The role of *Candida albicans* AP-1 protein against host derived ROS in in vivo models of infection

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*Candida albicans* is a major fungal pathogen of humans, causing mucosal infections that are difficult to eliminate and systemic infections that are often lethal primarily due to defects in the host’s innate status. Here we demonstrate the utility of *Caenorhabditis elegans*, a model host to study innate immunity, by exploring the role of reactive oxygen species (ROS) as a critical innate response against *C. albicans* infections. Much like a human host, the nematode’s innate immune response is activated to produce ROS in response to fungal infection. We use the *C. albicans cap1* mutant, which is susceptible to ROS, as a tool to dissect this physiological innate immune response and show that cap1 mutants fail to cause disease and death, except in bli-3 mutant worms that are unable to produce ROS because of a defective NADPH oxidase. We further validate the ROS-mediated host defense mechanism in mammalian phagocytes by demonstrating that chemical inhibition of the NADPH oxidase in cultured macrophages enables the otherwise susceptible cap1 mutant to resist ROS-mediated phagolysis. Loss of CAP1 confers minimal attenuation of virulence in a disseminated mouse model, suggesting that CAP1-independent mechanisms contribute to pathogen survival in vivo. Our findings underscore a central theme in the process of infection—the intricate balance between the virulence strategies employed by *C. albicans* and the host’s innate immune system and validates *C. elegans* as a simple model host to dissect this balance at the molecular level.

Introduction

The incidence of invasive fungal infections has escalated in recent years, primarily in hospital settings.¹ 90% of these infections are caused by various *Candida* species,² 50% of which are fatal.³ The estimated annual cost of treating nosocomial Candida infections exceeds $1 billion per year and has an attributable mortality of about 5,000 deaths per year in the United States.⁴ ⁶ *Candida* species are the fourth leading cause of bloodstream infections (BSI), with *C. albicans* responsible for about half the cases. *C. albicans* form robust biofilms on medical implants, such as intravenous catheters, prosthetic joints, or artificial heart valves, which can seed potentially lethal disseminated infections.⁷ ⁹ Approximately 75% of women have at least one episode of vaginitis caused by Candida in their lifetime¹⁰ and oropharyngeal thrush and esophagitis are common in both infants and in patients with AIDS.¹¹ *Candida* species also cause superficial infections on mucosal surfaces in the body, including the mouth, upper gastrointestinal (GI) and urogenital tract. The frequency of these superficial infections combined with the treatment challenges posed by disseminated infections make *C. albicans* an important pathogen for further study.

A variety of in vitro, ex vivo and in vivo models have been employed to study the interaction between the host and this fungal pathogen. In vitro studies of hyphal morphogenesis and biofilm formation, among many others, have yielded important insights into virulence.¹² ¹³ Ex vivo models, such as co-culturing *C. albicans* with isolated macrophages, neutrophils, epithelial or endothelial cells, and even intact, perfused organs, have demonstrated that *C. albicans* has very complex responses to host cell contact, which can differ dramatically between cell types.¹⁴ ¹⁹ A murine model of disseminated candidiasis has been frequently used to validate the role of specific genes on overall virulence. There is a general appreciation that each of these models has provided important insights into fungal pathogenesis. Recently, invertebrate models have become additional tools to dissect the roles of components of the antifungal host defense system, including flies (*Drosophila melanogaster*), wax moth larvae (*Galleria mellonella*) and the nematode *Caenorhabditis elegans*.²⁰ ²² *C. elegans* has emerged as a useful model to study infectious disease for several reasons. First, facets of its innate immune system are conserved in humans²³ ²⁴ and the nematode reacts to pathogens in a manner similar to mammals, such as activation of specific signal transduction pathways.²⁵ ²⁶ A rich body of literature
demonstrates that human pathogens, both bacteria and fungi, also infect *C. elegans* in ways that are mechanistically similar to humans. For example the opportunistic human pathogens *Pseudomonas aeruginosa* and *Serratia marcescens* produce toxins that are required for pathogenesis in disparate eukaryotic hosts. Mutant studies in Salmonella Typhimurium, typically thought to have a narrow host range, shows a direct correlation in virulence between humans and *C. elegans*. More recently a comparative study in *C. elegans* using fungal pathogens of the genus *Cryptococcus* showed that only the human pathogen *C. neoformans*, but not other related yeasts (*C. kurtzii* or *C. laurentii*), killed the nematode. These studies further demonstrated that a virulence factor such as Kin1, first identified in nematodes, was also important in mammals. More recent whole genome analyses of *C. elegans* infected with *C. albicans* reveal that the nematode induces immune defenses with known antifungal properties. Studies of *C. elegans* infected with bacterial pathogens reveal that the generation of reactive oxygen species (ROS) is an important part of the nematode’s defense response, a hallmark shared with mammalian innate immune responses. We previously reported a *C. elegans*-based assay to study several aspects of disease progression, namely Dar (deformity in the anal region), an early marker of infection; intestinal distension, resulting from colonization of the intestine; swelling in the vulva, representing infection of other epithelial layers; and ultimately death of the host worm. This assay mimics an infection, because a small amount of the pathogen is introduced along with the food. Furthermore, the assay is amenable to unbiased, high-throughput screens because it does not require manual handling of individual animals. Developed initially using *Saccharomyces cerevisiae*, here we have adapted the assay for *C. albicans*.

