

Filamentation protects *Candida albicans* from amphotericin B-induced programmed cell death via a mechanism involving the yeast metacaspase, *MCA1*

David J. Laprade, Melissa S. Brown[#], Morgan L. McCarthy[#], James J. Ritch, and Nicanor Austriaco*

Department of Biology, Providence College, 1 Cunningham Square, Providence, Rhode Island 02918, U.S.A..

[#]These two authors contributed equally to this work.

* Corresponding Author:

Rev. Nicanor Pier Giorgio Austriaco, O.P., Ph.D., Department of Biology, Providence College, Providence, RI 02918, U.S.A; Tel: +1 401-865-1823; Fax: +1 401-865-2959; E-mail: naustria@providence.edu

ABSTRACT The budding yeast *Candida albicans* is one of the most significant fungal pathogens worldwide. It proliferates in two distinct cell types: blastospores and filaments. Only cells that are able to transform from one cell type into the other are virulent in mouse disease models. Programmed cell death is a controlled form of cell suicide that occurs when *C. albicans* cells are exposed to fungicidal drugs like amphotericin B and caspofungin, and to other stressful conditions. We now provide evidence that suggests that programmed cell death is cell-type specific in yeast: Filamentous *C. albicans* cells are more resistant to amphotericin B- and caspofungin-induced programmed cell death than their blastospore counterparts. Finally, our genetic data suggests that this phenomenon is mediated by a protective mechanism involving the yeast metacaspase, *MCA1*.

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Abbreviations:

AMB – amphotericin B,

CAS – caspofungin,

FBS – fetal bovine serum,

GlcNAc – N-acetylglucosamine,

ROS – reactive oxygen species.

INTRODUCTION

The budding yeast *Candida albicans* has emerged as one of the most significant fungal pathogens globally [1]. As an opportunistic pathogen capable of life-threatening systemic infections, *C. albicans* poses a serious threat to immunocompromised individuals, including AIDS patients, cancer patients undergoing chemotherapy, organ transplant recipients, and patients with advanced diabetes [2-4]. Worldwide, invasive candidiasis is currently regarded as the fourth most common cause of nosocomial infections with an estimated mortality rate of 35% [5, 6]. Significantly, resistance to therapies traditionally used to treat candidiasis such as triazoles and amphotericin B is rising [7, 8]. Thus, there is a pressing need to develop more effective anti-fungal treatments.

There are a number of physiological characteristics of *C. albicans* known to contribute to its virulence. Most notably, the organism's ability to undergo a reversible mor-

phological transition from round, budding cells called 'blastospores,' to elongated cells attached end-to-end, called 'filaments,' is linked to its ability to infect a host: cells unable to become filamentous or vice versa have been shown to be avirulent in mouse and *C. elegans* models [9-18]. The process by which *C. albicans* undergoes the transition from blastospores to filaments is known as 'filamentation'. Within the filamentous form, we further individuate two distinct cellular morphologies. Pseudo-hyphal cells are attached end-to-end, exhibit constrictions at the septa, and have an elongated cell wall, while true hyphal cells of *C. albicans* are distinguished by the emergence of small cellular protrusions called 'germ tubes'. While a recent study has shown that virulence can be decoupled from cell type in *C. albicans*, the connection between cell type and pathogenicity remains an important one [19].

Interestingly, there is growing evidence to support the claim that the drugs commonly used to treat patients suf-

