Candida albicans White and Opaque Cells Undergo Distinct Programs of Filamentous Growth

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Abstract

The ability to switch between yeast and filamentous forms is central to Candida albicans biology. The yeast-hyphal transition is implicated in adherence, tissue invasion, biofilm formation, phagocyte escape, and pathogenesis. A second form of morphological plasticity in C. albicans involves epigenetic switching between white and opaque forms, and these two states exhibit marked differences in their ability to undergo filamentation. In particular, filamentous growth in white cells occurs in response to a number of environmental conditions, including serum, high temperature, neutral pH, and nutrient starvation, whereas none of these stimuli induce opaque filamentation. Significantly, however, we demonstrate that opaque cells can undergo efficient filamentation but do so in response to distinct environmental cues from those that elicit filamentous growth in white cells. Growth of opaque cells in several environments, including low phosphate medium and sorbitol medium, induced extensive filamentous growth, while white cells did not form filaments under these conditions. Furthermore, while white cell filamentation is often enhanced at elevated temperatures such as 37°C, opaque cell filamentation was optimal at 25°C and was inhibited by higher temperatures. Genetic dissection of the opaque filamentation pathway revealed overlapping regulation with the filamentous program in white cells, including key roles for the transcription factors EFG1, UME6, NRG1 and RFG1. Gene expression profiles of filamentous white and opaque cells were also compared and revealed only limited overlap between these programs, although UME6 was induced in both white and opaque cells consistent with its role as master regulator of filamentation. Taken together, these studies establish that a program of filamentation exists in opaque cells. Furthermore, this program regulates a distinct set of genes and is under different environmental controls from those operating in white cells.

Introduction

Morphological plasticity is key to the lifestyle of fungal pathogens such as Candida albicans, the most frequently isolated human fungal pathogen. The best-studied morphological switch in C. albicans is the transition between yeast and true hyphae or pseudohyphae (filamentous forms). Pseudohyphal cells are highly branched and consist of ellipsoidal cells with constrictions at the septa. In contrast, hyphal cells are less branched, have parallel sides, and lack constrictions at the septa [1,2]. The yeast-hyphal switch regulates C. albicans pathogenesis, as hyphal forms adhere to and invade epithelial cells during mucosal infections, resulting in extensive damage to host cells [2]. The switch to hyphae is also induced upon phagocytosis by macrophages, allowing pathogen evasion from immune capture [3,4]. Furthermore, the hyphal form is important for virulence in systemic models of disease, although it is not clear if the hyphal morphology per se or genes co-regulated with the morphological transition are critical for virulence [2].

The yeast-hyphal transition in C. albicans is induced in response to a wide variety of environmental stimuli including serum, neutral pH, nutrient limitation, high CO2 concentrations, and embedded conditions [2,5]. The transcriptional regulation of filamentation is complex, but many stimuli act via two major signaling pathways: a cyclic AMP-dependent pathway that depends on the Efg1 transcription factor, and a mitogen-activated protein kinase (MAPK) pathway that activates the Cph1 transcription factor [6,7]. In addition, most filamentation-inducing conditions require a temperature of 37°C (or higher) for efficient filamentous growth [2,5]. The temperature requirement appears to be mediated by Hsp90, as compromising Hsp90 activity promotes filamentation in response to serum at 30°C [8].

A second morphological switch involves the interconversion between white and opaque forms of C. albicans. This is an epigenetic switch that allows rapid and reversible switching between white cells that are round and opaque cells that are ellipsoidal [9]. Genes present at the Mating-Type Like (MTL) locus strictly regulate the white-opaque switch, so that only MTLα or MTLα strains can undergo the transition to the opaque form, while MTLα/α strains are permanently locked in the white state [10]. Opaque cells are the mating-competent form of C. albicans, undergoing mating approximately a million times more efficiently...
Author Summary

Candida albicans is the most common human fungal pathogen, capable of growing as a commensal organism or as an opportunistic pathogen. Perhaps the best-studied aspect of C. albicans biology is the transition between the single-celled yeast form and the multicellular filamentous form. This transition is necessary for virulence, as cells locked in either state are avirulent. Here, we demonstrate that the yeast-filament transition is tightly regulated by another morphological switch, the white-opaque phenotypic switch. White cells undergo filamentation in response to a wide range of established physiological cues, while opaque cells do not. We further show that opaque cells can indeed undergo filamentation, but that they do so in response to different environmental cues than those of white cells. We define the genetic regulation of filamentous growth in opaque cells, as well as the transcriptional profile of these cell types, and contrast them with the established program of filamentation in white cells. Our results reveal a close relationship between the white-opaque switch and the yeast-hyphal transition, and provide further evidence of the morphological plasticity of this pathogen. They also establish that epigenetic switching allows two fungal cell types with identical genomes to respond differently to environmental cues.

than white cells [10]. In addition to mating, the white-opaque switch also regulates multiple other facets of C. albicans biology. White cells are more virulent in systemic infections than opaque cells, while conversely opaque cells are better colonizers in skin infections than white cells [11,12]. The two cell states also interact differently with immune cells, as opaque cells are less susceptible to phagocytosis by macrophages [13]. It is therefore apparent that white and opaque cells are differentially programmed in many aspects of their behavior, including their interaction with the host.

In this work, we compare the ability of the two phenotypic states, white and opaque, to undergo filamentation. Studies on filamentation have almost exclusively utilized cells in the white state. In this form, cells are readily induced to form hyphae or pseudohyphae in response to diverse stimuli. In contrast, opaque cells do not undergo filamentation in response to these stimuli and typically grow only in the budding yeast form [14]. One indication that opaques may form filamentous growth in opaque cells, as well as the transcriptional profile of these cell types, and contrast them with the established program of filamentation in white cells. Our results reveal a close relationship between the white-opaque switch and the yeast-hyphal transition, and provide further evidence of the morphological plasticity of this pathogen. They also establish that epigenetic switching allows two fungal cell types with identical genomes to respond differently to environmental cues.

Materials and Methods

Media and reagents

Synthetic complete dextrose medium (SCD) and yeast extract peptone dextrose medium (YPD) were made as described previously [19]. YPD plates containing 200 µg/mL nourseothricin were used for selection of strains that were resistant to nourseothricin (Werner Bioagents, Jena, Germany) as previously described [20]. SCD low phosphate (LP) medium was made with yeast nutrient base (YNB) w/o phosphate (cat. #CYN6701, Formedium LTD, Histonstunton, England) and KH₂PO₄ was supplemented to a final concentration of 10 µM. LP medium was adjusted to pH 4.7 with 5 M HCl before autoclaving. Synthetic low ammonium dextrose (SLAD) medium was made as described previously [21]; 2% agar was washed five times with distilled water, autoclaved with 1.7 g/L YNB w/o ammonia, and then supplemented with ammonium sulfate and dextrose to final concentrations of 50 µM and 2%, respectively. Sorbitol medium (SOR) was SCD supplemented with 1 M sorbitol. Minimal (MIN) medium consisted of 7 g/L YNB and 2% dextrose. Spider medium contained 1.35% agar, 1% nutrient broth, 0.4% potassium phosphate, and 2% mannitol (pH 7.2). N-acetyl glucosamine containing medium (GlcNAc) was modified Lee's medium [22] without glucose but supplemented with 1.25% N-acetyl glucosamine (Sigma). Neutral pH medium was made by buffering SCD with 150 mM HEPES (pH 7.0). Calcofluor white stain was obtained from Fluuka Biochemika and geldanamycin from A.G. Scientific, Inc.