We use genetic and pharmacological tools to alter the intricate balance between the host and the pathogen and demonstrate that ablating either the ability of the worm to produce ROS or *C. albicans* to detoxify it (via mutation of the Cap1 transcription factor) has dramatic effects on the outcome of this infection. Results in the worm were recapitulated in macrophage co-cultures, validating this model. Surprisingly, the *C. albicans cap1Δ* mutant retained limited virulence in the disseminated murine bloodstream model, suggesting that additional layers of regulation of antioxidant defense exist in the context of a mammal. This work thus provides an avenue to investigate fungal pathogenesis and has allowed us to identify further complexity in the pathogenic *C. albicans*-host interaction.

**Results**

**Generation of CAP1 homozygous and complemented strains.** The original *cap1ΔΔ* mutant strain CJD21 was generated by Raymond and colleagues using an approach that was subsequently shown to be inappropriate for animal experiments due to the variability of expression of the *URA3* selectable marker. We selected a *ura3*-derivative of the *cap1ΔΔ* strain CJD21 on 5-FOA, then integrated either *URA3* or *URA3-CAP1* at the *RPS10* locus using plasmid Clp10, a strategy shown to stably express *URA3* during infection. This generated a *cap1ΔΔ* mutant strain (AGC2) and a complemented strain (AGC4); the mutant strain is phenotypically identical to the original CJD21 during in vitro challenge with ROS (data not shown).

**In vivo virulence assay for Candida infection.** Here we report a pathogenesis assay to test various fungal pathogens of the Candida genus, including *C. albicans*, *C. dubliniensis*, *C. krusei*, *C. tropicalis* and *C. glabrata*. We adapted a previously described pathogenesis assay where a small quantity of *S. cerevisiae*, was introduced with the nematode’s standard diet of *E. coli* OP50. *E. coli* was attenuated to avoid interactions with the fungal species under investigation. We decreased the concentration of Candida in the feeding mixture by 30-fold because of the increased pathogenicity of *C. albicans* species, and even at this lower fungal burden, most species induced Dar in 100% worms, compared with *S. cerevisiae*, which induced 0% Dar at the same concentration of inoculum (Table 1). We uncovered molecular mechanisms of fungal virulence and the reciprocal innate immune response of nematodes, which is also conserved in mammals.