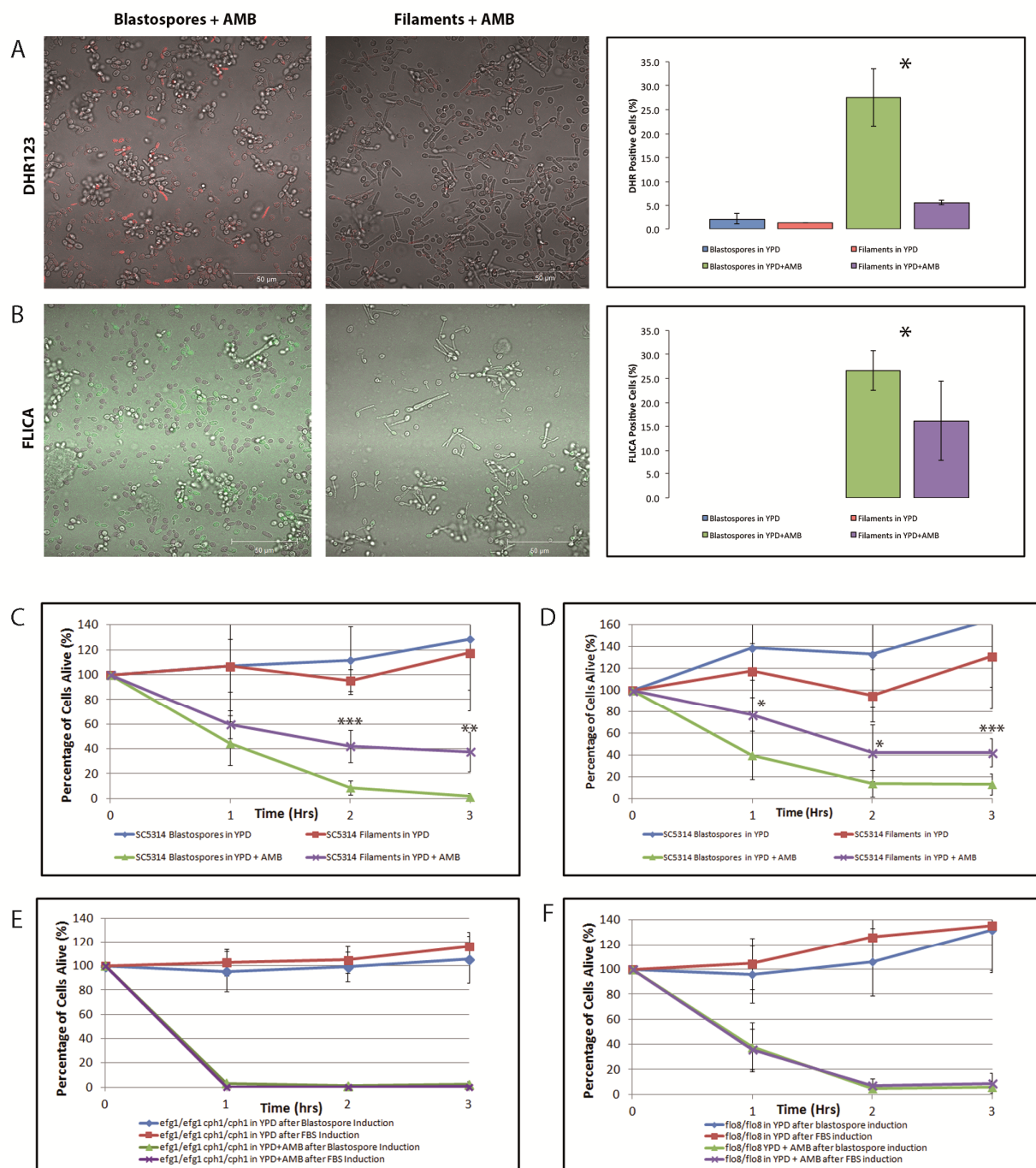


FIGURE 1: Filamentous *C. albicans* cells are more resistant than blastospores to AMB-induced programmed cell death. Exposure to amphotericin B leads to the generation of reactive oxygen species (ROS) and to caspase activation in *C. albicans* cells. Representative confocal scanning laser fluorescence images of wild-type SC5314 *C. albicans* cells treated with 8 $\mu\text{g/ml}$ AMB for 3 hours in YPD. Staining with dihydrorhodamine 123 (DHR123) confirms the presence of ROS (A) and with the FLICA assay for activation of intracellular caspases (B). Error bars indicate standard deviations for trials with at least three independent cultures, where at least 300 cells were counted for each trial. No FLICA positive cells were observed in the no drug controls. A single asterisk indicates statistical significance ($p < 0.05$) as compared to treated controls. Statistical significance was determined with the unpaired Student's t-test. Scale bar: 50 μm . Viability curves compare survival of the following cells exposed to AMB: (C) wild type blastospores and wild type filaments induced using 10% FBS; (D) wild type blastospores and wild type filaments induced using 0.5 g/l GlcNAc; (E) $\Delta\Delta\text{efg1/efg1 cph1/cph1}$ cells in YPD and $\Delta\Delta\text{efg1/efg1 cph1/cph1}$ cells following filamentous induction in YPD + 10% FBS, and (F) $\Delta\Delta\text{flo8/flo8}$ cells in YPD and $\Delta\Delta\text{flo8/flo8}$ cells following filamentous induction in YPD + 10% FBS. Note that after 3 hr, cells cultured in rich media without any drugs were able to grow and to divide, hence the relative viability levels that are greater than 100%. Error bars indicate standard deviations for trials with at least three independent cultures. A single, double, and triple asterisk indicates a significance of $p < 0.05$, $p < 0.005$, and $p < 0.0005$, respectively, as compared to treated controls. Statistical significance was determined with the unpaired Student's t-test.

fering from *C. albicans* infections, induce cell death [20, 21]. Specifically, *C. albicans* cells cultured in media containing the common anti-fungal drugs, amphotericin B (AMB) and caspofungin (CAS), undergo an apoptotic-like programmed cell death [22–25]. Programmed cell death is a cell suicide program that is essential for homeostasis, development, and disease prevention in many multi-cellular organisms [26–29]. When it occurs in yeast, programmed cell death is accompanied by the nicking of DNA, the accumulation of reactive oxygen species (ROS), and the intracellular activation of the fungal caspases [30–37].

