



Candida albicans Ras1 Inactivation Increases Resistance to Phagosomal Killing by Human Neutrophils

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ABSTRACT Host phagocytic cells are crucial players in initial defense against *Candida albicans* infection. *C. albicans* utilizes MAP kinases and Ras1 stress response signaling pathways to protect itself from killing by immune cells. In this study, we tested the importance of these pathways in *C. albicans* phagocytosis by neutrophils and subsequent phagosomal survival. Phagocytosis was influenced by *C. albicans* morphology, so hyphal length of $>10\ \mu\text{m}$ reduced the phagocytic index (PI) 2- to 3-fold in human neutrophils. Primary human neutrophils killed 81% of phagocytosed *C. albicans*, while primary mouse neutrophils killed 63% of yeasts. We found that both the *C. albicans* Cek1 and Hog1 pathways were required for survival of phagocytosed yeast, whereas deletion of *C. albicans* RAS1 resulted in an 84% increase in survival within neutrophils compared to that of the wild type (WT). The absence of Ras1 did not alter reactive oxygen species (ROS) production by *C. albicans*; however, phagocytosed *C. albicans* $\Delta/\Delta\text{ras1}$ cells reduced ROS release by neutrophils by 86%. Moreover, *C. albicans* $\Delta/\Delta\text{ras1}$ cells had increased resistance to hydrogen peroxide as a result of high levels of catalase activity. This phenotype was specific to Ras1, since these effects were not observed in the absence of its partner Cyr1 or with its downstream target Efg1. In addition, *C. albicans* $\Delta/\Delta\text{ras1}$ cells had a significantly increased resistance to nonoxidative killing by human neutrophil peptide 1 (HNP-1) that was reversed by restoring cellular cAMP levels. These data show that *C. albicans* Ras1 inactivation leads to fungal resistance to both oxidative and nonoxidative mechanisms of neutrophil phagosomal killing.

KEYWORDS *Candida albicans*, human neutrophils, phagocytosis, Ras1

Candida albicans is an opportunistic fungus colonizing the oral cavity in over half of healthy individuals (1); however, when the immune system is compromised, this normally commensal organism may become pathogenic and cause oropharyngeal candidiasis (OPC). Recruitment of phagocytic cells is considered a crucial early response to mucosal invasion by *C. albicans*. In a murine model of OPC, neutrophils migrate to the superficial epithelium and are essential for clearance of fungi within 1 day of infection (2). Humans and mice with innate immune deficiencies of neutrophils or macrophages have an elevated risk of invasive candidiasis and increased mortality rates (3, 4).

Professional phagocytes, including neutrophils and macrophages, function in engagement and phagocytosis of invading microbes, although their relative importance in killing and clearance of *C. albicans* differs. Following recognition of *C. albicans*, the two cell types engulf yeast through the process of phagocytosis at similar rates (5). However, once phagocytosed, neutrophils are more efficient at killing *C. albicans* than macrophages (80% and 60% killing rates, respectively) (6), and human neutrophils have significantly higher *C. albicans* killing activity than murine neutrophils (7). The high efficiency of human neutrophil killing of *C. albicans* is likely due to production of small

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reactive oxygen species (ROS) as well as human neutrophil peptide 1 (HNP-1), although the relative contributions of oxidative and nonoxidative mechanisms are unknown.

Killing of engulfed microbes within neutrophil phagosomes is initiated with a powerful oxidative burst produced by the NADPH-oxidase complex (NOX2) that is assembled on the membrane of the maturing phagosome. The NOX2 complex forms superoxide in the lumen of the phagosome, which goes on to form other small ROS and reactive nitrogen species (RNS), including H_2O_2 , NO_2^\bullet , and OCl^- . These compounds are highly concentrated within the phagosomal lumen so that ROS and RNS are efficient at killing fungi engulfed within this space (reviewed in reference 8). Despite the similarities between human and mouse neutrophil ROS release, production of ROS by human granulocytes is significantly higher than their mouse counterpart driven by a higher myeloperoxidase activity, which relates to the higher killing activity (7). In addition, human neutrophil phagosomes maintain NOX2 activity for an extended period (9) and are capable of delivering their preformed granules containing antimicrobial components by fusion with the maturing phagosome. HNP-1, commonly known as alpha-defensin, is one of the most abundant cationic peptides contained in azurophilic granules of human neutrophils. The presence of HNP-1 makes human neutrophils highly efficient in controlling *C. albicans* compared to mouse neutrophils, which lack alpha-defensins (7).

Although phagocytosis and subsequent killing are highly efficient and controlled processes, *C. albicans* and other pathogenic microbes are often able to adapt and resist these immune defenses in order to survive encounters with neutrophils (10–12). For example, *C. albicans* activates signal transduction pathways, particularly mitogen-activated protein kinase (MAP kinase) and Ras1-GTPase, which are crucial for *C. albicans* sensing of environmental conditions and initiating survival responses. *C. albicans* has three major MAP kinase signal transduction pathways involved in cellular and biological processes that have been widely studied (reviewed in references 13 and 14). The Cek1 pathway is a crucial contributor to cell wall biogenesis, mating, and morphogenesis (15–17). The *C. albicans* Hog1 pathway responds to osmotic and oxidative conditions, with Hog1 deletion mutants being more susceptible to hydrogen peroxide exposure (18) as well as being hyperfilamentous (18). The third MAP kinase is Mkc1, which functions during cell wall biogenesis and invasive growth and has an important role during biofilm formation (17).

In addition to the three MAP kinase pathways, the Ras1-cAMP pathway is also linked to morphogenesis and responses of *C. albicans* to environmental stress. This pathway is composed of the small Ras1-GTPase protein, the adenylyl cyclase Cyr1, and the downstream transcription factor Efg1. Although Cyr1 was originally thought to be a downstream component of the Ras1-cAMP pathway and fully dependent upon Ras1 activity, recent studies indicate a parallel and complex model in which the two components regulate each other (19, 20). Cyr1 senses high intracellular levels of ATP and triggers the exchange of Ras1-bound GDP for GTP, causing Ras1 activation (19). After Cyr1/Ras1 activation, ATP is converted to cAMP, which derepresses protein kinase A (PKA), leading to the activation of Efg1. Efg1, in turn, is important for filamentation and other biological processes (reviewed in reference 21). A variety of extracellular stimuli are capable of activating the Ras1 pathway, including low pH and serum. Furthermore, both Ras1 and Cyr1 are needed for virulence in a disseminated model of murine candidiasis (22, 23).

The connections between the MAP kinase signaling pathways and innate immunity have been broadly studied, and each pathway has been found to contribute to the initiation and maintenance of systemic infection in murine candidiasis (16, 24, 25). Galán-Díez et al. demonstrated that in the absence of *CEK1*, *C. albicans* has higher exposed β -glucan, which correlates with increased phagocytosis by human macrophages (26). Also, deletion of selected regulator components of the *C. albicans* Hog1 pathway results in increased susceptibility to human neutrophil killing (27). However, nothing is currently known about the relationship between *C. albicans* Ras1 and its interplay with immune cells, other than evidence from a recent transcriptomic study of

C. albicans phagocytosed by human neutrophils indicating that certain Ras1 pathway components were downregulated (28), suggesting that dampening of Ras1 signaling is important for survival of phagocytosed *C. albicans*.

In addition to being important regulators of the Ras1 pathway, levels of cAMP and ATP also affect major yeast virulence attributes. Increased intracellular levels of cAMP trigger *C. albicans* hyperfilamentation (29), while reduced cAMP initiates increased expression of genes required for stress defenses, leading to increased survival (30, 31). Additionally, low intracellular ATP levels in *C. albicans* lead to altered cell wall composition and physical state of the membrane and are associated with increased resistance to cationic antifungal peptides (32). Thus, it is likely that the Ras1 pathway is at least partially linked to these known cAMP and ATP effects on virulence and plays an important role in survival of *C. albicans* organisms that have been internalized by neutrophils.

In this study, we investigated the role of the fungal MAP kinases and Ras1 pathway in phagocytosis and intracellular killing of yeast by human neutrophils. We found that both Hog1 and Cek1 MAP kinases were required for survival of *C. albicans* phagocytosed by primary human neutrophils. However, *C. albicans* *RAS1* knockouts had significantly increased survival within neutrophils compared to wild-type (WT) cells. This increased survival of *C. albicans* *RAS1* knockouts was due, at least partially, to their increased resistance to both oxidative stress and HNP-1 as well as inhibition of ROS production by human neutrophils. Therefore, the Ras1 pathway plays a crucial role during *C. albicans* defense against killing by neutrophils.

RESULTS

Efficiency of phagocytosis of *C. albicans* is determined by hyphal length. Since phagocytosis is dependent upon the morphology of the target cell (33), we first examined the effect of *C. albicans* hyphal length on phagocytosis by human blood-derived neutrophils (H-PMNs) after 30 min of incubation at a multiplicity of infection (MOI) of 3. The phagocytic index (PI) of *C. albicans* with preformed hyphae was significantly reduced compared to that of yeast form cells (Fig. 1A). The PI of *C. albicans* with an average hyphal length of 10 μm was reduced 2-fold and increasing hyphal length further reduced the PI, so neutrophils had little to no success at phagocytosis of *C. albicans* cells with hyphae with an average length of 50 μm (Fig. 1A and C). Yeast cells were efficiently engulfed by H-PMNs and were frequently observed to contain 3 or 4 yeast cells (Fig. 1C). H-PMNs showed remarkable plasticity in engulfment of *C. albicans* hyphal cells with a length of 10 μm , so the overall dimensions of the neutrophil were increased almost two times the original size during phagocytosis of two hyphal cells (Fig. 1C). As we found that hyphal formation reduced the PI, for further experiments only yeast form cells of *C. albicans* were used, although some mutant strains had a hyperfilamentous phenotype, so some hyphae were formed. Next, we assessed the relative abilities of primary mouse and human neutrophils to kill internalized phagocytosed *C. albicans* after 2.5 h. We found that H-PMNs had a higher rate of killing (only 19% of *C. albicans* yeast survived) than did primary murine-bone marrow derived neutrophils (M-PMNs), in which 37% of internalized *C. albicans* organisms survived (Fig. 1B). Therefore, H-PMNs were used for all subsequent experiments based on this high killing rate for *C. albicans*.

