 susceptibility testing. Cells pretreated with GlcNAc at 37°C and incubated with the antibody showed reduced Hst 5 killing, similar to that of cells treated with Glc at 30°C and the antibody. (B) Cells pretreated with tunicamycin (5 µg/ml for 1 h at 37°C) had significantly ($P<0.05$) increased Hst 5 killing compared with cells treated with tunicamycin or Hst 5 alone. (C) Cells exposed to GlcNAc at 37°C had higher cytosolic levels of Biotin-labelled Hst 5 (BHst 5) after 30 min compared with cells treated with Glc at 30°C. (D) Propidium iodide (PI) uptake was visualized by time-lapse confocal microscopy after addition of Hst 5 to cells pretreated with GlcNAc at 37°C or Glc at 30°C. *C. albicans* pretreated with GlcNAc at 37°C

showed a higher rate of PI uptake and higher cell death at 6 min, compared with cells pretreated with Glc at 30°C.

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