In multicellular organisms, the response to programmed cell death is cell-type specific, and the rate of cell death varies widely from tissue to tissue and cell-type to cell-type within the plant or animal [26]. In this paper, we provide evidence that suggests that programmed cell death is also cell-type specific in yeast: filamentous *Candida* cells are more resistant to amphotericin B- and caspofungin-induced programmed cell death than their blastospore counterparts. Finally, our genetic data suggests that this phenomenon is mediated by a mechanism involving the yeast metacaspase *MCA1*.

RESULTS AND DISCUSSION

In recent years, it has become evident that programmed cell death occurs in unicellular organisms. For example, in the pathogenic fungus *Candida albicans* exposure to acetic acid, hydrogen peroxide, AMB, CAS, and farnesol leads to cell death accompanied by hallmark features of mammalian programmed cell death [22–24, 36, 38]. In multicellular organisms, the response to programmed cell death is cell-type specific, and the rate of cell death varies widely from tissue to tissue and cell type to cell type within the plant or animal [26]. To determine whether or not different forms of yeast respond differently to stimuli that induce programmed cell death, we first investigated whether or not filamentous cells manifest the markers of programmed cell death when they are cultured in media containing AMB. In this study, the clinical isolate SC5314—the parent of strains widely used for molecular analysis—was used as the wild type strain [39]. Briefly, overnight cultures of wild type cells in YPD were resuspended in YPD or YPD containing 10% fetal bovine serum (YPD+FBS) to obtain either blastospores or hyphal cells respectively (Supplemental Figure 1) [10–12]. These cells were then resuspended in YPD containing 8 $\mu\text{g}/\text{ml}$ AMB for 3 hours. Dihydrorhodamine 123 and FLICA staining confirmed that both these AMB-treated blastospores and filamentous cells accumulated ROS and activated caspases, respectively—two classic markers of programmed cell death – and were undergoing cell death as revealed by staining with propidium iodide (Figure 1). With both markers, however, there were fewer marker-positive filamentous cells as compared to blastospore controls, suggesting that the former cell type was more resistant to AMB.

Next, we compared the viability of wild-type *Candida albicans* cells in the blastospore and filamentous forms

when cultured in media containing 8 $\mu\text{g}/\text{ml}$ AMB with control cultures grown in YPD alone. Clonogenic survival assays are routinely used to assay programmed cell death in yeast [10, 23, 24, 40, 41]. As shown in Figure 1C, hyphal cells had a higher viability when cultured in media containing AMB than their blastospore counterparts ($p < 0.005$). This data suggests that filamentation protects *Candida* cells from AMB-induced programmed cell death and that this type of programmed cell death is cell-type specific in yeast.