Plasmids and strains

C. albicans strains used in this study are listed in Table 1 and oligonucleotides in Table S1. All strains are derived from SC5314 unless stated otherwise. To construct the ACT1-WOR1 plasmid, the promoter of the ACT1 gene was PCR amplified with oligonucleotides 610 and 611 and the WOR1 ORF with oligonucleotides 2421 and 2422. The two fragments were then combined in a single fusion PCR reaction using oligos 610 and 2422 and cloned into plasmid pSFS2A [20] between ApaI and XhoI restriction sites. A modified pSFS2A plasmid was also constructed in which the SAT1 gene was replaced with the gene for hygromycin B resistance. A region of plasmid pSFS2A was PCR amplified with oligos 1669 and 1652 and the gene for hygromycin B resistance amplified from plasmid pYM70 [23] with oligos 1651/1653. The two PCR products were combined by fusion PCR using oligos 1669/1651 and cloned between HindIII and PstI restriction sites in the pSFS2A backbone. The resulting ACT1-WOR1 plasmids (either with SAT1 or HYG markers) were digested with BglII to linearize in the ACT1 region and integrated into the endogenous ACT1 locus to obtain constitutive WOR1 expression. Correct integration was confirmed by PCR.

To target RFG1 for deletion, a fusion PCR product was created as described previously [24]. Briefly, oligos 984/992 and 985/993 were used to PCR the 5' and 3' homologous flanks of RFG1 and these flanks combined with a selectable marker (LEU2) by fusion PCR [24]. The pSFS2 (g4l::SAT1 flipper) plasmid [25] (a gift from David Kadosh, University of Texas San Antonio) was used to suppressed filamentous growth. Genetic analysis and transcriptional profiling of filamentous opaque cells revealed both similarities and differences between the filamentation programs in opaque and white cells. These studies establish nutrient-controlled filamentous growth in C. albicans opaque cells and indicate fundamental differences between the environmental cues regulating filamentation in white and opaque cells.
**Table 1. Strains used in this study.**

| Strains | Genotype | Mating Type | Source |
|---------|----------|-------------|--------|
| RBY717  | MTLaMTLa* (white) | a/a | [67] |
| RBY731  | MTLaMTLa* (opaque) | a/a | [67] |
| CAY1550 | MTLa/MTLa::FRT tup1::hisG/tup1::p405-URA3 ura3/ura3 (white) | a/Δxs Derived from BCa2-10 | [41] |
| CAY1552 | MTLa/MTLa::FRT ngl1::hisG/rgng1::hisG-URA3-hisG ura3/ura3 (white) | Δ/xs Derived from BCa23-3 | [46] |
| CAY1571 | umec:LEU2/umec:His1 arg4:ARG4 arg4::ARG4 leu2/leu2 his1/1 pAct1-WOR1::SAT1-FLIP* (opaque) | 0/0 Derived from DK312 | [25] |
| CAY1616 | MTLa/MTLa::FRT tup1::hisG/tup1::p405-URA3 ura3/ura3 pAct1-WOR1::SAT1-FLIP (opaque) | a/Δxs Derived from BCa2-10 | [41] |
| CAY1618 | MTLa/MTLa::FRT ngl1::hisG/rgng1::hisG-URA3-hisG ura3/ura3 pAct1-WOR1::SAT1-FLIP (opaque) | 0/0 Derived from BCa23-3 | [46] |
| CAY2091 | MTLa/MTLa::pSAT1-FLIP cph2::LEU2/cph2::His1 arg4:ARG4 arg4::ARG4 leu2/leu2 his1/1 (white) | a/Δxs This study |
| CAY2214 | cph1::LEU2/cph1::FRT leu2/leu2 his1/1 arg4:ARG4* (white) | a/a This study |
| CAY2646 | tec1::FRT/tec1::FRT* (white) | a/a This study |
| CAY2688 | tec1::FRT/tec1::FRT* (opaque) | a/a This study |
| CAY2723 | ras1::LEU2/ras1::ARG4 leu2/leu2 his1/1 arg4:ARG4 gal1/gal1* (white) | a/a This study |
| CAY2795 | ras1::LEU2/ras1::ARG4 leu2/leu2 his1/1 arg4:ARG4 gal1/gal1* (opaque) | a/a This study |
| CAY2791 | cph1::LEU2/cph1::FRT cph1::His1 arg4:ARG4 leu2/leu2 his1/1 arg4:ARG4* (white) | a/a This study |
| CAY2822 | cph1::LEU2/cph1::FRT cph1::His1 arg4:ARG4 leu2/leu2 his1/1 arg4:ARG4 pAct1-WOR1::SAT1* (opaque) | a/a This study |
| CAY2903 | MTLa/MTLa::pAct1-WOR1::SAT1-FLIP* (opaque) | a/a This study |
| CAY3151 | cph1::LEU2/cph1::His1 arg4:ARG4 leu2/leu2 his1/1 arg4:ARG4 pAct1-WOR1::SAT1* (opaque) | a/a This study |
| CAY3292 | MTLa/MTLa::FRT efg1::LEU2/efg1::His1 arg4:ARG4 arg4::ARG4 leu2/leu2 his1/1 pAct1-WOR1::SAT1* (opaque) | a/Δxs This study |
| CAY3294 | MTLa/MTLa::FRT cph2::LEU2/cph2::His1 arg4:ARG4 arg4:ARG4 leu2/leu2 his1/1 pAct1-WOR1::SAT1* (opaque) | a/Δxs This study |
| CAY3296 | MTLa/MTLa::FRT cph2::LEU2/cph2::His1 arg4:ARG4 arg4:ARG4 leu2/leu2 his1/1 pAct1-WOR1::SAT1* (opaque) | a/Δxs This study |
| CAY3298 | cph1::LEU2/cph1::His1 arg4:ARG4 arg4::ARG4 leu2/leu2 his1/1 pAct1-WOR1::SAT1* (opaque) | a/a This study |
| CAY3329 | cph1::LEU2/cph1::His1 arg4:ARG4 arg4::ARG4 leu2/leu2 his1/1 pAct1-WOR1::SAT1* (opaque) | a/a This study |
| CAY3522 | cph1::LEU2/cph1::His1 arg4:ARG4 arg4:ARG4* (white) | a/Δxs This study |
| CAY3524 | cph1::LEU2/cph1::His1 arg4:ARG4 arg4:ARG4* leu2/leu2 his1/1* (white) | a/a This study |
| CAY3526 | cph1::LEU2/cph1::His1 arg4:ARG4 arg4:ARG4* leu2/leu2 his1/1 MTLa/MTLa::pSAT1-FLIP* (opaque) | a/a This study |
| CAY3528 | cph1::LEU2/cph1::His1 arg4:ARG4 arg4:ARG4* (white) | a/a This study |
| CAY3619 | pRAS1::GFP::RAS1 (opaque) | a/a This study |
| CAY3621 | pRAS1::GFP::RAS1::FLIP* (opaque) | a/a This study |
| CAY3697 | umec:LEU2/umec:His1/UM66:HYG arg4:ARG4 arg4::ARG4 leu2/leu2 his1/1 pAct1-WOR1::SAT1-FLIP* (opaque) | Δ/xs This study |
| CAY3749 | mlta1::HisG/MTLa ara3/ara3 pRAS1::GFP::RAS1 (white) | Δ/xs Derived from CHY257 | [10] |
| CAY3751 | MTLa/mlta1::HisG mth2::HisG ura3/ara3 pRAS1::GFP::RAS1 FRT* (white) | a/Δxs Derived from CHY439 | [10] |
| CAY4193 | hgc1::FRT/hgc1::FRT* (opaque) | a/a This study |
| CAY4197 | hgc1::FRT/hgc1::FRT::HGC1::SAT1* (opaque) | a/a This study |
| CAY4291 | MTLa/MTLa::FRT tup1::hisG/tup1::p405-URA3 pAct1-WOR1::HYG pPop4-GFP::SAT1 (opaque) | a/Δxs This study |
| CAY4353 | MTLa/MTLa::FRT tup1::hisG/tup1::p405-URA3 pPop4-GFP::SAT1 (white) | a/a This study |
| CAY4356 | MTLa/MTLa::FRT tup1::hisG/tup1::p405-URA3 pWH11-mCherry::SAT1 (white) | a/Δxs This study |
| CAY4479 | MTLa/MTLa::pSAT1 cph2::LEU2/cph2::His1 leu2/leu2 his1/1 arg4:ARG4* (white) | a/a This study |
| CAY4384 | MTLa/MTLa::FRT cph1::LEU2/cph1::His1 arg4:ARG4 leu2/leu2 his1/1 arg4:ARG4 pAct1-WOR1::SAT1* (opaque) | a/Δxs This study |
| CAY4492 | MTLa/MTLa::FRT tup1::hisG/tup1::p405-URA3 pAct1-WOR1::HYG pWH11-mCherry::SAT1 (opaque) | a/Δxs This study |
| CAY4502 | MTLa/MTLa::HYG/MTLa::His1/MTLa          | Δ/xs Derived from MBY208 | [68] |
| CAY4504 | His1/1::FRT tetR U666/FLP-CaNaT1 tetO-U666 (white) | Δ/xs Derived from MBY208 | [68] |
| CAY4522 | efg1::LEU2/efg1::His1 arg4:ARG4 leu2/leu2 his1/1* (white) | a/Δxs Derived from DK312 | [25] |