MAP kinases and Ras1 are important contributors to *C. albicans* survival within human neutrophils. *C. albicans* MAP kinase signaling pathways are involved in resistance to oxidative and environmental stresses; however, little is known about any role these pathways play in modulating survival within professional phagocytic cells. Therefore, *C. albicans* mutants in which the major stress response signaling pathways had been knocked out ($\Delta/\Delta\text{cek1}$, $\Delta/\Delta\text{hog1}$, $\Delta/\Delta\text{mkc1}$ and $\Delta/\Delta\text{ras1}$) were tested for their ability to be phagocytosed and survive within H-PMNs. Significant differences ($P < 0.001$, one-way analysis of variance [ANOVA]) were found in the PIs of *C. albicans* *HOG1* and *RAS1* strains and WT cells, while no differences in phagocytosis were measured in *MKC1* or *CEK1* knockouts (Fig. 2A). The PI of *C. albicans* $\Delta/\Delta\text{hog1}$ and $\Delta/\Delta\text{ras1}$ mutants

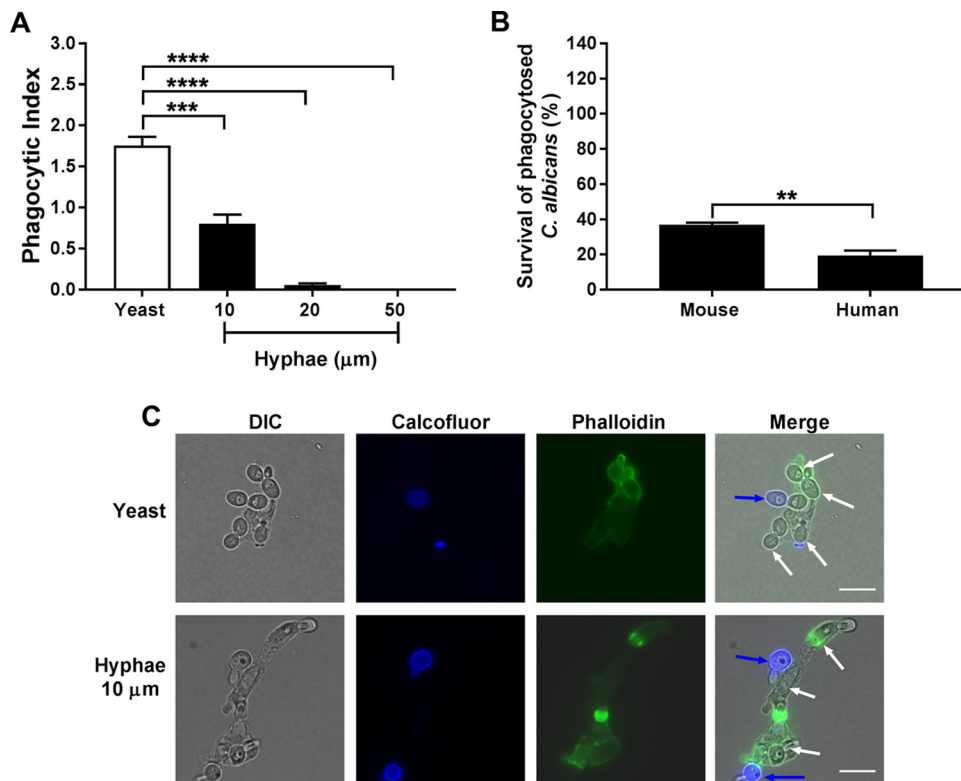


FIG 1 Initial assessment of *C. albicans* phagocytosis and survival by neutrophils. (A) Human blood-derived neutrophils (H-PMNs) were infected with *C. albicans* yeast or 10-, 20-, or 50- μm hyphae at an MOI of 3 for 30 min. Extracellular fungal cells were stained using calcofluor, and H-PMNs were stained with phalloidin. Unstained intracellular *C. albicans* cells were counted in at least 100 H-PMNs, and the phagocytic index was calculated by obtaining the ratio of the total number of ingested *C. albicans* cells and the total number of neutrophils counted. (B) Murine or human primary neutrophils were coincubated with *C. albicans* at an MOI of 0.1 for 3 h. Cells were lysed and internalized *C. albicans* was released, plated in agar, and incubated for 48 h to obtain viable CFU. Survival was calculated as follows: (recovered *C. albicans* CFU after neutrophil lysis/total number of phagocytosed *C. albicans* cells) \times 100. Results are the means \pm SEs from at least three independent experiments performed in duplicate. Significance was calculated using one-way ANOVA with *post hoc* Dunnett's multiple-comparison test (A) or unpaired Student's *t* test (B). **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$. (C) Representative yeast and hyphal (average of 10 μm) phagocytosis by H-PMNs. Nonphagocytosed *C. albicans* cells are shown by calcofluor white staining (blue arrows), H-PMNs by phalloidin staining (green), and phagocytosed *C. albicans* by white arrows in merged panels. Scale bars = 10 μm .

was reduced by 50% compared to that of the WT. Surprisingly, *C. albicans* $\Delta/\Delta\text{cek1}$ cells with defects in cell wall composition and thus expected to have defects in neutrophil recognition and uptake had no difference in phagocytosis compared to that of the WT (Fig. 2A).

Differences in the survival of *C. albicans* MAP kinase knockouts of phagocytosed yeast cells (Fig. 2B) were calculated independently of their PI (see Materials and Methods). As expected, survival of *C. albicans* $\Delta/\Delta\text{cek1}$ and $\Delta/\Delta\text{hog1}$ mutants (with knockouts of signaling pathways important for response to cell wall and oxidative/osmotic stressors, respectively) was significantly decreased in H-PMNs (13% and 6% survival, respectively) compared to that of WT cells. No difference in survival was found for *C. albicans* $\Delta/\Delta\text{mkc1}$ within H-PMNs. Unexpectedly, we observed dramatically increased survival of *C. albicans* $\Delta/\Delta\text{ras1}$ cells (106%) that was not observed for any other MAP kinase mutant. These differences were not related to the production of hyphae within phagosomes, since hyperfilamentous *C. albicans* $\Delta/\Delta\text{hog1}$ cells produced hyphae after phagocytosis (Fig. 2C) but were killed more efficiently than WT cells, while *C. albicans* $\Delta/\Delta\text{ras1}$ cells remained as the yeast form (Fig. 2C) yet were highly resistant to killing.

***C. albicans* Ras1 is important for resistance to phagosomal killing by human neutrophils.** We next examined whether other *C. albicans* Ras1 pathway components,

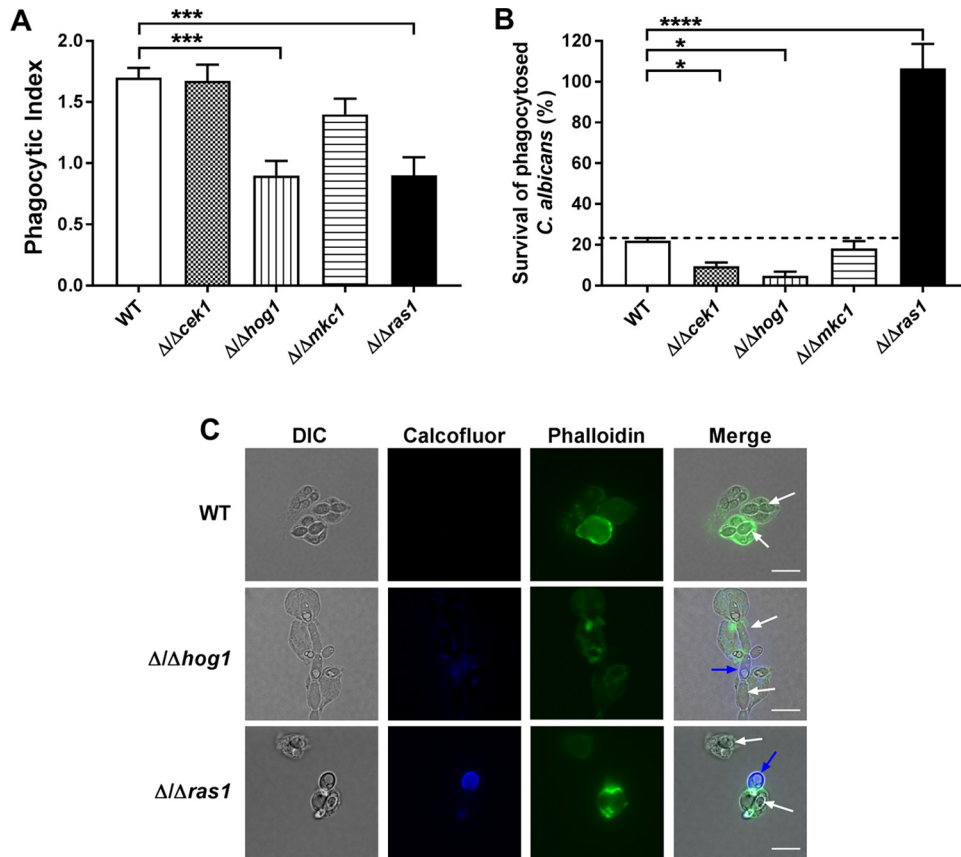


FIG 2 *C. albicans* *CEK1* and *HOG1* deletion mutants have decreased survival within human neutrophils, while *RAS1* deletion mutants have increased survival. (A) H-PMNs were infected with WT, $\Delta\Delta cek1$, $\Delta\Delta hog1$, $\Delta\Delta mkc1$, and $\Delta\Delta ras1$ *C. albicans* strains at an MOI of 3 for 30 min. Extracellular fungal cells were stained using calcofluor white, and H-PMNs were stained with phalloidin. Unstained intracellular *C. albicans* cells were counted in at least 100 phagocytic cells, and the phagocytic index was calculated by obtaining the ratio of the total number of ingested *C. albicans* and the total number of phagocytes counted. (B) To evaluate survival, H-PMNs were infected with the same *C. albicans* strains in an MOI of 0.1 for 3 h. After coincubation, phagocytic cells were lysed and internal *C. albicans* was released, plated in agar, and incubated for 48 h to obtain viable CFU. Survival was calculated as follows: (recovered *C. albicans* CFU after neutrophil lysis/total number of phagocytosed *C. albicans* cells) \times 100. Results represent the means \pm SEs from at least three independent experiments carried out in duplicate. Significance was obtained using one-way ANOVA with *post hoc* Dunnett's multiple-comparison test. *, $P < 0.05$; ***, $P < 0.001$; ****, $P < 0.0001$. (C) Representative phagocytosis of *C. albicans* WT, $\Delta\Delta hog1$, and $\Delta\Delta ras1$ after 30 min of infection with H-PMNs. Nonphagocytosed *C. albicans* cells are shown by calcofluor white staining (blue arrows), H-PMNs by phalloidin staining (green), and phagocytosed *C. albicans* by white arrows in merged panels. Scale bars = 10 μ m.