However, because hyphae were induced by culturing blastospores in media containing FBS [11], it is possible that the differences in clonogenic survival rate could be attributed to culture conditions—namely, the presence of FBS—rather than to filamentation. To rule out this alternative explanation for our observations, we repeated our assays with a filamentation induction protocol that used N-acetylglucosamine (GlcNAc) instead of FBS [42, 43]. As shown in Figure 1D, GlcNAc-induced filamentous cells were also more resistant than their blastospore counterparts to AMB-induced cell death. Still, it could be argued that the difference in survival rate observed between the two cell types was only due to the variable presence of either FBS or GlcNAc. To respond to this concern, we repeated our experiments with Can36, a SC5314-derived mutant yeast strain lacking *CPH1* and *EFG1*, two putative transcription factors necessary for filamentation in *Candida* [12]. As expected, this strain was unable to undergo filamentation in media containing 10% FBS (Supplemental Figure 1). However, as shown in Figure 1E, the viability of the $\Delta\Delta\text{cph1}/\text{cph1}$ *efg1/efg1* mutant yeast cells cultured in FBS and exposed to AMB was indistinguishable from that of mutant yeast cells cultured in media with AMB alone. Finally, we repeated our assay a fourth time with *CCF3*, a SC5314-derived $\Delta\Delta\text{flo8}/\text{flo8}$ strain that is also unable to undergo filamentation when cultured in FBS [10]. Again, this non-filamentous mutant was unable to survive when cultured in the presence of AMB regardless of whether or not it was first cultured in the presence of FBS [Figure 1F]. Complementation of the $\Delta\Delta\text{flo8}/\text{flo8}$ strain confirmed that this phenotype, along with the inability to undergo filamentation, are both dependent upon the null $\Delta\Delta\text{flo8}/\text{flo8}$ mutation as others had previously shown [10]. Thus, we conclude that the resistance pattern noted in both non-filamentous mutants is not related to secondary effects of the mutations distinct from their inability to undergo filamentation, and that FBS itself is unable to protect yeast cells from AMB-induced programmed cell death. Together, these experiments suggest that filamentation protects yeast cells against AMB-induced programmed cell death.

To investigate the mechanism behind this anti-cell death phenomenon, we decided to focus on the yeast metacaspase, *MCA1*, a homolog of the mammalian caspases linked to apoptosis in metazoans. The *MCA1* homolog in *S. cerevisiae*, *YCA1*, has been implicated in programmed cell death: mutants lacking *YCA1* in *S. cerevisiae* exhibit lower levels of intracellular caspase activation and significantly decreased levels of programmed cell death when