*strains also contain the genotype ura3::imm434/URA3 ino1::imm434/RIO1.

\[\text{hgc1 mutant strains constructed in the P37005 strain background of } C. \text{ albicans.}\]

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delete the second copy of RFG1 and generate rfg1::LEU2/ rfg1::MATI double deletion mutants, as previously described [25]. The MATI marker was subsequently excised by growth on maltose medium [20] to obtain strain CAY2214. Fusion PCR was also used to delete CPRH1 or EFG1 from CAY2214, using the selectable markers HIS1 and ARG4 to generate rfg1/APH1 and rfg1/esg1 double mutants. Oligos 902/990 and 903/991 were used to PCR amplify the 5’ and 3’ flanks of the CPRH1 gene, and oligos 1213/2425 and 1216/1217 used to amplify the 5’ and 3’ flanks of the EFG1 gene. The MTIz locus was also deleted from esg1, czf1 and opb2 mutant strains acquired from the Fungal Genetics Stock Center (strains originally generated by Homann et al. [26]) using the plasmid pRB102, as previously described [27]. The MATI marker was subsequently excised by growth on maltose medium [20] to generate MTIa/MTIz::FRT strains. Correct integration of constructs was verified by PCR across 5’ and 3’ disruption junctions, and loss of the ORF was confirmed with primers internal to the open reading frame. Auxotrophic strains were also transformed with C. albicans LEU2, HIS1, or ARG4, PCR amplified by oligos 2490/2491, 2492/2493 or 2494/2495, respectively, to restore prototrophy to these strains.

White- and opaque-specific reporter constructs were generated as follows. For the opaque-reporter, the SAT1 gene was PCR amplified from pSFS2A [20] using primers 169/170 and cloned into pCR-Blunt II-TOPO (Invitrogen) between XhoI and XbaI restriction sites. A triple mCherry reporter was next integrated into this vector by PCR amplifying three copies of the mCherry gene from plasmid pADH77 [28] with oligonucleotides 1846/1847, 1848/1849 and 1850/1851. The three mCherry PCR products were stitched together using BamHI/A/I, AFL/A/I and StuI/SalI restriction sites and integrated between BamHI and XhoI sites in the vector backbone to generate plasmid pRB224. The C. albicans OP4 promoter was then PCR amplified using oligos 1974/1975 and cloned between KpnI and SacI restriction sites in pRB224 to generate the final reporter construct pRB227. This plasmid was linearized in the OP4 gene with SgrI and transformed into C. albicans as an opaque-specific reporter. For the white-cell reporter, the C. albicans WH11 gene promoter was PCR amplified with oligos 1384/1396 and the GFP gene amplified from pADH77 [28] with oligos 1385/1386. These PCR products were fused by PCR with oligos 1384 and 1386, digested with Apal/SalI and cloned into pSFS2A to generate pRB168. This plasmid was linearized within the WH11 gene with AarI and transformed into C. albicans to generate a white-specific reporter.

An EFG1 complementation plasmid was constructed by PCR amplification of EFG1 using oligos 1839/1839, and cloning between ApaI and KpnI sites in the modified pSFS2A for hygromycin B resistance. The resulting EFG1 addback plasmid pRB326 was linearized by HpaIII and integrated into the endogenous EFG1 locus in the rfg1 mutant strain CAY3292. Similarly, oligos 1832/1833 were used to PCR amplify UME6 and the PCR product cloned between ApaI and KpnI sites in pSFS2A (hygromycin B) to generate plasmid pRB328. This plasmid was linearized by Smal and integrated into the endogenous UME6 locus in the opaque ume6 mutant strain CAY1571 to obtain strain CAY3697.

Construction of a UME6 overexpressing strain was achieved using derivatives of strain MBY208, a gift of David Kadosh. MBY208 contains a construct expressing high levels of an Eichhorstia calci tet repressor-Saccharomyces cerevisiae Hap4 activation domain fusion protein, as well as a second construct expressing the UME6 gene under the control of the E. calci tet operator [29]. The MTIz locus was deleted in MBY208 using a derivative of pRB102 (contains hygromycin B marker) to generate white (CAY4504) and opaque (CAY4502) strains.

RNA sample preparation
Single opaque or white colonies were inoculated into liquid LP and SOR media at room temperature. Cells were harvested by centrifugation after 12 hours (LP) or 16 hours (SOR), and pellets frozen in liquid nitrogen. Total RNA was extracted from cell pellets (8–10 OD) following the RiboPure Yeast Kit protocol (Applied Biosystems, Bedford, MA). RNA was treated with DNaseI (Applied Biosystems) to eliminate DNA contamination and re-extracted with phenol/chloroform. For quality control, RNA was analyzed using an Agilent 2100 Bioanalyzer to check RNA integrity.

Hybridization of cDNA to microarrays and data analysis
Aminoallyl-labeled cDNA synthesis and hybridization to microarrays was previously described by Tuch et al. [30]. Arrays were scanned on a GenePix 4000 scanner (Axon Instruments), data quantified using GENEPIX PRO version 3.0 and normalized using Goulphar [http://transcriptome.ens.fr/goulphar]. Pairwise average linkage clustering analysis was performed using CLUSTER and visualized by TREEXVIEW [31]. Significance Analysis of Microarrays (SAM, http://www-stat.stanford.edu/~tibs/SAM/) and R (Ver 2.15.2, http://www.r-project.org/) were used to screen the statistically significant genes induced in filamentous opaque cells in LP or SOR medium versus SCD medium (four replicates each). The parameters used for screening and SAM results are provided in the supplemental data (see tables in Text S1 and Text S2). The Candida genome database (www.candidagenome.org) and the Yeast Genome Database (http://www.yeastgenome.org/) were used to facilitate further analysis. Array data has been uploaded to GEO (accession number GSE42963).