**Disease phenotypes of *C. elegans* upon *C. albicans* infection.** Subsequent studies focused on *C. albicans* because it is the most prevalent infectious species in this genus and adequate genomic and molecular tools have been developed. To visualize infection, disease progression and death, we exposed nematodes to *C. albicans* and observed them daily over a 6 d period. The Dar phenotype was clearly visible in every worm on Day 4 (Fig. 1 and Table 1). Swelling in the vulvar region was also noted in the worms infected with *C. albicans* (Fig. 2B). The worm succumbed to the infection following these phenotypic observations. To observe colonization of the intestinal lumen, worms were exposed to mCherry-labeled *C. albicans* and observed micrographs of worms infected with *C. albicans* indicated that the intestinal lumen was colonized 2 d post-exposure compared with uninfected worms. Considerable intestinal distension was observed in infected worms on subsequent days (Fig. 2A) compared with the uninfected control population. In general, the disease phenotypes were more robust with *C. albicans* compared with *S. cerevisiae*, even with lower inoculum for infection. To further validate our assay we tested known mutants *efg1ΔΔ* and *cpb1ΔΔ*—previously documented virulence factors

| Strains         | % Dar | n  |
|-----------------|-------|----|
| *C. albicans* (SC 5314) | 100    | 114 |
| *C. albicans*   | 100   | 111 |
| *C. dubliniensis* | 100   | 122 |
| *C. glabrata*   | 59.7 ± 1.1 | 119 |
| *C. parapsilosis* | 4.9 ± 0.3 | 133 |
| *C. krusei*     | 100   | 115 |
| *C. tropicalis* | 100   | 134 |
| *S. cerevisiae* (BY4741) | 0     | 76  |

± standard error
that regulate hyphal transition of *C. albicans* and have been shown to be important in in vivo infections of mice and nematodes. The *efg1Δ/Δ* and *cph1Δ/Δ* single mutants showed decreased Dar 10% and 50%, respectively, compared with the cognate wild type, while the *efg1Δ/Δ cph1Δ/Δ* double mutant failed to induce any Dar (0% Dar). These results recapitulate the pattern of virulence in mice, where the *cph1Δ/Δ* mutant is slightly attenuated, the *efg1Δ/Δ* mutant is significantly attenuated, and the double mutant is completely avirulent. Accordingly, our assay is suitable for exploring virulence strategies of *C. albicans* and the reciprocal host defenses that may be correlated with aspects of innate immunity that is conserved in mammals.

**CAP1** is required to establish and sustain infection in nematodes. We compared the Dar response of worms exposed to null mutant (*cap1Δ/Δ*) and wild-type strain (**CAP1/CAP1**) as well as a **CAP1**-complemented strain (**cap1Δ/Δ + **CAP1**), where a single wild-type copy of **CAP1** has been re-introduced. The wild-type strain was able to induce Dar in 100% of the worms while the *cap1Δ/Δ* homozygous mutant showed a significant reduction in Dar induction (**Fig. 3A**). The **CAP1**-complemented strain showed an intermediate Dar response that is commonly observed for complemented strains. This result indicates that Cap1 function is required for full virulence in the nematode model.

To test whether the mechanism by which Cap1 promotes virulence is related to the production of ROS by the host, we

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**Figure 1.** *C. albicans* induces deformed anal region (Dar) in wild-type worms. Worms were exposed to *E. coli* as control (uninfected) and *C. albicans* (shown are two examples of infected worms) and pictures were taken on Day 4 (arrow indicates the Dar region). Scale = 20 µm.

**Figure 2.** Different phenotypes are observed due to *Candida* infection. (A) Exposure to *C. albicans* causes intestinal distention in the worms over time (Days 2, 3 and 4). DIC (Nomarski) pictures of worms feeding on *E. coli* was taken as control and the DIC, RFP and merged pictures of worms infected with *C. albicans* are shown over 3 d. (B) Vulva swelling is observed (arrow points to the vulvar region) by Day 4 when worms are exposed to Candida compared with worms exposed *E. coli* as control (uninfected). Scale = 20 µm.
tested mutants in the bli-3 gene which contains the only NADPH oxidase moiety in C. elegans genome. We and others previously reported that Bli-3 produces ROS in response to pathogenic insult. To test the hypothesis that Cap1 is responsible for counteracting this oxidative environment upon C. albicans infection, we exposed bli-3 mutant worms to cap1Δ/Δ, CAP1-complemented and wild-type CAP1/CAP1 strains. As shown in Figure 3B, the Dar phenotype of bli-3 mutant worms infected with the cap1Δ/Δ null mutant is indistinguishable from that of the wild type and complemented counterparts, suggesting that Cap1 function is not required for infection if the host cannot produce ROS. Thus, Cap1 is dispensable when the ROS production part of the host’s defense repertoire has been compromised, as in the bli-3 mutant.