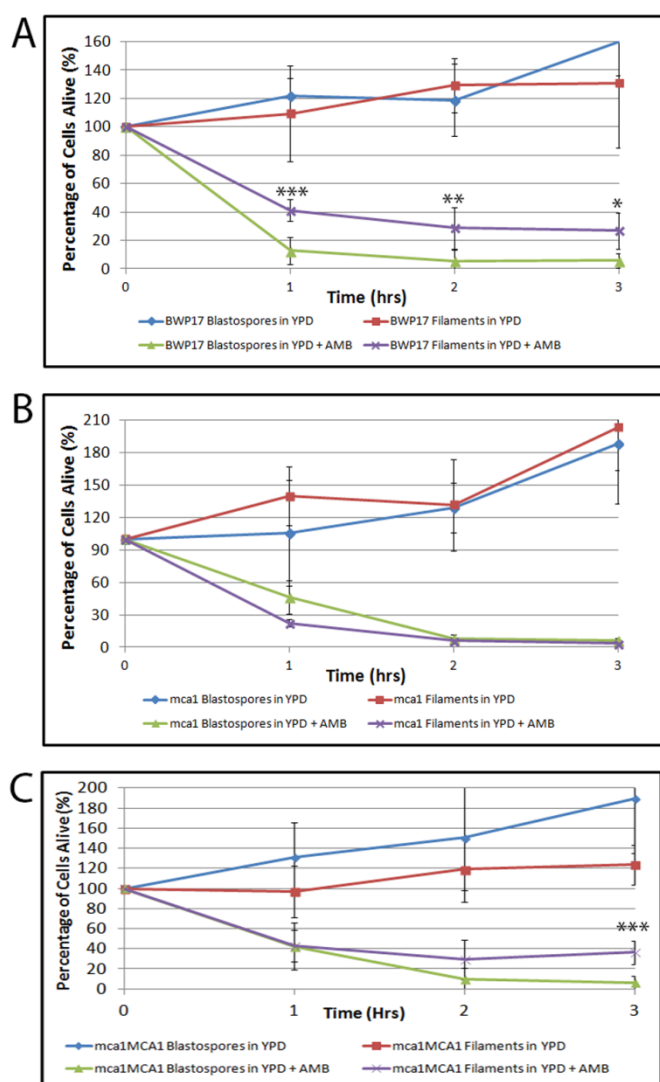


FIGURE 2: Filamentous *C. albicans* cells are more resistant than blastospores to AMB-induced programmed cell death in an *MCA1*-dependent manner. Viability curves compare survival of the following cells exposed to AMB: **(A)** wild type (BWP17) blastospores and BWP17 filaments induced using 10% FBS; **(B)** BWP17-derived $\Delta\Delta mca1/mca1$ blastospores and $\Delta\Delta mca1/mca1$ filaments induced using 10% FBS; and **(C)** $\Delta\Delta mca1/mca1::MCA1$ blastospores and $\Delta\Delta mca1/mca1::MCA1$ filaments induced using 10% FBS. Error bars indicate standard deviations for trials with at least three independent cultures. Note that after 3 hr, cells cultured in rich media without any drugs were able to grow and to divide, hence the relative viability levels that are greater than 100%. A single, double, and triple asterisk indicates statistical significance of $p < 0.05$, $p < 0.005$, and $p < 0.0005$, respectively, as compared to treated controls. Statistical significance was determined with the unpaired Student's t-test.

exposed to hyposomatic stress [32, 44]. We compared the survival rate of the wildtype BWP17 blastospores and filaments with their BWP17-derived $\Delta\Delta mca1/mca1$ mutant counterparts. Wildtype and all *mca1* mutants were able to undergo filamentation when exposed to 10% FBS (Supplemental Figure 2). As shown in Figure 2, $\Delta\Delta mca1/mca1$ blastospores and hyphal cells had indistinguishable survival rates when cultured in media containing AMB. This data suggests that *MCA1* is involved in the resistance of filamentous cells to AMB-induced programmed cell death. Complementation of the null $\Delta\Delta mca1/mca1$ mutant restored the original difference in viability that we had observed between blastospore and hyphal cells cultured in AMB-containing media, suggesting that the original $\Delta\Delta mca1/mca1$ phenotype could be linked to the original loss-of-function mutation in *MCA1*. In sum, our data suggests that filamentation protects *C. albicans* cells from AMB induced cell death and that this phenotype is dependent upon the yeast metacaspase, *MCA1*. Given that *MCA1* has previously been thought to have a pro-death function, it is not yet clear how Mca1p functions in this protective capacity in filamentous cells. However, it is intriguing that sever-

al recent papers have revealed that the Mca1p homolog has a non-death role in *S. cerevisiae* and possibly, in *C. albicans* as well [36, 45-49].

Finally, we wanted to determine if filamentation protected *Candida* cells from another anti-fungal drug known to induce programmed cell death. Thus, we compared the viability of blastospores and hyphal cells in media containing 0.05 $\mu\text{g/ml}$ caspofungin (CAS), an echinocandin known to trigger cell death, in *Candida albicans* [22, 23]. As shown in Figure 3, filamentation also appears to protect yeast cells from CAS-induced cell death suggesting the protective effects of filamentation may be a general phenomenon in *Candida albicans*. Watamoto *et al.* have proposed that filamentous *Candida* cells are resistant to AMB and to nystatin because they are able to form biofilms [17, 50]. In light of our findings, we also propose that planktonic hyphal cells may in themselves be relatively more resilient to these drugs—and possibly other anti-fungal drugs as well—because of their heightened resistance to programmed cell death.