Sample preparation and light microscopy
White and opaque cells were streaked on thin agar plates. After 22 hours of growth, a small square (~1 cm²) was cut from the plate and stained with calcofluor white. Digital images of cells were collected with Infinity analyzer software and an Infinity 2 digital camera (Lumenera Corporation, Ottawa, Canada). DIC and fluorescent images were collected with a Zeiss Inverted Microscope (Axio Observer. Z1) fitted with an AxioCam HR. Images were processed with AxioVision Rel. 4.8 (Zeiss, Germany).

Scanning electron microscopy
C. albicans opaque cells were grown on solid SOR or LP media at 25°C for 22 hours. C. albicans white cells were grown in liquid YPD + 10% serum at 37°C for 2 hours. Cells were resuspended in water and attached to poly-L-lysine coated-coverslips. Cells were fixed with 2.5% (w/v) glutaraldehyde in 0.1 M Na-cacodylate buffer, pH 7.4 at 4°C, and washed with 0.1 M Na-cacodylate buffer, pH 7.4. The cells were postfixed with 1% aqueous osmium tetroxide in 0.1 M Na-cacodylate buffer, pH 7.4 at 25°C for 90 minutes, and washed with 0.1 M Na-cacodylate buffer, pH 7.4. Following fixation, cells were dehydrated gradually using a gradient ethanol series and subsequently dried in a critical point dryer. The samples were then coated with 20 nm gold palladium (60:40) in an Emitech K550 sputter coater. Cells were imaged with a Hitachi S-2700 scanning electronic microscopy and collected with Quartz PCI software.

Results
Opaque cells do not undergo filamentation under conditions that induce hyphal growth in white cells
Filamentation of C. albicans white cells occurs in response to a wide variety of environmental stimuli, including neutral pH,
Filamentous Growth in \textit{C. albicans} Opaque Cells

Opaque cells undergo efficient filamentation in response to unique environmental cues

We next screened a series of \textit{in vitro} culture conditions to identify environments that induce robust filamentous growth in opaque cells. Opaque-locked strains (overexpressing \textit{WOR1}) or natural opaque strains were cultured on a variety of media, and colony and cell morphologies examined for evidence of filamentation. At least three distinct environmental cues were found to activate a program of opaque cell filamentation.

First, growth of opaque cells on medium containing the sugar sorbitol (synthetic complete dextrose medium supplemented with 1 M sorbitol; SOR medium) produced highly wrinkled colonies containing cells that were highly filamentous (Figure 2A). Filamentation was induced within 24 hours, and colony and cell morphologies are shown at 4 days of growth at 25°C. Staining of the cell walls with calcofluor white revealed that filaments consisted of cells with parallel sides and no constrictions at the septa, similar to true hyphae formed by white cells (Figure 2A).

Second, growth on minimal medium (MIN medium; synthetic medium which lacks amino acids) induced filamentous growth producing highly wrinkled colonies (Figure 2B). Colony phenotypes were even more marked in minimal medium lacking nitrogen (SLAD medium) or minimal medium containing low phosphate concentrations (LP medium), as these conditions induced extensive peripheral filamentation around the edges of the colonies (Figure 2C and D). Filamentation was induced within 24 hours and colonies are shown after 4 days of growth at 25°C. Filaments from MIN, SLAD, and LP resembled pseudohyphae rather than true hyphae, as cells were highly branched and elongated, with slight constrictions at each of the branch points (Figure 2B-D).

Third, growth of opaque cells in the presence of the carbon source N-acetyl glucosamine (GlcNAC medium; Lee’s medium containing 1.25% GlcNAC) induced efficient filamentation. Filamenting opaque cells resembled pseudohyphal cells with constrictions present at the septa (Figure 2E, cells shown after 24 hours and colonies after 4 days at 25°C). GlcNAC and SLAD have previously been reported to be activators of hyphal growth in white cells [37,38], but we found that this required extended incubation for 5 days or longer at 37°C. In fact, white cells did not undergo filamentation under any of the conditions that efficiently induce opaque cell filamentation (compare white and opaque cell and colony morphologies in Figure 2).

Filamentous phenotypes were similar when using either natural opaque cells or those locked into the opaque state by constitutive \textit{WOR1} expression (compare Figure 2 and Figure S1). In addition, removal of cells from the filamenting opaque colonies gave rise to regular opaque colonies when incubated on SCD medium (data not shown). This result indicates that opaque cells did not switch to the white state when grown under filament-inducing conditions but were stably maintained as opaque cells.

The results described above were achieved using culture on solid media, but filamentation was also observed in liquid culture. For example, growth of opaque cells in liquid SOR or LP medium was found to efficiently induce filamentous growth (Figure S2B and C). In contrast filamentation in MIN or SLAD media was considerably reduced compared to that on solid media (Figure S2D and E). We therefore establish that opaque cells can filament in response to different environmental cues, and can do so when grown both in liquid culture and on agar plates.

The structure of filamentous opaque cells was further analyzed by scanning electron microscopy and compared to that of hyphal white cells (Figure 3). These images confirmed that opaque cells grown on SOR medium resembled hyphal cells (parallel sides with no constrictions) while those grown on LP medium resembled pseudohyphal cells (constrictions between elongated buds). These results establish that opaque cells can undergo a program of filamentous growth including either pseudohyphal-like or hyphal-like cells. Furthermore, we show that the environmental cues regulating filamentation in white and opaque cells are distinct, with different cues inducing filamentous growth in the two phenotypic states.

Differential regulation of white/opaque filamentation by temperature

The yeast-hyphal switch in \textit{C. albicans} white cells is strongly influenced by temperature, with most filamentation-inducing conditions occurring at elevated temperatures (e.g. serum induction of hyphae is increased at 37°C compared to 30°C [8]), although white cells also undergo filamentation at 25°C in response to certain cues [39,40]. To determine if opaque cell filamentation is temperature-dependent, filament-inducing conditions were tested at 25°C, 30°C, and 37°C. Opaque filamentation was most efficient at 25°C, with decreased filamentation observed at 30°C, and even less filamentation at 37°C (Figure 4). We confirmed that the lack of filamentation at 37°C was not due to opaque cells switching back to white by using opaque-locked strains that have constitutive \textit{WOR1} expression. The thermal regulation of filamentation was similar when compared for SOR, MIN, SLAD and LP media, indicating that elevated temperatures generally inhibit opaque cell filamentation (Figure 4B-E). Again, white cells did not filament efficiently under any of the tested media conditions even when incubated at 37°C (Figure 4A-E), further demonstrating the specificity of the program of opaque filamentation.

The heat shock protein Hsp90 has been implicated as a key regulator of temperature sensing in \textit{C. albicans}. Elevated temperatures compromise Hsp90’s functional capacity and this is thought to promote filamentation of white cells [8]. Pharmacological inhibition of Hsp90 (e.g. using the drug geldanamycin) therefore stimulates filamentation in white cells [8]. We used geldanamycin (GdA) to test if loss of Hsp90 also affects filamentation in opaque cells. White and opaque cultures were treated with geldanamycin in liquid YPD medium at 25°C and 30°C. Inhibition of Hsp90 caused efficient induction of filamentation in white cells at 30°C,
Figure 1. Conditions inducing white cell filamentation do not induce opaque cell filamentation. Environmental cues that induce efficient filamentation in *C. albicans* white cells (RBY717) include serum (A), high temperature (B), Spider medium (C and F), neutral pH medium (D), and Lee’s medium (E). None of these environmental cues induce filamentous growth in opaque cells. Cell photographs were taken after 5 hours incubation at 37°C except for YPD supplemented with serum (2 hour incubation). Colonies grown on Spider medium were incubated at 30°C for 4 days. doi:10.1371/journal.ppat.1003210.g001
Figure 2. Novel environmental cues induce filamentation in *C. albicans* opaque cells. Several culture conditions are described that induce filamentous growth in opaque-locked *a* cells (CAY2903). These include growth on (A) sorbitol (SOR), (B) minimal (MIN), (C) low nitrogen (SLAD), (D) low phosphate (LP) or (E) N-acetyl glucosamine (GlcNAc) medium at 25°C. These culture conditions do not induce filamentation in white *a* cells (RBY717). Panels show colony morphologies from mixed white/opaque populations (solid arrow, white colony; dashed arrow, opaque colony). Additional panels show white cells, opaque cells, and calcofluor white (CW)-strained opaque cells.