We and others previously showed that Dar is an early indicator of an eventually lethal infection. To test the hypothesis that cap1Δ/Δ mutants are avirulent, we measured the life span of worms infected with the cap1Δ/Δ null mutant, complemented and wild-type strains relative to uninfected worms. The average lifespan of C. elegans infected with wild-type C. albicans (CAP1/CAP1) or a CAP1-complemented strain was significantly shorter (median survival = 6 d) than those infected with the cap1Δ/Δ null mutant (median survival = 13 d) (Fig. 3C). These data indicate that Cap1 is required for the infection to persist and ultimately kill the host. To test the hypothesis that Cap1 is required to neutralize host ROS, we challenged an ROS-deficient bli-3 mutant host with the C. albicans cap1Δ/Δ mutant. Lifespan plots reveal that the kinetics of killing are indistinguishable between the cap1Δ/Δ mutant and the wild type or complemented strains (Fig. 3D), suggesting that ROS is the primary defense of nematodes against C. albicans and that C. albicans requires Cap1 for a lethal infection to persist. The lifespan of uninfected bli-3 mutants is the same as their wild-type counterparts (Fig. 3), eliminating confounding factors other than ROS production as a likely cause of death of infected worms.

In vitro, Cap1 regulates the response to oxidative stress, which is a key component of the human innate immune response to fungal infections by neutrophils. To correlate these studies, performed at 30°C and 37°C, with our in vivo infection, performed at 20°C, we compared the ROS sensitivity profiles of
cap1Δ/Δ, CAP1-complemented and the cognate wild-type strain at all three temperatures using hydrogen peroxide. The results indicate that ROS sensitivity of the cap1Δ/Δ strain is independent of temperature and that all C. albicans strains used in this study show the same phenotype at all temperatures (data not shown).

Cap1 is responsible for neutralizing ROS produced by phagocytes. Part of the mammalian host defense against Candida infection is production of ROS within phagosomes that have engulfed the fungal pathogen. Wild-type C. albicans can effectively neutralize ROS to survive and eventually cause Candida infection is production of ROS within phagosomes that have engulfed the fungal pathogen. 

Figure 4. Cap1 is required for survival in macrophages. Macrophages were exposed to different strains of Candida in a ratio of 1:15 macrophages with and without DPI. Percentage survival was calculated by dividing the CFUs obtained for Candida grown with macrophages to Candida alone. Data were then normalized to the wild type. Percent survival of cap1Δ/Δ mutant is significantly less **p < 0.05) than the wild type and the complemented Cap1 strain.

Discussion

Mammalian biology has been effectively modeled using a variety of species that present substantial advantages in complexity, genetic tractability, ethical considerations and cost. The last decade has seen the acceptance of invertebrates, such as C. elegans, as relevant hosts that can contribute to the molecular understanding of microbial pathogenesis. C. elegans has proven an effective model for several systemic pathogens such as Cryptococcus, Pseudomonas and Enterococcus, among others, even though it differs from mammalian infections in several respects, including the site of infection (gut vs. bloodstream), growth temperature (25–30°C vs. 37°C) and the absence of an adaptive immune response. We previously demonstrated that the model yeast S. cerevisiae can cause pathology in the nematode. In this work we show that this assay is far more robust when using pathogenic Candida species and that the virulence of these species in the worm roughly correlates with virulence in humans.
Thus, our cap1 MATa; his3 null mutants are C. elegans RPS10/rps1::URA3-CAP10 alters fungal growth medium was 72 38 cph1 and are based on SC5314 and its cap1 65 Interestingly these strains null expression. In humans, SOD4 Wild type Clinical isolates A.B. Onderdonk in, in vitro is required for virulence of C. albicans and their induction in the context of the mammalian host. Indeed, the superoxide dismutases SOD4 and SOD5 are both more highly expressed in hyphal cells, which can be induced in response to several host-associated cues. Thus, our C. elegans model may allow analysis of critical fungal responses to the innate immune system that could be masked by phenotypic redundancy in the intact mammal. In conclusion, host-generated ROS is a critical component of the ancient innate immune response against C. albicans in a variety of species and proper regulation of anti-oxidant defenses is an important virulence factor in many species including, as we show here, in C. albicans.