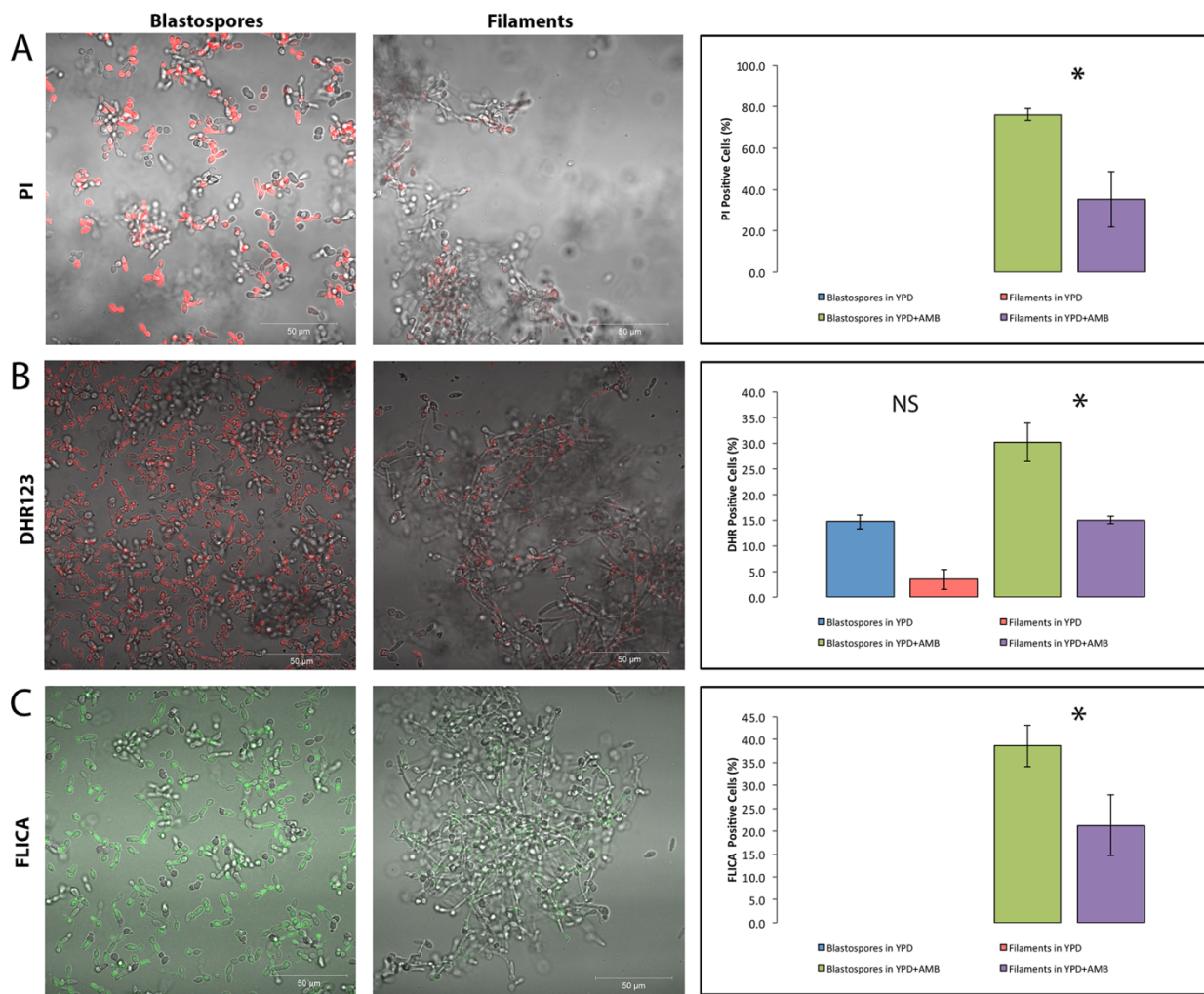


FIGURE 3: Filamentous *C. albicans* cells are more resistant than blastospores to caspofungin-induced programmed cell death. Representative confocal scanning laser fluorescence images of wild-type SC5314 *C. albicans* cells treated with 0.05 μg/ml caspofungin for 3 hours in YPD. Propidium iodide stains dead cells, dihydrorhodamine 123 (DHR123) indicates the presence of reactive oxygen species (ROS), and the FLICA assay stains for cells with activated intracellular caspases. No PI or FLICA positive cells were observed in the no drug controls. Error bars indicate standard deviations for trials with at least three independent cultures, where at least 300 cells were counted for each trial. A single asterisk indicates statistical significance ($p < 0.05$) as compared to treated controls. Statistical significance was determined with the unpaired Student's t-test.

MATERIALS AND METHODS

Media and Growth Conditions

C. albicans cells were grown in yeast extract/peptone/dextrose broth (YPD) made according to standard recipes [51]. Cells were inoculated from single colonies growing on YPD plates into 20 ml YPD and grown under shaking at 30°C until the culture attained an OD₆₀₀ value of 2.00 A. Once the culture had reached OD₆₀₀≈2.0 A, cells were harvested and then resuspended in fresh media at a concentration of 3x10⁷ cells/ml (OD₆₀₀≈1.26 A). For blastospore induction, cells from the original culture were resuspended in fresh YPD, transferred to a sterile flask, and then grown under shaking at 30°C for 3 hours. For hyphal induction, harvested cells were resuspended in either YPD + 10% fetal bovine serum (HyClone) pre-warmed to 37°C (YPD + FBS) or YPD + N-acetylglucosamine at a concentration of 0.5 g/l GlcNAc (Sigma-Aldrich;

YPD+GlcNAc), transferred into a fresh flask, and placed in an incubator with shaking at 37°C for 3 hours [11, 12, 42, 43, 52].

Viability Assays