including its interacting partner Cyr1, or downstream signaling components (Efg1) were needed for resistance to H-PMN killing. To further evaluate the specificity of Ras1 for these phenotypes, a Ras1 overexpression strain (*RAS1OE*) and complemented *ras1/RAS1* strain also were examined. Inhibited phagocytosis of *C. albicans* $\Delta\Delta ras1$ cells by H-PMNs was reversed in the complemented *ras1/RAS1* strain, while a small but significant defect in phagocytic uptake of *RAS1OE* cells was retained (Fig. 3A). However, the PIs of $\Delta\Delta cyr1$ and $\Delta\Delta efg1$ deletion mutants did not differ. Thus, altering intracellular levels of Ras1 likely resulted in changes in the cell surface that affected phagocytic recognition, while Cyr1 or Efg1 did not influence these interactions.

Next, we compared the high resistance to neutrophil killing of *C. albicans* $\Delta\Delta ras1$ cells with the resistance of other strains (Fig. 3B). As expected, complementation of *RAS1* (*ras1/RAS1*) restored neutrophil killing to WT levels, while *C. albicans* $\Delta\Delta cyr1$ and *RAS1OE* cells showed no significant difference in survival compared to that of WT cells (survival rates of 15% and 14.8%, respectively). On the other hand, *C. albicans* $\Delta\Delta efg1$ cells had increased survival within H-PMNs (72%, compared to 25% for WT); however,

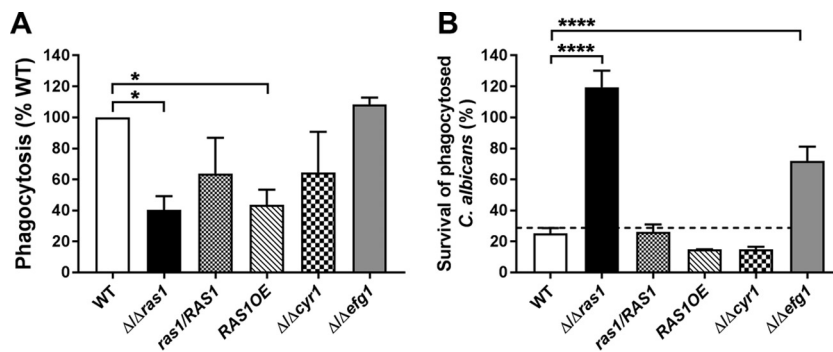


FIG 3 *RAS1* and *EFG1* deletions improve survival of *C. albicans* within human neutrophils. (A) H-PMNs were infected with *C. albicans* WT, $\Delta/\Delta ras1$, *ras1/RAS1*, *RAS1OE*, $\Delta/\Delta cyr1$, and $\Delta/\Delta efg1$ strains for 30 min. Extracellular fungal cells were stained using calcofluor white, unstained *Candida* cells were counted, and the PI was calculated by obtaining the ratio of the total number of ingested *C. albicans* cells and the total number of phagocytes counted. Phagocytosis of the WT was set as 100%. (B) Survival of *C. albicans* strains within H-PMNs was evaluated after 3 h of coinubation. H-PMNs were lysed, and internalized *C. albicans* cells were released, plated in agar, and incubated for 48 h to obtain viable CFU. Survival was calculated as follows: (recovered *C. albicans* CFU after neutrophil lysis/total number of phagocytosed *C. albicans* cells) \times 100. Results represent the means \pm SEs from at least three independent experiments carried out in duplicate. Significance was calculated using one-way ANOVA with *post hoc* Dunnett's multiple-comparison test. *, $P < 0.05$; ****, $P < 0.0001$.

this was approximately half the survival rate of *C. albicans* $\Delta/\Delta ras1$ cells, suggesting that the mechanism of survival within phagosomes contributed by Efg1 differs from that of Ras1.

***C. albicans* $\Delta/\Delta ras1$ cells suppress ROS production in human neutrophils.**

Shortly after completion of phagosome formation, a powerful oxidative burst comprised of reactive oxygen species (ROS) and reactive nitrogen species (RNS) is released into the lumen of the phagosome (34). Therefore, we next measured whether *C. albicans* $\Delta/\Delta ras1$ cells were able to suppress ROS production within neutrophils. *C. albicans* $\Delta/\Delta ras1$ cells were compared to WT, complemented, and overexpression strains for the ability to stimulate H-PMN ROS generation using a luminol-based chemiluminescence assay (Fig. 4A). Since *C. albicans* cells themselves are able to release ROS to the extracellular environment in response to some stimuli (35), ROS production by *C. albicans* strains alone was measured under the same conditions (Fig. 4B) and these values were subtracted from the total values for ROS generated by neutrophils stimulated with yeasts. Neutrophils stimulated with WT yeast produced ROS by 10 min and had maximal ROS production by 50 min, which was temporally similar to ROS production by the positive-control phorbol myristate acetate (PMA)-stimulated neutrophils that produced almost twice the levels of ROS (in agreement with previous reports [36, 37]). Surprisingly, H-PMNs stimulated with *C. albicans* $\Delta/\Delta ras1$ had substantially delayed detectable ROS production (no ROS was evident until after 40 min), and levels of ROS were reduced by an average of 86% of that of the WT (Fig. 4A and C). Moreover, detectable ROS production remained suppressed by neutrophils when neutrophils were incubated with *C. albicans* $\Delta/\Delta ras1$ through 150 min of experimental observation. This effect on ROS production was specific to the Ras1 knockout strain, as the complemented strain and *C. albicans* $\Delta/\Delta efg1$ (Fig. 4A, blue line and gray line, respectively) elicited ROS production similar to that of the WT. Furthermore, stimulation of H-PMNs with the *RAS1OE* overexpression strain resulted in a nearly immediate and large burst of ROS production (Fig. 4A, red dotted line), suggesting that Ras1 overexpression elicits changes in the cell that trigger immediate neutrophil activation and ROS production. This suppression of neutrophil ROS production by *C. albicans* $\Delta/\Delta ras1$ cells was not due to any differences in ROS production by the yeasts themselves, as all strains except for the *RAS1* overexpression strain had similar levels of ROS production (Fig. 4B). Quantification of total ROS production in four independent experiments confirmed that H-PMNs produced significantly less detectable ROS when stimulated with *C. albicans*

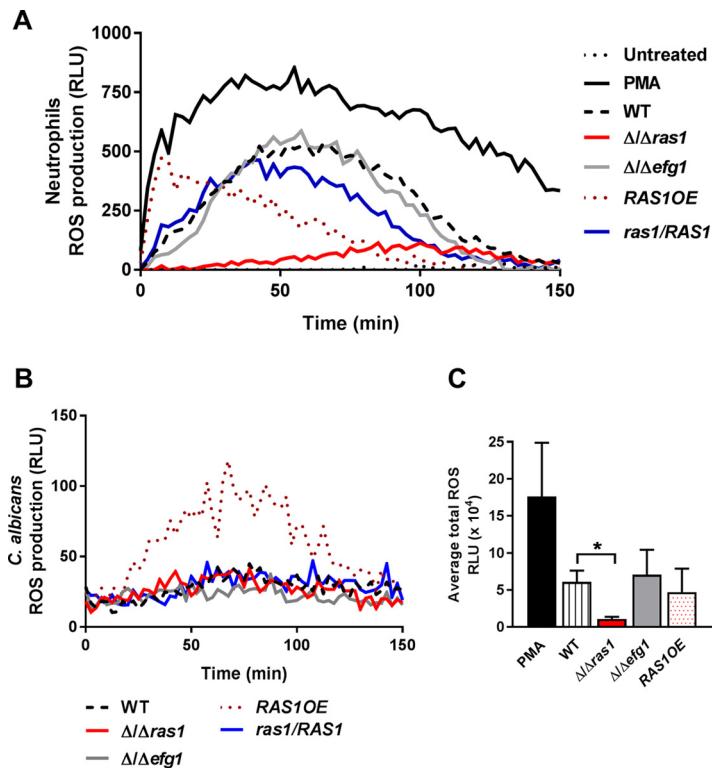


FIG 4 *C. albicans* $\Delta/\Delta ras1$ suppressed ROS production. (A) Human neutrophil ROS production upon coinocubation with *C. albicans* WT, $\Delta/\Delta ras1$, $\Delta/\Delta efg1$, RAS1OE, and *ras1/RAS1* complemented strains was detected with luminol chemiluminescence over 150 min at 37°C. As controls, neutrophils were left untreated (negative) or treated with PMA (positive). The graph is a representation of one biological replicate. (B) *C. albicans* WT, $\Delta/\Delta ras1$, $\Delta/\Delta efg1$, RAS1OE, and *ras1/RAS1* complemented strain ROS production was detected using luminol as indicated for panel A. (C) Total ROS production was obtained by calculating the area under the curve of four independent experiments performed in duplicate. Shown are the means \pm SDs from four independent experiments performed in duplicate. Significance was calculated using one-way ANOVA with *post hoc* Dunnett's multiple-comparison test. *, $P < 0.05$.