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but only a very limited morphological response in opaque cells (Figure S3). In fact, opaque cells were more susceptible to geldanamycin treatment than white cells, with the drug causing cell death in a large proportion of the population. Surviving opaque cells exhibited mixed morphologies, indicating that Hsp90 does not play as dominant a role in regulating opaque cell filamentation as it does in white cells.

The role of MAPK and cAMP signal transduction pathways in opaque filamentation

Multiple signaling pathways regulate filamentation in *C. albicans* white cells, including the MAPK pathway and the cAMP pathway that mediate filamentation in response to serum, temperature, CO2, and starvation [2,5]. The *C. albicans* MAPK pathway consists of a series of conserved kinases including Cst20, Hst7, and Cek1 that are homologous to *S. cerevisiae* Ste20, Ste7, and Kss1, respectively. The terminal Cek1 kinase activates Cph1, a transcription factor (homologous to *S. cerevisiae* Ste12) responsible for inducing filamentous growth [6]. The cAMP pathway is similarly activated in response to multiple environmental cues and mediates filamentation via the transcription factor, Efg1 [7]. Together, Cph1 and Efg1 can be regarded as master regulators of *C. albicans* filamentation, and mutants lacking both Cph1 and Efg1 are highly defective in filamentation in white cells [41].

To evaluate the role of the MAPK and cAMP pathways in opaque filamentation, we constructed *MTLα/α Δcph1/Δcph1* and *Δefg1/Δefg1* mutants to analyze MAPK and cAMP signaling, respectively. Construction in an *MTL* homozygous strain was
Figure 4. Differential thermal regulation of filamentous growth in white and opaque cells. White (RBY717) and opaque (CAY2903) cells were cultured on SCD or filamentation-inducing media (SOR, MIN, SLAD, or LP) at 25°C, 30°C, or 37°C for 4 days. Opaque filamentation was optimal in the order 25°C > 30°C > 37°C. In contrast, white cell filamentation increased as temperatures increased, as evidenced by weak filamentation of white colonies on SCD medium at 37°C (top panels). (A) SCD, (B) SOR, (C), MIN, (D), SLAD, or (E) LP medium.

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necessary to allow for strains to switch between white and opaque. Loss of Cph1 had little, if any, effect on opaque filamentation under any of the inducing conditions (SOR, MIN, LP, or GlcNAc media), indicating that the MAPK pathway does not play a major role in opaque filamentation. As shown in Figure 5, opaque \( \Delta cph1/\Delta cph1 \) colonies appeared similar to wildtype colonies; they exhibited extensive peripheral filamentation and consisted of pseudohyphal or hyphal cells, depending on the inducing medium. We also found that deletion of \( CPH1 \) had only a very modest effect on filamentation phenotypes in white cells (either \( MTL^a/\alpha \) or \( MTL^a/\alpha \) strains, see Figure S4), indicating that the MAPK pathway plays a relatively minor role in regulating filamentation compared to that of the cAMP pathway, consistent with previous observations [41].

The role of \( EFG1 \) in white and opaque cell filamentation was also addressed. We note that loss of \( EFG1 \) has been shown to increase switching from white to opaque [42–44], and that switching in \( \epsilon fg\) mutants is regulated by pH [45]. We observed distinct white and opaque \( \epsilon fg\) colonies on SOR, SLAD, and LP media, and loss of Efg1 resulted in decreased opaque cell filamentation under each of these culture conditions. For example, \( \Delta \epsilon fg1/\Delta \epsilon fg1 \) mutants were unable to form hyphal-like cells when grown on SOR medium and instead grew as chains of opaque cells (Figure 5A). Similarly, whereas wildtype opaque cells formed highly elongated pseudohyphae when grown on SLAD or LP media, \( \epsilon fg1 \) mutants formed chains of normal looking opaque cells on these media (Figure 5B and C). \( \epsilon fg1/\epsilon fg1 \) mutants also produced smoother colonies compared to filamentous wildtype colonies (Figure 5A–C). Reintegration of the \( EFG1 \) gene into the mutant background restored filamentation, confirming that the mutant phenotype was due to the loss of this gene (Figure 5A–C).

Loss of Efg1 was also shown to inhibit filamentation in white \( MTL^a \) cells (Figure S4), consistent with its role in white \( a/\alpha \) cells [7]. These results establish that Efg1 is a master regulator of filamentation in both \( C. albicans \) white and opaque cells.

The role of negative transcriptional regulators in white and opaque filamentation

Several negative regulators of filamentation play prominent roles in controlling the yeast-hyphal transition in white cells. These include the transcription factors Nrg1 and Rfg1, as well as the global repressor of gene transcription, Tup1. Loss of any one of these regulators results in white cells growing as hyphae or pseudohyphae under conditions that normally support yeast cell growth [36,46–49]. Somewhat paradoxically, \( \epsilon fg1 \) mutants display a defect in hyphae formation under nutrient-limiting conditions [48], while overexpression of \( RFG1 \) promotes pseudohyphal growth [50]. We constructed mutants in each of these factors in switching-competent (\( MTL^a \) homozygous) strains to define their role in opaque filamentation.

As shown in Figure 6, loss of Nrg1 or Tup1 resulted in filamentation of opaque cells even when cultured on SCD medium, a medium that normally does not induce filamentous growth. Wor1 was constitutively expressed in these cells to drive formation of opaque cells, as \( \epsilon fg1 \) mutants do not undergo stochastic switching to the opaque state, at least in the WO-1 strain.
Opaque colonies were extremely wrinkled in \textit{nrg1} and \textit{tup1} mutant strains, and consisted mostly of elongated pseudohyphal-like cells with constrictions at sites of cell division (Figure 6A and B). Since the highly filamentous phenotype of the \textit{tup1} mutant is similar between white and opaque cells, we also constructed fluorescence reporters to confirm the phenotypic state of these cells. \textit{pOP4-GFP} and \textit{pWh11-mCherry} constructs were employed to indicate the opaque or white state, respectively. As expected, the \textit{pOP4-GFP} signal was higher in opaque \textit{tup1} cells than in white cells, although basal \textit{OP4} expression was evident in white cells (Figure S5B), consistent with previous observations [51]. Conversely, the \textit{pWh11-mCherry} reporter was expressed in...
white cells but not opaque cells (Figure S3A). These results establish that opaque tuf1 mutants are maintained in the opaque state and are as filamentous as white tuf1 mutants.

In contrast to Ng1 and Tup1, loss of Rfg1 reduced filamentation in opaque cells under the conditions tested. For example, growth of tfg1 mutants on SOR, SLAD or LP media produced very weak filamentation compared to the wildtype strain (Figure 6C–E). In general, tfg1 mutants grew as chains of opaque cells but cell shape was no longer extremely elongated and was reminiscent of the phenotype of tfg1 mutants (compare Figure 6C–E and Figure 5A–C). We further examined the role of Rfg1 in the context of Cph1 and Ef1g that act in the MAPK and cAMP pathways, respectively. Opaque Δcph1/Δcph1 Δefg1/Δefg1 double mutants exhibited a similar phenotype to tfg1 mutants, indicating that Cph1 has no effect on opaque filamentation in the tfg1 background (Figure 6C–E). In addition, opaque ef1g/ef1g double mutants were analyzed, as both ef1g and tfg1 mutants exhibit reduced filamentation. It appeared that ef1g/ef1g opaque cells resembled ef1g and tfg1 single mutants, growing as chains of cells, and that some filamentation was still evident even in the absence of both transcription factors (Figure 6C–E).