Materials and Methods

Strains, media and growth conditions. The C. albicans strains used are listed in Table 2 and are based on SC5314 and its auxotrophic derivative CAI4-F2. C. albicans transformations were performed via electroporation. Fungal growth medium was
prepared as described previously and strains were grown overnight in yeast extract-peptone-dextrose (YPD) at 37°C. The *C. elegans* strains were grown at 20°C on nematode growth agar medium (NGM), spotted with *Escherichia coli* OP50 and maintained as described previously. E. coli OP50 was grown overnight in Luria broth at 37°C.

**Generation of cap1 mutant strains.** The existing cap1Δ/Δ strain CJD21 expresses *URA3* from the disrupted cap1 locus, a strategy that has been subsequently demonstrated to potentially affect virulence through mis-expression of the *URA3* marker. This can be overcome through ectopic integration of *URA3* at the *RPS10* locus using plasmid Cip10. To generate the cap1Δ/Δ mutant and complemented strains with *URA3* at *RPS10*, we grew CJD21 on YPD for two overnight passages in YPD, then plated to media containing 5-fluorouracil (5-FOA) to select for *ura3* auxotrophs. PCR amplified the *CAPl* open reading frame from genomic DNA of strain SC5314, -1,000 bp of 5’ UTR and -350 bp of 3’ UTR. This fragment was ligated between the XhoI and HinDIII sites in Cip10. This plasmid was digested with *Stud* and used to transform the 5-FOA-selected cap1Δ/Δ strain. In parallel, the empty Cip10 plasmid was digested with *Stud* and used to transform the same strain. After selection on SD-Ura, correct integration at *RPS10* was confirmed by PCR to generate cap1Δ/Δ strains with either *URA3* or *CAPl–URA3* expressed from the same genomic site as the mutant (AGC2) and complemented (AGC4) strains, respectively.

**Microscopic analysis of C. elegans.** A 2% agarose pad containing 0.01 M sodium azide as anesthetic was prepared on a slide. A 5 μl drop of M9 buffer was added to the pad. Worms exposed to wild-type Candida or RFP-labeled Candida were picked and transferred to the drop on the slide. Mounted worms were then covered with a coverslip and observed at 200× and 400× magnifications using an Axiosvision Zeiss microscope under differential interference contrast (Nomarski) and epifluorescence optics. An ApoTome attachment was used to enhance the fluorescence images.

**Egg preparation.** Three worms each in the L3/L4 stage were transferred to two NGM agar plates containing *E. coli* OP50 and grown at 20°C for 4 d. On the day of the experiment, worms were washed off the plates with M9 buffer and centrifuged at 900 × g for 2 min. The supernatant was removed and the worms were then re-suspended in a bleach solution [1:4 dilution of commercial bleach (5.25%) containing 0.25 M sodium hydroxide]. The worm suspension was mixed gently by inversion for 1 min and centrifuged for 2 min at 2,000 × g. The pellet was washed and centrifuged with M9 buffer at 2,000 × g for 2 min and then finally re-suspended in 500 μl M9 buffer. The egg suspension was diluted or concentrated with M9 buffer as required to obtain approximately 30–40 eggs/5 μl.