$\Delta/\Delta ras1$, while $\Delta/\Delta efg1$ and RAS1OE cells produced total ROS levels similar to those of the WT (Fig. 4C), suggesting that intraphagosomal survival of $\Delta/\Delta ras1$ cells is related to suppression of ROS production, while $\Delta/\Delta efg1$ cell survival is unrelated to ROS production.

***C. albicans* $\Delta/\Delta ras1$ cells have a higher tolerance to oxidative stress through increased catalase activity.** To demonstrate the importance of ROS production in *C. albicans* killing within neutrophils, we treated H-PMNs with the NADPH oxidase inhibitor diphenyleneidonium chloride (DPI) before adding *C. albicans*, expecting to see an increase in survival of *C. albicans* strains when ROS production was blocked (see Fig. S1 in the supplemental material). Indeed, we observed that upon ROS inhibition, *C. albicans* WT and $\Delta/\Delta ras1$ strain survival rates were significantly increased (66.4% and 99.6%, respectively) compared to those in untreated neutrophils (10% and 69.2%). However, there was still a difference in survival between WT and $\Delta/\Delta ras1$ cells within DPI-treated neutrophils (Fig. 5A), suggesting that changes in survival between the strains were only partially due to increased oxidative stress resistance. Furthermore, an increase in survival of the $\Delta/\Delta efg1$ mutant within DPI-treated neutrophils was not observed, further suggesting that the $\Delta/\Delta efg1$ mutant survival is governed by mechanisms other than ROS resistance (Fig. 5A). Therefore, we compared the survival of *C. albicans* $\Delta/\Delta ras1$ and $\Delta/\Delta efg1$ cells upon exposure to both oxidative and nitrosative stresses. Since low levels of intracellular cAMP in *C. albicans* have been associated with increased expression of genes for resistance to oxidative stress, including catalase genes (38), we expected that $\Delta/\Delta ras1$, but not $\Delta/\Delta efg1$, cells would be more resistant to hydrogen peroxide (H_2O_2). Indeed, *C. albicans* $\Delta/\Delta ras1$ cells were significantly more

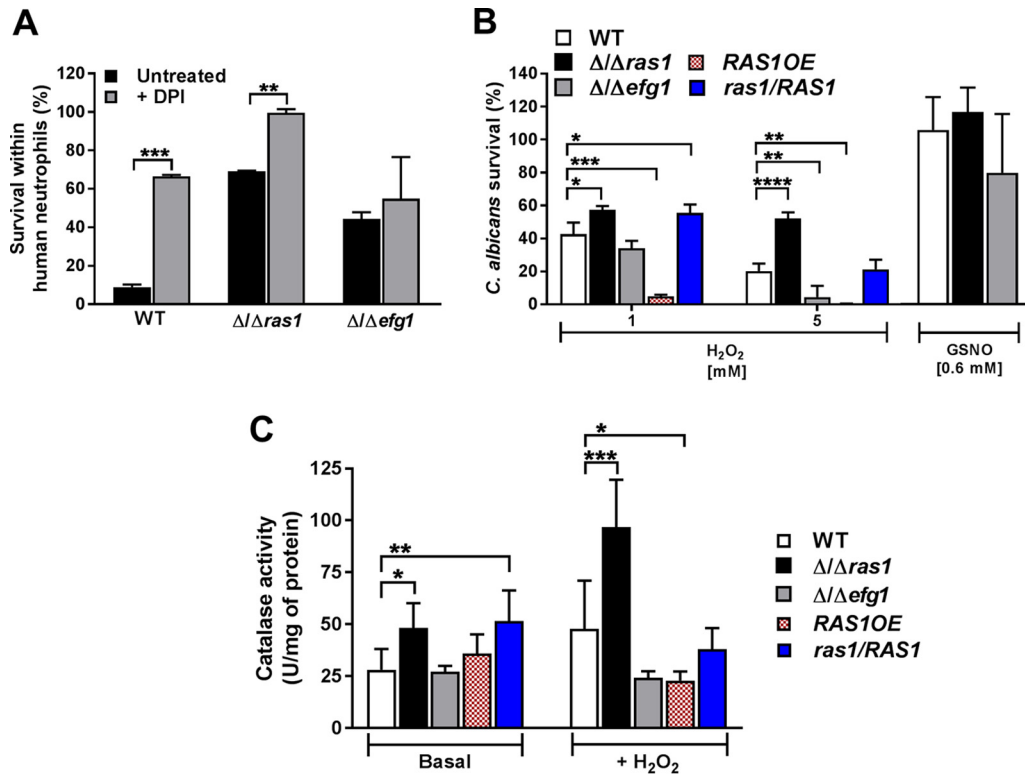


FIG 5 *C. albicans* $\Delta/\Delta ras1$ increased resistance to oxidative stress. (A) Human blood-derived neutrophils were treated with diphenyleiiodonium chloride (DPI) for 15 min at 37°C, followed by infection with *C. albicans* WT, $\Delta/\Delta ras1$, or $\Delta/\Delta efg1$ strains for 3 h. H-PMNs were lysed and internalized *C. albicans* cells were released, collected, and plated on agar for 48 h to obtain CFU. Survival was calculated as follows: (recovered *C. albicans* CFU after H-PMN lysis/total number of phagocytosed *C. albicans* cells) \times 100. Shown are the means \pm SDs from three independent experiments performed in duplicate. (B) *C. albicans* strains were treated with the indicated concentrations of H_2O_2 or *S*-nitrosoglutathione (GSNO) for 2 h at 30°C. Control cells were left untreated. After incubation, *C. albicans* cells were diluted and plated on agar, and CFU were obtained after 48 h. Survival was calculated as follows: (CFU of treated plate/CFU control plate) \times 100. Shown are the means \pm SDs from three independent experiments performed in duplicate. (C) Catalase activity was measured colorimetrically at 570 nm using whole-cell extracts isolated from mid-log-phase cells incubated for 1 h at 30°C without (basal) or with 5 mM H_2O_2 . Shown are means \pm SDs from three independent experiments performed in duplicate. Significance was calculated using unpaired Student *t* tests (A) or one-way ANOVA with *post hoc* Dunnett's multiple-comparison tests (B and C). *, *P* < 0.05; **, *P* < 0.01; ***, *P* < 0.001; ****, *P* < 0.0001.

resistant to H_2O_2 at both 1 mM and 5 mM than the WT, while *C. albicans* $\Delta/\Delta efg1$ cells had a susceptibility similar to that of the WT at 1 mM but higher susceptibility at 5 mM (Fig. 5B). Additionally, the effect observed in the absence of Ras1 was restored in the complemented *ras1/RAS1* strain, which showed a survival rate similar to that of the WT at 5 mM (survival rates of 21% and 20%, respectively). Moreover, *C. albicans* RAS1OE cells were significantly less resistant to H_2O_2 at both concentrations tested (Fig. 5B). Cell sensitivity to nitrosative stress using *S*-nitrosoglutathione (GSNO) was also tested; however, all strains were completely resistant to this nitrosative stress after 2 h of treatment (Fig. 5B). Thus, ROS, but not RNS, are important determinants for survival of *C. albicans* $\Delta/\Delta ras1$ within neutrophils.

C. albicans is able to induce catalase expression as a mechanism to detoxify ROS and resist phagocytic killing (39, 40), and catalase levels are higher in cells with reduced levels of cAMP (31, 41). Therefore, we hypothesized that *C. albicans* $\Delta/\Delta ras1$ cells would have higher basal catalase expression and an increased catalase response to H_2O_2 -mediated oxidative stress compared to the WT. As expected, *C. albicans* $\Delta/\Delta ras1$ had 2-fold-higher levels of basal catalase activity as well as a 2-fold increase in activity in response to H_2O_2 compared to the WT (Fig. 5C). In contrast, *C. albicans* $\Delta/\Delta efg1$ mutant levels of basal and induced catalase activity were similar to those of WT cells, showing that higher catalase activity is specific to *C. albicans* $\Delta/\Delta ras1$ cells. Furthermore, *C.*

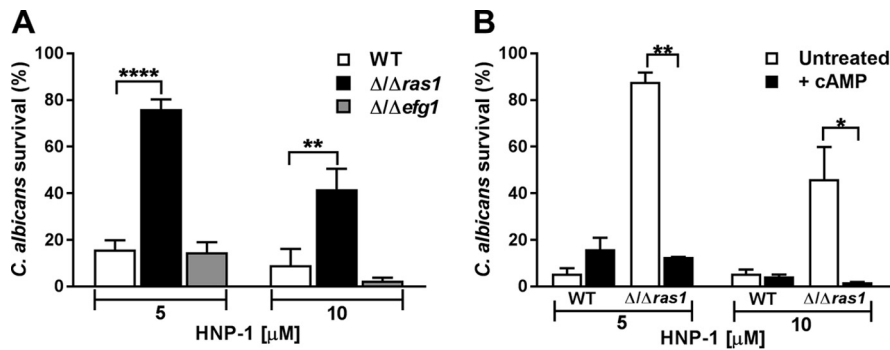


FIG 6 *C. albicans* $\Delta/\Delta ras1$ cells have increased resistance to human neutrophil peptide 1 (HNP-1) via a cAMP-dependent mechanism. (A) *C. albicans* WT, $\Delta/\Delta ras1$, and $\Delta/\Delta efg1$ strains were treated with 5 or 10 μM human neutrophil peptide 1 for 1 h at 37°C. Cells were diluted in NaPB, plated on agar, and incubated for 48 h to obtain viable CFU. (B) *C. albicans* WT and $\Delta/\Delta ras1$ strains were pretreated with 10 mM cAMP during exponential growth. After pretreatment, cells were incubated with HNP-1 as described above. Control cells were left untreated for all experiments. Survival was calculated as follows: (CFU of treated plate/CFU control plate) \times 100. Shown are means \pm SDs from three independent experiments performed in duplicate. Significance was calculated using one-way ANOVA with *post hoc* Dunnett's multiple-comparison tests (A) or unpaired Student *t* test (B). *, $P < 0.05$; **, $P < 0.01$; ****, $P < 0.0001$.

albicans overexpressing Ras1 had decreased catalase activity under treatment compared to that of the WT (Fig. 5C).