Together, these results establish roles for Ng1, Tup1, and Rfg1 in both white and opaque filamentation programs. Loss of Ng1 or Tup1 transcriptional repressors results in activation of the program of filamentous growth in white and opaque cells. However, whereas Rfg1 is both a positive and negative regulator of filamentation in white cells depending on the conditions, we observe only a positive role for Rfg1 in promoting opaque cell filamentation.

Analysis of the Ume6-Hgc1 pathway in opaque filamentation

Recent studies have uncovered Ume6 as a key transcriptional regulator of hyphal and pseudohyphal growth in C. albicans white cells. In particular, it was shown that high levels of UME6 expression drive hyphal formation (and increase virulence), whereas intermediate levels of UME6 expression resulted in pseudohyphal growth [29]. The cyclin-related protein Hgc1 is an important downstream target of Ume6 and mediates agar invasion, hyphal extension, and formation of true septa [53]. Hgc1 functions as part of a Hgc1/Cdc28 complex that promotes filamentation by phosphorylating Rga2, a Cdc42 GAP protein, which in turn activates Cdc42 and drives actin polymerization [54]. In addition, both UME6 and HGCI are negatively regulated by the Ng1 and Tup1 transcription factors discussed above [25,55]. Mutants in UME6 and HGCI are constructed and tested in MTT/α strain backgrounds to distinguish their contribution to opaque filamentation phenotypes.

Deletion of UME6 resulted in a marked decrease in opaque cell filamentation under each of the tested media conditions. Thus, growth of opaque ume6 mutants on SOR, LP, or MIN media generated chains of cells but cells were no longer highly elongated and no hyphal-like cells were observed. In fact, opaque ume6 cells resembled those of tfg1 and tfg1 mutants (compare Figure 7 and Figure 6C–D). These results show strong parallels between white and opaque cells, as deletion of UME6 also compromises white cell filamentation [25,55].

In white cells, UME6 overexpression also drives cells into the hyphal form [29]. We therefore addressed whether UME6 overexpression is sufficient to induce opaque cell filamentation by using a strain in which UME6 is under the control of the E. coli tet operon (tetO). The constructed strain also expresses an E. coli tet repressor–Saccharomyces cerevisiae Hap4 activation domain fusion protein. As a result, the UME6 gene is turned off in the presence of doxycycline, but is highly induced in the absence of doxycycline [29]. We found that overexpression of UME6 induced filamentous growth in both white and opaque cells, establishing UME6 as a master regulator of filamentation in both phenotypic states (Figure S6).

The role of HGCI in opaque cell filamentation was also examined. These mutants were constructed in the P37005 background of C. albicans that is a natural MTI/α isolate. The wildtype P37005 opaque cells underwent filamentation in response to inducing conditions, including on SOR and LP media. In contrast, P37005 Δhgc1/Δhgc1 mutants showed a marked defect in opaque filamentation, as these strains were unable to form filaments when grown on SOR or LP media (Figure 7E and F). The defect in hgc1 mutant filamentation was restored by complementation with the wildtype HGCI gene (Figure 7E and F). Together, these results indicate a shared role for UME6 and HGCI in promoting filamentation in both white and opaque cells.

Analysis of additional white cell filamentation regulators for roles in opaque cell filamentation

The Ras1 protein plays a prominent role in white cell filamentation and acts upstream of both the MAPK and cAMP pathways. Mutants in ras1 show a severe defect in hyphal growth in white cells under multiple conditions, while strains expressing a dominant active Ras1 mutation (Ras1G13V) show enhanced hyphal formation [56]. Here, we tested the phenotype of Δras1/Δras1 mutants in opaque cells and observed a defect in opaque filamentation on SOR and LP media (Figure S7), consistent with cAMP signaling being necessary for filamentous growth (compare to the ef1g mutant phenotype, Figure 5). Curiously, expression of the constitutively active Ras1G13V allele also partially suppressed filamentation on LP and SOR media (Figure S7). Thus, either loss of RAS1 function or hyperactive Ras1 activity appears to decrease filamentation of opaque cells.

Other pathways that regulate white cell filamentation include the Cph2/Tec1 pathway. Both of these factors are transcription factors and Cph2 is necessary for Tec1 expression, which in turn upregulates genes involved in hyphal development [57,58]. Mutants in cph2 and tec1 were examined in the opaque phase but these mutants had no significant effect on filamentous growth under any of the tested conditions (Figure S8).

White cells are also induced to undergo filamentation when embedded in soft agar, and this program is dependent on the Czf1 transcription factor [59]. Opaque cells similarly underwent increased filamentation when cultured under embedded conditions (data not shown). Embedded conditions therefore represent an environmental cue that is conducive to inducing filamentation in both white and opaque cells. Interestingly, embedded growth is also one of the few filament-inducing conditions that is effective at 25°C for white cells [59]. As Czf1 promotes white filamentation under embedded conditions, opaque czf1 mutants were analyzed. This required overexpression of WOR1 to generate opaque cells, as czf1 mutants exhibit very low rates of white-to-opaque switching [44]. Opaque czf1 mutants produced an unusual ‘hyper-branching’ phenotype when grown on the surface on several media, including SLAD, LP and SOR medium (Figure S9). czf1 mutant opaque cells grew as highly branched chains that were clearly distinguishable from all other filamentation phenotypes. Czf1 is therefore a regulator of filamentation in opaque cells, even when grown under non-embedded conditions.

Transcriptional profiling of filamentous opaque cells

Gene expression of filamentous white cells has been defined during growth in serum medium at 37°C [36,60]. We set out to
Comparison of filamenting and non-filamenting opaque cells revealed that 1188 genes were differentially expressed (by SAM) between LP and SCD medium, while 341 genes were differentially regulated between SOR and SCD medium. More specifically, 445 genes were induced and 743 genes repressed (>3-fold) in LP medium, while 143 genes were induced and 198 genes repressed in SOR medium. Presumably, these expression changes include many genes that are regulated by the nutritional change, as well as genes that directly mediate the transition from yeast to filamentous growth.

Comparison of gene expression profiles revealed that a core set of 48 genes was induced during filamentous growth of opaque cells in both SOR and LP medium (see Table S2). These genes were compared to those induced during white cell filamentation in serum at 37°C (Figure 8). In general, overlap between white and opaque filamentation profiles was limited, with most genes specific to one program or the other. Thus, many of the genes characteristic of hyphal formation in white cells, including ALS3, HYY1, PHR1, and SAP5, were not induced in filamentous opaque cells (Figure 8B). In fact, of the 55 genes induced in white cell hyphae, only 11 were induced in filamentous opaque cells in LP and SOR media. Significantly, one gene that was highly induced during both white and opaque filamentation was the key transcriptional regulator UME6. This result is consistent with the requirement for this factor for filamentous growth in both white and opaque cells.