**Pathogenesis assay.** *E. coli* and Candida strains were grown overnight at 37°C. Culture aliquots were centrifuged at full speed for 1 min in a table top microcentrifuge and the supernatant removed. Pellets were washed twice in sterile deionized water, and re-suspended to a final concentration of 200 mg/ml and 10 mg/ml, respectively. Next, a mixture of 10 μl of a 50 mg/ml streptomycin sulfate stock to inhibit *E. coli* growth, 7 μl of distilled water, 2.5 μl of *E. coli* and 0.5 μl of Candida was spotted on to each NGM plate. *E. coli* spotted plates were used as control. Finally, 5 μl of *C. elegans* egg suspension was transferred to each plate. Plates were then kept in a 20°C incubator and were observed for 5 d. All the experiments were done in triplicate. Student’s *t*-test was used to check the statistical significance of the differences observed between wild type and other Candida strains.

**C. elegans survival analysis.** Survival analysis was done on the *CAPl/CAPl, cap1Δ/Δ + CAPl* and *cap1Δ/Δ* strains and plates were prepared as described under egg preparation and pathogenesis assay. Starting on Day 3 following the initial assay, the number of worms alive, dead and dead on the rim was recorded each day. In order to differentiate between the first-generation worms and their offspring, worms were transferred every two days to new plates that were prepared the day before as previously discussed and incubated overnight at 20°C.

For data analysis, SPSS (IBM, Inc.) was used to generate Kaplan-Meier survival curves. In this experiment, each worm that died on the plate was entered as a “1,” indicating the event of death due to fungal disease took place. Worms that were found dead on the rim of the plate were censored and entered as a “0,” since death occurred for a non-related reason. Significance, as defined as a p value < 0.05, was assessed using the Gehan-Breslow test. This test assumes that data from earlier survival times are more accurate than later times and weights these data accordingly. Data were combined from three plates and another independent experiment gave the same results.

**Macrophage growth inhibition assay.** Macrophage cell line RAW 264.7 (ATCC) was used in the assay. The cell line was maintained in DMEM supplemented with 10% fetal bovine serum (FBS). The protocol was slightly modified from the original. Briefly, macrophages, on reaching 80–90% confluence, were scraped and brought up in DMEM supplemented with 10% FBS, 100 U/ml penicillin and 100 μg/ml streptomycin, then 2 × 10⁶ macrophage cells were plated on 35 mm² plates and allowed to adhere for 5 h. Candida strains were grown overnight at 37°C and diluted 1:10 and allowed to grow for another 5 h. Candida cells were then washed with supplemented DMEM and were added to the plates containing macrophages in a ratio 1:15 macrophage and to a final volume of 2 ml. The yeast strain was grown in parallel without macrophages to calculate percent survival. Plates were incubated overnight at 37°C and 5% CO₂. Cells were then brought up to 24 ml in a tube using 0.05% Triton X-100 (v/v) in water to osmotically lyse the macrophage cells. Dilutions were prepared and plated on YPD plates and grown overnight at 37°C. Colony forming units (CFUs) were counted and percent survival was calculated by taking the ratio of CFU from co-culture of Candida and macrophage to the CFU obtained for Candida alone.

Experiments involving diphenyleneiodonium chloride (DPI) were performed as mentioned above but with the addition of 0.05 μM DPI from a 31.8 mM stock solution in DMSO. Controls without DPI had the same concentration of DMSO. Statistical analysis was done using Student’s *t*-test.

**Mouse virulence assay.** Mouse virulence assays were performed as described previously. Female, adult (21–25 g)
ICR mice (Harlan) were maintained on a normal laboratory diet. C. albicans strains were passaged twice in overnight cultures in YPD, then diluted into fresh YPD and grown for 3 h at 30°C. Cells were collected by centrifugation, washed with water and re-suspended in PBS, then diluted and counted with a hemocytometer. Cells were injected with 100 µl of this suspension via the tail vein, with groups of 10 mice/strain. Animals were monitored 2–3 times daily for signs of infection and were euthanized when moribund. Survival data were analyzed with Prism5 (Graphpad Software) using the log rank test. All animal experiments were conducted in accordance with protocols approved by the University of Texas Health Science Center Animal Welfare Committee.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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