***C. albicans* $\Delta/\Delta ras1$ cell resistance to HNP-1 was reversed by cAMP.** In addition to oxidative mechanisms, human neutrophils utilize nonoxidative antimicrobial peptides localized within primary granules to kill pathogenic microorganisms. HNP-1 is one of the most abundant cationic peptides within granules of human neutrophils, but it is not present in murine neutrophils (42). To examine nonoxidative killing of *C. albicans* $\Delta/\Delta ras1$ cells by HNP-1, we first compared the *in vitro* susceptibility of *C. albicans* WT and $\Delta/\Delta efg1$ cells. Surprisingly, 76.1% of *C. albicans* $\Delta/\Delta ras1$ cells survived HNP-1 treatment (5 μM), compared to only 15.8% of WT cells, and treatment of yeast with 10 μM HNP-1 resulted in 9.2% survival of WT cells, compared to 41.7% of $\Delta/\Delta ras1$ cells (Fig. 6A). *C. albicans* $\Delta/\Delta efg1$ cells showed survival rates similar to those of the WT following treatment at both HNP-1 concentrations.

Since low levels of ATP have been associated with an increased rigidity of the *Candida* cell membrane, making it less susceptible to the action of cationic antimicrobial peptides (32), we hypothesized that increasing cAMP levels in *C. albicans* $\Delta/\Delta ras1$ cells would restore killing by HNP-1. As predicted, pretreatment of *C. albicans* $\Delta/\Delta ras1$ cells with cAMP completely restored HNP-1 killing to levels of WT cells at both tested HNP-1 concentrations (Fig. 6B).

DISCUSSION

C. albicans MAP kinases and Ras1 signaling pathways are important contributors to virulence in mouse models of candidiasis (16, 24, 25) and are required for adaptation and resistance to a variety of stress conditions. To identify which of these MAP kinases contribute to *C. albicans* survival in phagocytes, we systematically tested for defects in phagocytosis and survival of *C. albicans* knockouts of individual kinases. We found that human neutrophils had significantly reduced phagocytic indices for *C. albicans* $\Delta/\Delta hog1$ and $\Delta/\Delta ras1$ cells, suggesting that these mutants have significant alterations in surface cell wall composition involved in innate immune cell recognition. Although $\Delta/\Delta cek1$ cells have alterations in cell wall composition (26), we did not find a measurable reduction in phagocytosis, contrary to previous observations by Galán-Díez et al. (26).

We next tested for survival of *C. albicans* cells that were taken up by neutrophils, independent from their rate of phagocytosis. As expected from their established role in protection from oxidative stress (43) and cell wall stress defense (44), *HOG1* and *CEK1* knockouts had reduced survival in neutrophils. *C. albicans* $\Delta/\Delta mkc1$ cells, in contrast,

had phagocytosis and survival similar to those of WT cells, thus showing that the primary role of *MKC1* is unrelated to recognition and survival within immune cells. Unexpectedly, *C. albicans* $\Delta/\Delta ras1$ cell survival within the phagosome was increased to 106% within human primary neutrophils, suggesting that $\Delta/\Delta ras1$ cells not only survived but also potentially replicated within human neutrophils. To our knowledge, this is the only example of a *C. albicans* gene knockout resulting in complete survival of yeast within neutrophils.

Ras1 and Cyr1 are major regulators of fungal cellular cAMP levels, and these, in turn, control the expression of genes required for stress defense. Low levels of cAMP are linked with increased fungal survival following environmental oxidative stress, while high levels of cAMP have the opposite effect (30, 31, 38). Since *C. albicans* $\Delta/\Delta ras1$ cells have decreased intracellular cAMP (41), it is likely that altered cAMP levels in $\Delta/\Delta ras1$ contribute to increased survival within neutrophils. This seems not to be the case for *C. albicans* $\Delta/\Delta cyr1$ cells, which showed no improvement in survival compared to the WT despite very low cellular cAMP levels; however, these cells additionally have decreased resistance to osmotic/oxidative stresses, which may counteract any increased resistance to cell wall stressors and apoptosis (22, 31, 41, 45).

We also found that knockout of *EFG1* resulted in significantly improved survival of *C. albicans* in human neutrophils. It is not known whether *C. albicans* *EFG1* impacts cAMP levels or Ras1 activity, though we did not observe increased resistance to oxidative or nonoxidative mechanisms that could explain this phenotype. The resistance to phagosomal killing of *C. albicans* $\Delta/\Delta efg1$ cells could be based on the high redundancy in pathways regulated by Efg1 (46) and its complex regulatory network; however, this remains to be elucidated. In any case, its resistance to neutrophil killing appears to be through mechanisms other than those provided by Ras1.

Oxidative molecules released within the phagosome are crucial for killing engulfed pathogens, and we found that when ROS production was blocked by an NADPH oxidase inhibitor, survival of WT yeast cells increased 7-fold, but *C. albicans* $\Delta/\Delta ras1$ cells survival increased only 30% (Fig. 5A), suggesting that ROS contributed only about a third of the array of neutrophil killing mechanisms for these cells, compared with up to 80% for WT cells. Other crucial mechanisms for *C. albicans* $\Delta/\Delta ras1$ cell survival are suppression of ROS release by human neutrophils (Fig. 4A), increased catalase activity (Fig. 5C), and significant resistance to HNP-1 (Fig. 6A). In addition to inhibiting ROS release by neutrophils, as low cAMP levels stimulate fungal superoxide dismutase (SOD) production (38), it is possible that the lower cAMP levels in *C. albicans* $\Delta/\Delta ras1$ cells might increase the rate of ROS detoxification, rather than actual generation of phagosomal ROS. Alternatively, defective phagocytosis of *C. albicans* $\Delta/\Delta ras1$ cells (Fig. 2A) might affect the assembly of NOX2 and subsequent phagosome maturation within neutrophils, as *C. albicans* is capable of altering phagosome maturation and reducing ROS release in macrophages in order to resist killing (12). Therefore, whether the increased catalase production by *C. albicans* $\Delta/\Delta ras1$ we saw is sufficient to cause the reduction in detectable ROS production from neutrophils remains to be determined.

Most remarkable is our finding that *C. albicans* $\Delta/\Delta ras1$ cells displayed significant resistance to HNP-1 killing, since this antimicrobial peptide is generally considered to be a nonspecific membrane lytic peptide with a broad spectrum of activity against both bacteria and fungi. Although there is an association between ATP and cAMP levels of target microorganisms with their resistance to other antimicrobial peptides, such as histatin 5 (32, 47), this is the first evidence that *C. albicans* Ras1 can blunt the effects of HNP-1. We suggest a mechanism of fungal killing by HNP-1 that is dependent upon cellular cAMP, since inactivation of Ras1 which is linked to ATP and cAMP levels (19) reduced killing, while killing was completely restored upon replacement of cAMP. Furthermore, low ATP levels in *C. albicans* alter both the cell membrane state and entry of antifungal peptides (32), suggesting that the fungicidal mechanism of HNP-1 is dependent upon the energy status of the cell and requires active transport of HNP-1 rather than being a non-energy-requiring membrane-lytic peptide.

Downregulation of *C. albicans* Ras1 expression is an important mechanism that may

TABLE 1 *C. albicans* strains used in this study

Strain	Genotype	Reference
CAI-4 (wild type)	<i>ura3Δ::imm434/URA3</i>	56
Δ/Δ <i>cek1</i>	<i>ura3Δ::imm434/ura3Δ::imm434 cek1Δ::hisG-URA-hisG/cek1Δ::hisG</i>	16
Δ/Δ <i>hog1</i>	Δ <i>ura3::imm434/Δura3::imm434 his1::hisG/his1::hisG hog1::loxP-ura3-loxP/hog1::loxP-HIS1-loxP Clp20 (URA3 HIS1)</i>	57
Δ/Δ <i>mkc1</i>	CAI-4, <i>mkc1D::hisG/mkc1 D::hisG mkc1::pCK70 (URA3)</i>	58
Δ/Δ <i>ras1</i>	<i>ura3::λimm434/ura3::λimm434 ras1::hisG/ras1::hisG::URA3</i>	23
Δ/Δ <i>cyr1</i>	<i>ura3D::kimm434/ura3D::k imm434 cyr1D::hisG::cyr1 D::hisG</i>	59
Δ/Δ <i>efg1</i>	<i>ura3::kimm434/ura3::kimm434efg1::hisG/efg1::hisGHisGura3::kimm434/ura3::kimm434efg1::hiG</i>	60
<i>ras1/RAS1</i>	<i>ura3::λimm434/ura3::λimm434 ras1::hisG/ras1::hisG::RAS1-URA3</i>	61
<i>ras1/ras1-G13V (RAS1OE)</i>	<i>ura3Δ::λimm434/ura3Δ::λimm434 ras1::hisG/ras1::hisG::ras1-G13V-URA3</i>	61

be used by fungal cells to survive within phagocytes. Other pathogenic organisms resist intraphagosomal killing and then use immune cells as a vehicle to disseminate infection (48, 49). Indeed, the yeast *Cryptococcus neoformans* resists macrophage killing (50) and then uses phagocytes to disseminate to other body sites (51), as illustrated by decreased dissemination during infection upon phagocytic depletion (52). It is possible that *C. albicans* employs similar mechanisms in which Ras1 downregulation reduces its phagosome killing and allows fungal dissemination through neutrophils. Thus, Ras1 may be a key modulator of fungal cell survival within the host, and this pathway needs further study to understand its role in *C. albicans* infection.