In general, most hyphal-specific genes in white cells were not induced in filamenting opaque cells, and conversely opaque cells expressed filamentation genes not induced in white cells. In fact, several genes induced during opaque filamentation were repressed in white hyphal cells, including MVN22, OSM2, PCK1, and SAP98 (Figure 8B), while data for a number of opaque filamentation genes was not present in the white expression data set. Several opaque-specific filamentation genes are of interest, including PGA430 and PGA431 that encode putative GPI-anchored cell wall proteins. Upregulation of these genes suggests that filamentous opaque cells could exhibit altered adherence compared to yeast cells. A similar phenomenon has been observed in white cells, where expression of hyphal-specific surface proteins (e.g. ALS3, ALS10, ECE1, and HWP1) mediates increased adhesion of hyphae to host cells and promotes biofilm formation [61,62]. We note that HWP1 expression was increased in both white and opaque filamentous cells (Figure 8B) and was also induced in opaque cells forming polarized mating projections [16], consistent with the model that HWP1 expression is directly regulated by actin dynamics during the morphological transition from yeast to polarized growth [63].

Together, these analyses reveal that the gene expression profiles of filamentous white and opaque cells are distinct, with only a limited number of factors induced in both programs of filamentation. However, the induction of UME6 in both gene profiles is consistent with a key role for this transcription factor in regulating filamentation in both white and opaque cell types.

Discussion

C. albicans is multimorphic – it grows in a variety of morphological forms and the ability to switch between these forms underlies its ability to colonize and infect diverse niches in the mammalian host. Here, we address the relationship between two distinct forms of phenotypic plasticity; white-opaque switching and the transition between yeast and filamentous forms. We report that white and opaque cells can both undergo filamentation but do so in response to distinct environmental cues. Furthermore, we define the transcriptional profile of filamentous opaque cells and reveal marked differences with genes regulated by the yeast-hyphal transition in white cells. We also compare and contrast the signal transduction pathways that regulate filamentation in C. albicans white and opaque cell types.

Differential regulation of filamentous growth in C. albicans white and opaque cells

Our results demonstrate that the white-opaque switch plays a key role in regulating the yeast-hyphal transition in C. albicans. Thus, two of the best-studied programs regulating morphogenesis in C. albicans are interconnected, indicating overlap of the regulatory mechanisms involved in these programs. We show that opaque cells can form filamentous cells and do so in response to different environmental cues than those that induce filamentous growth in white cells. Thus, whereas serum, neutral pH, nutrient deprivation and high temperature are signals that induce filamentation in white cells, these stimuli do not induce filamentation in opaque cells (Figure 1). In contrast, opaque cells undergo filamentation in response to distinct cues, including sorbitol or low phosphate medium that do not induce filamentous growth in white cells (Figure 2). These results establish that white and opaque states are differentially programmed with respect to the integration of environmental signals for filamentous growth.

Genetic regulation of opaque filamentation

The regulation of filamentous growth in C. albicans white cells has been the subject of extensive studies [reviewed in [2,5]]. We examined whether the regulatory pathways controlling white cell filamentation, including MAPK and cAMP pathways, also function to regulate opaque cell filamentation. We show that...
Efg1, the terminal transcription factor in the cAMP pathway, and Ume6, a transcription factor that acts downstream of Efg1 [29,53,55], are key regulators of filamentation in opaque cells. Furthermore, overexpression of UME6 was sufficient to induce opaque cell filamentation, as it is in white cells [29]. We also observed roles for the positive regulator Hgc1 and the negative regulator.
regulators Nrg1-Tup1 in opaque cell filamentation, similar to their established roles in white cell filamentation. Filamentous growth in white cells can be either positively or negatively regulated by Rfg1 depending on the conditions [48], and we found that rfg1 mutants were defective for filamentation in opaque cells. Thus, this transcription factor also plays a role in regulating filamentation in both white and opaque cells.

We also examined several other regulators of white cell filamentation for potential roles in opaque cell filamentation. Cph1, the master regulator of the MAPK pathway, did not influence opaque cell filamentation and we also observed that this factor has only a subtle role in white cell filamentation in the wildtype strain background. Similarly, Cph2 and Tec1 did not affect opaque filamentation, while deletion of Czf1 resulted in a hyperbranching phenotype specifically in opaque cells at 25°C. Taken together, our findings indicate that white and opaque filamentation occurs in response to different environmental stimuli, and generates different transcriptional responses (discussed below), but that genetic regulation of these programs involves many of the same signaling pathways in both phenotypic states (see model in Figure 9).

Contrasting temperature regulation of filamentation in white and opaque cells

Filamentous growth in white cells is often dependent upon elevated temperatures, with several hyphal-inducing conditions requiring a temperature of 37°C for efficient filamentation [2,5]. We found that filamentous growth in opaque cells exhibited the opposite dependence on temperature; opaque cells underwent filamentation efficiently at 25°C while filamentation was reduced at 30°C or 37°C. This was not due to opaque cells switching back to the white form at 37°C, as opaque cells were stably maintained by constitutive expression of WOR1, the master regulator of the opaque state [33–35]. In white cells, the molecular chaperone Hsp90 has recently been implicated in thermal regulation of the yeast-hyphal transition, as raised temperatures compromised Hsp90-mediated repression of hyphal formation [8]. In contrast to white cells, we found that inhibition of Hsp90 failed to elicit a large-scale change in morphology, although this could be due, at least in part, to the increased sensitivity of opaque cells to geldanamycin. These results reveal that elevated temperatures and/or decreased Hsp90 activity typically promote hyphal growth in white cells, while lower temperatures promote filamentation in opaque cells. Our findings have direct implications for pathogenicity by white/opaque cells, as they are likely to filament in different niches in response to different environmental cues, as discussed below.

Polarized growth and opaque filamentation

While this study revealed a novel program of filamentous growth in opaque cells, it is noted that polarized growth in opaque cells is not a new phenomenon. Opaque cells are the mating-competent form of C. albicans and respond to pheromones by forming mating projections that can be many times the length of the original opaque cell [16,17]. Furthermore, opaque mating projections contain a Spitzenkörper-like structure in which the myosin regulatory light chain protein, Mlc1, localizes to a characteristic ball at the growing tip of the cell [18,64]. The fact that opaque cells can form a Spitzenkörper indicates that these cell types undergo highly polarized growth by a mechanism similar to that in truly filamentous fungi. The discovery of environmental conditions that induces opaque filamentation now opens the door for further exploration of the regulation of opaque filamentation.

Comparison of white and opaque gene expression during filamentation

Gene expression profiles of filamenting white and opaque cells were distinct, with only 11 genes induced in both programs, while a further 44 genes were induced specifically in white hyphal cells and 37 genes induced only in opaque filamentous cells (Figure 8). One gene that was highly induced in both white and opaque states was the transcription factor UME6. As discussed above, UME6 is a critical regulator of filamentous growth in opaque cells, similar to its established role in white cells, and expression profiling confirms that this gene is co-regulated by morphogenesis in both cell types.

Figure 9. Regulation of filamentous growth in C. albicans white and opaque cells. The central programs regulating filamentation in white cells are shown, including the two core pathways of MAPK and cAMP-PKA signaling. White cell filamentation is also negatively regulated by the transcription factors Tup1 and Nrg1, and positively regulated by Ume6 in concert with the cyclin-like protein Hgc1. Filamentation in opaque cells is regulated by many of the same signaling pathways. In particular, Efg1 and Ume6 appear to be master regulators of filamentous growth in both white and opaque cells. However, there was no detectable role for MAPK signaling through Cph1, or for signaling via Cph2/Tec1, under the conditions used for inducing opaque filamentation.

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Interestingly, comparison of white-opaque regulated genes reveals that several regulators of filamentation are differentially expressed between white and opaque cells. For example, UME6, HGCl, NRG1, and EFG1, are white-opaque regulated genes; the first two genes are expressed at significantly higher levels in opaque cells, while the latter two genes show elevated expression in white cells (see Figure S10). EFG1 is itself a master regulator both of the white-opaque phenotypic switch and of filamentous growth [3,43,44]. It is therefore apparent that the transcriptional regulation of white/opaque phenotypes and that of filamentous growth are highly integrated, as previously suggested [65]. Presumably, the differential expression of these key regulators is at least partially responsible for the different propensities of white and opaque cells to filament in response to different environmental cues.