Blastospores and hyphal cells were harvested and resuspended at a concentration of 1x10⁷ cells/ml in fresh YPD, and placed in 15 ml conical tubes. Cells were then exposed to AMB (Sigma) at a concentration of 5 μg/ml or 8 μg/ml (from a 1 mg/ml stock in dimethyl sulfoxide) for 3 hours, with shaking, at 25°C [24]. At t=0, 1, 2, and 3 hours of AMB exposure, serial dilutions of the cell cultures were done on YPD plates. The plates were then placed in a 30°C incubator for 24 to 48 hours, or until single colonies were distinguishable. Colonies for each time point were counted and then compared as a percentage of the number of colonies that formed on the t=0 plate. For each time point, three independent cultures were tested.

Notably, we confirmed our clonogenic assays by directly visualizing dead filamentous cells using propidium iodide (50 µg/ml) and then counting them with a Zeiss LSM700 fluorescent microscope. For the experiments with caspofungin, blastospores and filaments were cultured in the drug at a concentration of 0.05 µg/ml (from a 1 mg/ml stock in dimethyl sulfoxide) for 3 hours, with shaking, at 25°C. The viability of the cells was determined by culturing them in propidium iodide (50 µg/ml) and then counting them visually with a Zeiss LSM700 fluorescent microscope. Again, three independent cultures were tested, and at least 300 cells were counted for each determination. Statistical significance for all experiments was determined with the unpaired Student's t-test.

In Vivo Detection of ROS Accumulation and Caspase Activation

Intracellular ROS accumulation was examined after treatment with AMB or caspofungin using 5 µg/ml of dihydrorhodamine 123 (DH123; Sigma Aldrich) [24]. Activated caspases were detected in *C. albicans* cells after treatment with AMB or CAS using a FLICA apoptosis detection kit (ImmunoChemistry Technologies, LLC) according to the manufacturer's specifications [38]. After exposure to either DHR123 or the FLICA reagent, *C. albicans* cells were harvested and examined using a Zeiss 700 Confocal Laser Scanning Microscope.

SUPPLEMENTAL MATERIAL

All supplemental data for this article are available online at www.microbialcell.com.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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