MATERIALS AND METHODS

***C. albicans* strains and culture conditions.** *C. albicans* strains genotypes are indicated in Table 1. The *C. albicans* CAI-4 wild type (WT) and Δ/Δ *cek1* mutant were kindly provided by Malcolm Whiteway (Concordia University, Montreal, Canada), the Δ/Δ *hog1* mutant was provided by Janet Quinn (Newcastle University, Newcastle, United Kingdom), the Δ/Δ *mkc1* mutant was provided by Carol Kumamoto (Tuft University), the Δ/Δ *ras1* and *ras1/ras1-G13V (RAS1OE)* mutants and *ras1/RAS1* complemented strain were provided by Deborah Hogan (Dartmouth College), and the Δ/Δ *cyr1* and Δ/Δ *efg1* mutants were provided by Amy Piispanen (Dartmouth College). All *C. albicans* strains were cultured for 12 h in yeast extract-peptone-dextrose medium (YPD; BD Difco) broth supplemented with 50 μ g/ml of uridine (Sigma-Aldrich) at 30°C in an orbital shaker at 220 rpm. Cultures were diluted to an optical density at 600 nm (OD₆₀₀) of 0.3 to 0.4 in fresh YPD medium and then recultured to an OD₆₀₀ of 0.7 to 0.8. *C. albicans* cells were pelleted by centrifugation at 2,500 \times *g* for 5 min, washed thrice with phosphate-buffered saline (PBS; pH 7.4; Corning), and suspended in RPMI 1640 (Corning) or other indicated media. To induce hypha formation, *C. albicans* yeast (OD₆₀₀ = 0.3 to 0.4) were cultured in fresh yeast nitrogen base (YNB; MP Biomedicals) supplemented with 1.25% *N*-acetylglucosamine (GlcNAc; Sigma-Aldrich) and incubated for 2 to 4 h at 37°C. Cell suspensions were vortexed vigorously to avoid clumping, which was confirmed microscopically. Hyphal length was assessed in a Zeiss Axio Observer Z1 inverted fluorescence microscope (Carl Zeiss, Germany) and measured using ZEN 2011 (blue edition) software.

Isolation of human neutrophils. Human blood-derived neutrophils (H-PMNs) were isolated from venous blood, collected into VACUETTE EDTA tubes (Greiner Bio-One) from healthy donors that had provided informed consent as approved by the University at Buffalo institutional review board (IRB; protocol 626714). Cells were isolated by density gradient centrifugation using 1-step Polymorphs (Accurate Chemicals & Scientific Corporation) following the manufacturer's instructions. H-PMNs were suspended at a concentration of 2×10^6 /ml in RPMI 1640 supplemented with L-glutamine (Corning) and 10% fetal bovine serum (FBS; Seradigm) and used immediately. Purity was confirmed by Wright-Giemsa (Polysciences, Inc.) and 4',6-diamidino-2-phenylindole (DAPI; Sigma-Aldrich) staining.

Isolation of mouse neutrophils. Isolation of mouse bone marrow-derived neutrophils (M-PMNs) was performed as previously described, with modifications (53). Long bones from 6- to 8-week-old female C57BL/6J mice (Jackson Laboratory) were collected and washed in α -MEM medium (Gibco, Life Technologies) to remove any tissue debris. Bone marrow was flushed out, washed by centrifugation at 800 \times *g* for 5 min, and suspended in 1 ml of α -MEM medium. The cell suspension was carefully layered on top of a discontinuous Percoll gradient (55%, 60%, and 80%) and centrifuged at 1,200 \times *g* for 30 min at 4°C. The lower band (M-PMN) was collected and cells were washed with Hanks' balanced salt solution (HBSS) without calcium (Corning) at 800 \times *g* for 5 min. Red blood cell lysis was achieved using red blood cell lysis buffer (BioLegend) for 5 min at room temperature (RT; 21°C) with gentle shaking. After a final wash with HBSS without calcium, cells were resuspended in RPMI 1640 at a density of 2×10^6 /ml. Cell viability was evaluated by trypan blue (Sigma-Aldrich) exclusion, and purity was determined by Wright-Giemsa and DAPI staining.

Evaluation of *C. albicans* uptake by PI. For phagocytosis assays, H-PMNs and M-PMNs were seeded in 12-well plates and *C. albicans* resuspended in RPMI 1640 to an OD₆₀₀ of 0.7 to 0.8 was added at a multiplicity of infection (MOI) of 3 and incubated for 30 min at 37°C (in a 5% CO₂ environment) to allow phagocytosis. Nonadherent neutrophils were collected after *C. albicans* phagocytosis and placed on positively charged slides (Globe Scientific Inc.) for 15 min at RT to allow cells to attach. Following

attachment, cells were gently washed with ice-cold PBS to remove medium, and 4 mg/ml of calcofluor white in PBS (CW; Sigma-Aldrich) was added for 2 min on ice to stain nonphagocytosed *C. albicans*. Next, cells were washed with ice-cold PBS to remove excess CW and fixed with 4% paraformaldehyde (Electron Microscopy Sciences) for 30 min at RT. After fixation, cells were permeabilized with 0.1% Triton X-100 (Fisher Bioreagents) for 5 min and stained with 4 mg/ml of Alexa Fluor 488-conjugated phalloidin (Invitrogen) for 5 min. After a final wash, positively charged slides were covered with number 1 cover glass (Knittel Glaser) using fluorescent mounting medium (Dako). Cells were counted using a Zeiss Axio Observer Z1 inverted fluorescence microscope (Carl Zeiss, Germany). A minimum of 100 phagocytic cells were observed for each experiment, and *C. albicans* cells that were partially or not stained with CW were counted as phagocytosed. The phagocytic index (PI) was calculated as the ratio of the total number of phagocytosed *C. albicans* cells and the total number of neutrophils counted. Assays were performed in duplicates, and experiments were repeated at least thrice.

Intracellular survival assay of *C. albicans* strains within neutrophils. Survival assays were performed following the protocol described previously (54). Briefly, fungal and phagocytic cells were incubated in RPMI 1640 medium for 3 h at 37°C and 5% CO₂ at an MOI of 0.1. This MOI was used to ensure that approximately 100% of added *C. albicans* WT cells were phagocytosed, as determined by microscopy PI determination as described above. The total number of phagocytosed *C. albicans* cells was calculated by multiplying the PI by the total number of neutrophils added. After 3 h of incubation, sterile water and 0.25% SDS (Thermo Fisher Scientific) were added to lyse neutrophils and release phagocytosed *C. albicans*. Cell suspensions were collected, vortexed vigorously to avoid cell clumping, and diluted. Finally, *C. albicans* cells were plated on YPD BD Difco agar and incubated for 48 h at 30°C to obtain viable CFU. Percent survival was determined as follows: (recovered *C. albicans* CFU after phagocytic cell lysis/total number of phagocytosed *C. albicans*) × 100. To block ROS release, phagocytic cells were treated before addition of *C. albicans* with 20 μM diphenyleneiodonium chloride (DPI; Sigma-Aldrich) for 15 min at 37°C and 5% CO₂ (55). Assays were performed in duplicates, and results are representative of those from at least three independent experiments.

ROS production assay. ROS production by H-PMNs was evaluated as described previously, with minor modifications (36). Human neutrophils (0.5 × 10⁶/ml) were resuspended in RPMI 1640 without phenol red, supplemented with 5% FBS, and seeded (100 μl) in a 96-well plate (Corning Inc.). To avoid nonspecific activation, the plate was treated with 0.05% albumin (Sigma-Aldrich) at 4°C for 1 h and then washed twice with 200 μl of Dulbecco's phosphate-buffered saline (DPBS; Corning Inc.) prior to seeding of neutrophils. *C. albicans* strains were grown as described above, resuspended in RPMI 1640 at a concentration of 5 × 10⁶/ml, and then added to each well at an MOI of 5. A control for each strain was performed without neutrophils. Immediately after addition of *C. albicans*, luminol (50 μl of 200 μM stock) and 16 U of horseradish peroxidase (HRP) (both from Sigma-Aldrich) were added to wells to detect total ROS production. Luminescence was measured using an integration time of 1 s at intervals of 2.5 min over 2.5 h at 37°C using a FlexStation 3 multimode microplate reader (Molecular Devices). Relative light units (RLU) were obtained by subtracting the luminescence of the wells that contained only *C. albicans* without neutrophils from luminescence in experimental wells. Unstimulated and PMA-stimulated (750 ng/ml) neutrophils were used as negative and positive controls, respectively.

Oxidative and nonoxidative resistance assays. For cAMP experiments, overnight cultures of *C. albicans* were diluted to an OD₆₀₀ of 0.3 to 0.4 in YPD medium was supplemented with 10 mM dibutyryl-cAMP (Sigma-Aldrich) and then cultured to an OD₆₀₀ of 0.7 to 0.8. *C. albicans* cells were then washed three times with 10 mM sodium phosphate buffer, pH 7.4 (NaPB), and then the cells (1.5 × 10⁶/ml) were treated with 5 μM or 10 μM HNP-1 (AnaSpec Inc.) for 1 h at 37°C. After treatment, cells were serially diluted in NaPB and plated on YPD, and surviving cells were counted after incubation for 48 h at 30°C. Oxidative stress resistance was tested by incubation of *C. albicans* cells (1 × 10⁷/ml) in YPD medium supplemented with 1 mM or 5 mM H₂O₂ (Sigma-Aldrich) or 0.6 mM S-nitrosoglutathione (GSNO; Sigma) for 2 h at 30°C with shaking. Percentage of survival was calculated as follows: (CFU from treated plate/CFU from untreated control plate) × 100.