The role of white and opaque filamentation during infection

The yeast-hyphal transition is critical for infection by C. albicans white cells, where hyphae are more adherent and invasive than yeast-form cells. Despite this, questions remain as to the exact role of the hyphal structure during infection, and whether it is the genes that are co-regulated with the morphological switch that are critical for virulence or the hyphal structure itself [2]. In contrast, little is known about the potential for opaque cell filaments to promote pathogenesis. Early studies indicated that opaque cells are more effective at causing skin infections than white cells, and that opaque filamentation was induced on human skin epithelium [11,12,15]. Mating between opaque cells has also been shown to occur in this niche, indicating that the skin may represent a natural site for opaque colonization [66].

It is also possible that opaque filamentation occurs in other environmental niches, including those not associated with colonization and infection of the mammalian host. Future studies will examine both in vitro and in vivo conditions to determine those capable of inducing the program of filamentation in opaque cells. It is therefore an open question as to the role of the filamentous program in C. albicans opaque cells in infection and disease, and whether this program parallels that in white cells in promoting tissue destruction and pathogenesis.

Supporting Information

Figure S1 Natural opaque cells undergo filamentation in response to environmental cues. Culture conditions induce filamentous growth in wildtype opaque cells as well as in opaque-locked cells (see Figure 2). Strain RBY731 (opaque form of strain RBY717) was grown on (A) SCD, (B) low phosphate (LP), (C) low nitrogen (SLAD), or (D) sorbitol (SOR) medium at 25°C. These culture conditions do not induce filamentation in white cells. Panels show colony morphologies from mixed white/opaque populations (solid arrow, white colonies; dashed arrow, opaque colonies) after 4 days growth. Additional panels show DIC images of white and opaque cells, as well as calcofluor white (CW)-stained cells after 22 hours growth. Scale bar, 10 μm. (TIF)

Figure S2 Opaque cells undergo filamentation in liquid culture media. Opaque cells (strain CAY2903) undergo filamentation when cultured in liquid media, in addition to growth on solid media (Figure 2). Images were taken after 16 hours incubation at 25°C. (A) SCD, (B) LP, (C) MIN, and (D) SLAD medium. Opaque cell filamentation is strongest in liquid SOR and LP media. (TIF)

Figure S3 Contrasting Hsp90-mediated regulation of morphogenesis in white and opaque cells. White cells (RBY717) treated with the Hsp90 inhibitor geldanamycin (GdA) were induced to undergo filamentous growth at 30°C, but not 25°C. In contrast, opaque cells (CAY2905) did not undergo efficient filamentation when incubated with GdA at either temperature. Cells were grown at 25°C (A and C) or 30°C (B and D) and treated with 10 μM GdA for 12 hours. (TIF)

Figure S4 Comparison of cph1 and efg1 mutant phenotypes in white MTLα/a and MTLα strains. All strains were grown on Spider medium at 30°C for 4 days and photographed. (A) Wildtype white a/a, (B) wildtype white a/a strain (RBY717), (C) cph1 white a/a strain (CN7241), (D) cph1 white a/Δα2 strain (CAY1479), (E) efg1 white a/a strain (CAY4522), (F) efg1 white a/Δα2 strain (CAY3526). In both MTL heterozygous and MTL homozygous strains the cph1 mutant had a subtle defect in filamentation while the efg1 mutant had a marked defect in filamentation. (TIF)

Figure S5 Deletion of TUP1 induces filamentous growth in both white and opaque cells. The role of the transcription factor Tup1 was tested in both white and opaque programs of filamentous growth using fluorescent reporters to confirm the phenotypic state of the cell. (A) White tup1 mutants expressing a white-specific reporter (pWHI1-mCherry) or an opaque-specific reporter (pOP4-GFP) in strains CAY4356 or CAY4353, respectively. Strong expression of the WHI1 gene (high mCherry levels) confirms that white tup1 mutants are undergoing filamentation. (B) Opaque tup1 mutants expressing white and opaque reporter constructs in strains CAY4492 and CAY4491. Strong expression of the opaque-specific OP4 reporter confirms that tup1 mutants are propagating in the opaque state and undergoing constitutive filamentous growth similar to that of white cells. Cells were grown for 16 h in SCD medium and photographed. Scale bar, 10 μm. (TIF)

Figure S6 Induction of UME6 expression induces filamentation in both white and opaque cells. The UME6 gene was placed under the control of the tetO operator in a strain expressing the E. coli tet repressor - S. cerevisiae Hap4 activation domain fusion protein. In the presence of Dox (doxycycline) the UME6 gene is repressed (A and C), while in the absence of Dox the UME6 gene is induced (B and D). In both white (CAY4504) and opaque (CAY4502) cells filamentous growth occurred when grown on YPD without doxycycline. Colonies were grown for 6 days at 25°C. Scale bar, 10 μm. (TIF)

Figure S7 Deletion or overexpression of RAS1 leads to a defect in opaque filamentation. Wildtype cells (expressing a Ras1-GFP fusion protein), Δras1/Δras1 mutants, or cells expressing a constitutively active Ras1 allele (G13V) were compared for their ability to undergo filamentation in the opaque state. Both ras1 mutants and strains expressing hyperactive Ras1 alleles showed decreased filamentation on LP and SOR medium relative to the control strain. Strains were incubated on media for 4 days at 25°C. Strains used were wildtype white cells (CAY3749), opaque cells (CAY3619), ras1 white cells (CAY2723), ras1 opaque cells (CAY2795), and constitutively active Ras1 white cells (CAY3751) and opaque cells (CAY3621). (TIF)

Figure S8 Analysis of the role of Cph2 and Tec1 in opaque filamentation. Mutants lacking (A) Tec1 and (B) Cph2...
were analyzed for opaque filamentation phenotypes. Neither of these factors appeared to play a significant role in filamentous growth in opaque cells. Strains were incubated on media for 22 hours (cell images) or 4 days (colony images) at 25°C. Cpf2 mutants used were CAY2091 (white cells) and CAY3296 (opaque cells). Tcz1 mutants used were CAY2646 (white cells) and CAY2688 (opaque cells). Solid arrow, white cells; dashed arrow, opaque cells. Scale bar, 10 μm.

Figure S9 Analysis of the role of Czf1 in opaque filamentation. Opaque cells (CAY3294) lacking the transcription factor Czf1 were found to exhibit a hyper-branching phenotype when grown on LP, SLAD and SOR medium. In contrast, white czf1 mutants (CAY3522) did not exhibit this phenotype. Solid arrow, white colonies; dashed arrow, opaque colonies. Cells were imaged after 22 h and colonies after 4 d at 25°C.

Fold change in expression is shown in parentheses. Data adapted from RNA-seq analysis in Tuch et al. [50].

Table S1 Oligonucleotides used in this study. (DOCX)

Table S2 Genes induced during filamentous growth in opaque cells. Table shows genes induced during filamentous growth of opaque cells in both LP and SOR media. (XLSX)

Text S1 SAM_for LP op v. SCD op. File includes SAM analysis of genes differentially expressed in LP medium compared to SCD medium for opaque cells. (XLSX)

Text S2 SAM_for SOR op v. SCD op. File includes SAM analysis of genes differentially expressed in SOR medium compared to SCD medium for opaque cells. (XLSX)

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Author Contributions

Conceived and designed the experiments: HS ADH RJB. Performed the experiments: HS ADH MPH. Analyzed the data: HS ADH MPH RJB. Wrote the paper: HS ADJ RJB.

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