Catalase assay. Catalase activity was measured using an EnzyChrom catalase assay kit (BioAssay Systems). *C. albicans* WT and mutant strains cultured as described above were incubated with or without 5 mM H₂O₂ for 1 h at 30°C and then harvested, and catalase activity was measured colorimetrically according to the manufacturer's instructions.

Statistical analysis. All data were analyzed by GraphPad Prism software version 7 (GraphPad Software, San Diego, CA), and a significance level (α) of 0.05 was used for all experiments.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <https://doi.org/10.1128/IAI.00685-18>.

SUPPLEMENTAL FILE 1, PDF file, 0.1 MB.

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REFERENCES

- Norris HL, Friedman J, Chen Z, Puri S, Wilding G, Edgerton M. 2018. Salivary metals, age, and gender correlate with cultivable oral *Candida* carriage levels. *J Oral Microbiol* 10:1447216. <https://doi.org/10.1080/20002297.2018.1447216>.
- Trautwein-Weidner K, Gladiator A, Nur S, Diethelm P, LeibundGut-Landmann S. 2015. IL-17-mediated antifungal defense in the oral mucosa is independent of neutrophils. *Mucosal Immunol* 8:221–231. <https://doi.org/10.1038/mi.2014.57>.
- Horn DL, Neofytos D, Anaissie EJ, Fishman JA, Steinbach WJ, Olyaei AJ, Marr KA, Pfaller MA, Chang C-H, Webster KM. 2009. Epidemiology and outcomes of Candidemia in 2019 patients: data from the prospective antifungal therapy alliance registry. *Clin Infect Dis* 48:1695–1703. <https://doi.org/10.1086/599039>.
- Qian Q, Jutila MA, Van Rooijen N, Cutler JE. 1994. Elimination of mouse splenic macrophages correlates with increased susceptibility to experimental disseminated candidiasis. *J Immunol* 152:5000–5008.
- Rudkin FM, Bain JM, Walls C, Lewis LE, Gow NA, Erwig LP. 2013. Altered dynamics of *Candida albicans* phagocytosis by macrophages and PMNs when both phagocyte subsets are present. *mBio* 4:e00810-13. <https://doi.org/10.1128/mBio.00810-13>.
- Vonk AG, Wieland CW, Netea MG, Kullberg BJ. 2002. Phagocytosis and intracellular killing of *Candida albicans* blastoconidia by neutrophils and macrophages: a comparison of different microbiological test systems. *J Microbiol Methods* 49:55–62. [https://doi.org/10.1016/S0167-7012\(01\)00348-7](https://doi.org/10.1016/S0167-7012(01)00348-7).
- Ermert D, Niemiec MJ, Rohm M, Glenthoj A, Borregaard N, Urban CF. 2013. *Candida albicans* escapes from mouse neutrophils. *J Leukoc Biol* 94:223–236. <https://doi.org/10.1189/jlb.0213063>.
- Dupré-Crochet S, Erard M, Nüße O. 2013. ROS production in phagocytes: why, when, and where? *J Leukoc Biol* 94:657–670. <https://doi.org/10.1189/jlb.1012544>.
- Nordenfelt P, Tapper H. 2011. Phagosome dynamics during phagocytosis by neutrophils. *J Leukoc Biol* 90:271–284. <https://doi.org/10.1189/jlb.0810457>.
- Pethe K, Swenson DL, Alonso S, Anderson J, Wang C, Russell DG. 2004. Isolation of *Mycobacterium tuberculosis* mutants defective in the arrest of phagosome maturation. *Proc Natl Acad Sci U S A* 101:13642–13647. <https://doi.org/10.1073/pnas.0401657101>.
- Flannagan RS, Cosio G, Grinstein S. 2009. Antimicrobial mechanisms of phagocytes and bacterial evasion strategies. *Nat Rev Microbiol* 7:355–366. <https://doi.org/10.1038/nrmicro2128>.
- Fernandez-Arenas E, Bleck CK, Nombela C, Gil C, Griffiths G, Diez-Orejas R. 2009. *Candida albicans* actively modulates intracellular membrane trafficking in mouse macrophage phagosomes. *Cell Microbiol* 11:560–589. <https://doi.org/10.1111/j.1462-5822.2008.01274.x>.
- Monge RA, Román E, Nombela C, Pla J. 2006. The MAP kinase signal transduction network in *Candida albicans*. *Microbiology* 152:905–912. <https://doi.org/10.1099/mic.0.28616-0>.
- Roman E, Arana DM, Nombela C, Alonso-Monge R, Pla J. 2007. MAP kinase pathways as regulators of fungal virulence. *Trends Microbiol* 15:181–190. <https://doi.org/10.1016/j.tim.2007.02.001>.
- Ramírez-Zavala B, Weyler M, Gildor T, Schmauch C, Kornitzer D, Arkowitz R, Morschhäuser J. 2013. Activation of the Cph1-dependent MAP Kinase signaling pathway induces white-opaque switching in *Candida albicans*. *PLoS Pathog* 9:e1003696. <https://doi.org/10.1371/journal.ppat.1003696>.
- Csank C, Schroppel K, Leberer E, Harcus D, Mohamed O, Meloche S, Thomas DY, Whiteway M. 1998. Roles of the *Candida albicans* mitogen-activated protein kinase homolog, Cek1p, in hyphal development and systemic candidiasis. *Infect Immun* 66:2713–2721.
- Ernst JF, Pla J. 2011. Signaling the glycoshield: maintenance of the *Candida albicans* cell wall. *Int J Med Microbiol* 301:378–383. <https://doi.org/10.1016/j.ijmm.2011.04.003>.
- Alonso-Monge R, Navarro-García F, Roman E, Negredo AI, Eisman B, Nombela C, Pla J. 2003. The Hog1 mitogen-activated protein kinase is essential in the oxidative stress response and chlamyospore formation in *Candida albicans*. *Eukaryot Cell* 2:351–361. <https://doi.org/10.1128/EC.2.2.351-361.2003>.
- Grahl N, Demers EG, Lindsay AK, Harty CE, Willger SD, Piispanen AE, Hogan DA. 2015. Mitochondrial activity and Cyr1 are key regulators of Ras1 activation of *C. albicans* virulence pathways. *PLoS Pathog* 11:e1005133. <https://doi.org/10.1371/journal.ppat.1005133>.
- Hollomon JM, Grahl N, Willger SD, Koeppen K, Hogan DA. 2016. Global role of cyclic AMP signaling in pH-dependent responses in *Candida albicans*. *mSphere* 1:e00283-16. <https://doi.org/10.1128/mSphere.00283-16>.
- Inglis DO, Sherlock G. 2013. Ras signaling gets fine-tuned: regulation of multiple pathogenic traits of *Candida albicans*. *Eukaryot Cell* 12:1316–1325. <https://doi.org/10.1128/EC.00094-13>.
- Rocha CRC, Schröppel K, Harcus D, Marcil A, Dignard D, Taylor BN, Thomas DY, Whiteway M, Leberer E. 2001. Signaling through adenylyl cyclase is essential for hyphal growth and virulence in the pathogenic fungus *Candida albicans*. *Mol Biol Cell* 12:3631–3643. <https://doi.org/10.1091/mbc.12.11.3631>.
- Leberer E, Harcus D, Dignard D, Johnson L, Ushinsky S, Thomas DY, Schroppel K. 2001. Ras links cellular morphogenesis to virulence by regulation of the MAP kinase and cAMP signalling pathways in the pathogenic fungus *Candida albicans*. *Mol Microbiol* 42:673–687.
- Diez-Orejas R, Moleró G, Navarro-García F, Pla J, Nombela C, Sanchez-Pérez M. 1997. Reduced virulence of *Candida albicans* *MKC1* mutants: a role for mitogen-activated protein kinase in pathogenesis. *Infect Immun* 65:833–837.
- Cheetham J, MacCallum DM, Doris KS, da Silva Dantas A, Scorfild S, Odds F, Smith DA, Quinn J. 2011. MAPKKK-independent regulation of the Hog1 stress-activated protein kinase in *Candida albicans*. *J Biol Chem* 286:42002–42016. <https://doi.org/10.1074/jbc.M111.265231>.
- Galán-Díez M, Arana DM, Serrano-Gómez D, Kremer L, Casasnovas JM, Ortega M, Cuesta-Domínguez A, Corbí AL, Pla J, Fernández-Ruiz E. 2010. *Candida albicans* β -glucan exposure is controlled by the fungal CEK1-mediated mitogen-activated protein kinase pathway that modulates immune responses triggered through dectin-1. *Infect Immun* 78:1426–1436. <https://doi.org/10.1128/IAI.1128.IAI.00989-09>.
- Du C, Calderone R, Richert J, Li D. 2005. Deletion of the *SSK1* response regulator gene in *Candida albicans* contributes to enhanced killing by human polymorphonuclear neutrophils. *Infect Immun* 73:865–871. <https://doi.org/10.1128/IAI.73.2.865-871.2005>.
- Niemiec MJ, Grumaz C, Ermert D, Desel C, Shankar M, Lopes JP, Mills IG, Stevens P, Sohn K, Urban CF. 2017. Dual transcriptome of the immediate neutrophil and *Candida albicans* interplay. *BMC Genomics* 18:696. <https://doi.org/10.1186/s12864-017-4097-4>.
- Bahn YS, Staab J, Sundstrom P. 2003. Increased high-affinity phosphodiesterase PDE2 gene expression in germ tubes counteracts *CAP1*-dependent synthesis of cyclic AMP, limits hypha production and promotes virulence of *Candida albicans*. *Mol Microbiol* 50:391–409. <https://doi.org/10.1046/j.1365-2958.2003.03692.x>.
- Wilson D, Tutulan-Cunita A, Jung W, Hauser NC, Hernandez R, Williamson T, Piekarska K, Rupp S, Young T, Stateva L. 2007. Deletion of the high-affinity cAMP phosphodiesterase encoded by *PDE2* affects stress responses and virulence in *Candida albicans*. *Mol Microbiol* 65:841–856. <https://doi.org/10.1111/j.1365-2958.2007.05788.x>.
- Harcus D, Nantel A, Marcil A, Rigby T, Whiteway M. 2004. Transcription profiling of cyclic AMP signaling in *Candida albicans*. *Mol Biol Cell* 15:4490–4499. <https://doi.org/10.1091/mbc.e04-02-0144>.
- Veerman EC, Valentijn-Benz M, Nazmi K, Ruissen AL, Walgreen-Weterings E, van Marle J, Doust AB, van't Hof W, Bolscher JG, Amerongen AV. 2007. Energy depletion protects *Candida albicans* against antimicrobial peptides by rigidifying its cell membrane. *J Biol Chem* 282:18831–18841. <https://doi.org/10.1074/jbc.M610555200>.
- Champion JA, Mitragotri S. 2006. Role of target geometry in phagocytosis. *Proc Natl Acad Sci U S A* 103:4930–4934. <https://doi.org/10.1073/pnas.0600997103>.
- Segal AW. 2005. How neutrophils kill microbes. *Annu Rev Immunol* 23:197–223. <https://doi.org/10.1146/annurev.immunol.23.021704.115653>.
- Rossi DCP, Gleason JE, Sanchez H, Schatzman SS, Culbertson EM, Johnson CJ, McNeese CA, Coelho C, Nett JE, Andes DR, Cormack BP, Culotta VC. 2017. *Candida albicans* *FRE8* encodes a member of the NADPH oxidase family that produces a burst of ROS during fungal morphogenesis. *PLoS Pathog* 13:e1006763. <https://doi.org/10.1371/journal.ppat.1006763>.
- Miramón P, Dunker C, Windecker H, Bohovych IM, Brown AJ, Kurzai O, Hube B. 2012. Cellular responses of *Candida albicans* to phagocytosis and the extracellular activities of neutrophils are critical to counteract carbohydrate starvation, oxidative and nitrosative stress. *PLoS One* 7:e52850. <https://doi.org/10.1371/journal.pone.0052850>.

37. Zhang X, Zhao S, Sun L, Li W, Wei Q, Ashman RB, Hu Y. 2017. Different virulence of *Candida albicans* is attributed to the ability of escape from neutrophil extracellular traps by secretion of DNase. *Am J Transl Res* 9:50–62.
38. Bahn YS, Molenda M, Staab JF, Lyman CA, Gordon LJ, Sundstrom P. 2007. Genome-wide transcriptional profiling of the cyclic AMP-dependent signaling pathway during morphogenic transitions of *Candida albicans*. *Eukaryot Cell* 6:2376–2390. <https://doi.org/10.1128/EC.00318-07>.
39. Nakagawa Y, Koide K, Watanabe K, Morita Y, Mizuguchi I, Akashi T. 1999. The expression of the pathogenic yeast *Candida albicans* catalase gene in response to hydrogen peroxide. *Microbiol Immunol* 43:645–651. <https://doi.org/10.1111/j.1348-0421.1999.tb02452.x>.
40. Pradhan A, Herrero-de-Dios C, Belmonte R, Budge S, Lopez Garcia A, Kolmogorova A, Lee KK, Martin BD, Ribeiro A, Bebes A, Yuceel R, Gow NAR, Munro CA, MacCallum DM, Quinn J, Brown AJP. 2017. Elevated catalase expression in a fungal pathogen is a double-edged sword of iron. *PLoS Pathog* 13:e1006405. <https://doi.org/10.1371/journal.ppat.1006405>.
41. Davis-Hanna A, Piispanen AE, Stateva LI, Hogan DA. 2008. Farnesol and dodecanol effects on the *Candida albicans* Ras1-cAMP signalling pathway and the regulation of morphogenesis. *Mol Microbiol* 67:47–62. <https://doi.org/10.1111/j.1365-2958.2007.06013.x>.
42. Eisenhauer PB, Lehrer RI. 1992. Mouse neutrophils lack defensins. *Infect Immun* 60:3446–3447.
43. Arana DM, Alonso-Monge R, Du C, Calderone R, Pla J. 2007. Differential susceptibility of mitogen-activated protein kinase pathway mutants to oxidative-mediated killing by phagocytes in the fungal pathogen *Candida albicans*. *Cell Microbiol* 9:1647–1659. <https://doi.org/10.1111/j.1462-5822.2007.00898.x>.
44. Eisman B, Alonso-Monge R, Roman E, Arana D, Nombela C, Pla J. 2006. The Cek1 and Hog1 mitogen-activated protein kinases play complementary roles in cell wall biogenesis and chlamydospore formation in the fungal pathogen *Candida albicans*. *Eukaryot Cell* 5:347–358. <https://doi.org/10.1128/EC.5.2.347-358.2006>.
45. Phillips AJ, Crowe JD, Ramsdale M. 2006. Ras pathway signaling accelerates programmed cell death in the pathogenic fungus *Candida albicans*. *Proc Natl Acad Sci U S A* 103:726–731. <https://doi.org/10.1073/pnas.0506405103>.
46. Lane S, Birse C, Zhou S, Matson R, Liu H. 2001. DNA array studies demonstrate convergent regulation of virulence factors by Cph1, Cph2, and Efg1 in *Candida albicans*. *J Biol Chem* 276:48988–48996. <https://doi.org/10.1074/jbc.M104484200>.
47. Jain P, Akula I, Edlind T. 2003. Cyclic AMP signaling pathway modulates susceptibility of *Candida* species and *Saccharomyces cerevisiae* to anti-fungal azoles and other sterol biosynthesis inhibitors. *Antimicrob Agents Chemother* 47:3195–3201. <https://doi.org/10.1128/AAC.47.10.3195-3201.2003>.
48. Gresham HD, Lowrance JH, Caver TE, Wilson BS, Cheung AL, Lindberg FP. 2000. Survival of *Staphylococcus aureus* inside neutrophils contributes to infection. *J Immunol* 164:3713–3722. <https://doi.org/10.4049/jimmunol.164.7.3713>.
49. Kubica M, Guzik K, Koziel J, Zarebski M, Richter W, Gajkowska B, Golda A, Maciag-Gudowska A, Brix K, Shaw L, Foster T, Potempa J. 2008. A potential new pathway for *Staphylococcus aureus* dissemination: the silent survival of *S. aureus* phagocytosed by human monocyte-derived macrophages. *PLoS One* 3:e1409. <https://doi.org/10.1371/journal.pone.0001409>.
50. Levitz SM, Nong SH, Seetoo KF, Harrison TS, Speizer RA, Simons ER. 1999. *Cryptococcus neoformans* resides in an acidic phagolysosome of human macrophages. *Infect Immun* 67:885–890.
51. García-Rodas R, Zaragoza O. 2012. Catch me if you can: phagocytosis and killing avoidance by *Cryptococcus neoformans*. *FEMS Immunol Med Microbiol* 64:147–161. <https://doi.org/10.1111/j.1574-695X.2011.00871.x>.
52. Charlier C, Nielsen K, Daou S, Brigitte M, Chretien F, Dromer F. 2009. Evidence of a role for monocytes in dissemination and brain invasion by *Cryptococcus neoformans*. *Infect Immun* 77:120–127. <https://doi.org/10.1128/IAI.01065-08>.
53. Ermert D, Urban CF, Laube B, Goosmann C, Zychlinsky A, Brinkmann V. 2009. Mouse neutrophil extracellular traps in microbial infections. *J Innate Immun* 1:181–193. <https://doi.org/10.1159/000205281>.
54. Pathirana RU, Friedman J, Norris HL, Salvatori O, McCall AD, Kay J, Edgerton M. 2018. Fluconazole-resistant *Candida auris* is susceptible to salivary histatin 5 killing and to intrinsic host defenses. *Antimicrob Agents Chemother* 62:e01872-17. <https://doi.org/10.1128/aac.01872-17>.
55. Ellis JA, Mayer SJ, Jones OT. 1988. The effect of the NADPH oxidase inhibitor diphenyleiiodonium on aerobic and anaerobic microbicidal activities of human neutrophils. *Biochem J* 251:887–891. <https://doi.org/10.1042/bj2510887>.
56. Fonzi WA, Irwin MY. 1993. Isogenic strain construction and gene mapping in *Candida albicans*. *Genetics* 134:717–728.
57. Smith DA, Nicholls S, Morgan BA, Brown AJP, Quinn J. 2004. A conserved stress-activated protein kinase regulates a core stress response in the human pathogen *Candida albicans*. *Mol Biol Cell* 15:4179–4190. <https://doi.org/10.1091/mbc.e04-03-0181>.
58. Kumamoto CA. 2005. A contact-activated kinase signals *Candida albicans* invasive growth and biofilm development. *Proc Natl Acad Sci U S A* 102:5576–5581. <https://doi.org/10.1073/pnas.0407097102>.
59. Lindsay AK, Deveau A, Piispanen AE, Hogan DA. 2012. Farnesol and cyclic AMP signaling effects on the hypha-to-yeast transition in *Candida albicans*. *Eukaryot Cell* 11:1219–1225. <https://doi.org/10.1128/EC.00144-12>.
60. Lo HJ, Kohler JR, DiDomenico B, Loebenberg D, Cacciapuoti A, Fink GR. 1997. Nonfilamentous *C. albicans* mutants are avirulent. *Cell* 90:939–949. [https://doi.org/10.1016/S0092-8674\(00\)80358-X](https://doi.org/10.1016/S0092-8674(00)80358-X).
61. Piispanen AE, Bonnefoi O, Carden S, Deveau A, Bassilana M, Hogan DA. 2011. Roles of Ras1 membrane localization during *Candida albicans* hyphal growth and farnesol response. *Eukaryot Cell* 10:1473–1484. <https://doi.org/10.1128/EC.05153-11>.