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Bcr1 plays a central role in the regulation of opaque cell filamentation in *Candida albicans*

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Summary

The human fungal pathogen *Candida albicans* has at least two types of morphological transitions: white to opaque cell transitions and yeast to hyphal transitions. Opaque cells have historically not been known to undergo filamentation under standard filament-inducing conditions. Here we find that Bcr1 and its downstream regulators Cup9, Nrg1 and Czf1 and the cAMP-signaling pathway control opaque cell filamentation in *C. albicans*. We have shown that deletion of *BCR1*, *CUP9*, *NRG1* and *CZF1* results in opaque cell filamentation under standard culture conditions. Disruption of *BCR1* in white cells has no obvious effect on hyphal growth, suggesting that Bcr1 is an opaque-specific regulator of filamentation under the conditions tested. Moreover, inactivation of the cAMP-signaling pathway or disruption of its downstream transcriptional regulators, *FLO8* and *EFG1*, strikingly attenuates filamentation in opaque cells of the *bcr1/bcr1* mutant. Deletion of *HGCI*, a downstream gene of the cAMP-signaling pathway encoding G1 cyclin-related protein, completely blocks opaque cell filamentation induced by inactivation of *BCR1*. These results demonstrate that Bcr1 regulated opaque cell filamentation is dependent on the cAMP-signaling pathway. This study establishes a link between the white-opaque switch and the yeast-filamentous growth transition in *C. albicans*.

Keywords

Candida albicans; Bcr1; opaque cell filamentation; cAMP signaling pathway

Introduction

Fluctuations in environmental conditions often lead to increased phenotypic plasticity and functional robustness of living organisms. *Candida albicans*, a major fungal pathogen of humans, has two known phenotypic transition systems, the “white” and “opaque” morphological switch, and the “yeast” and “filamentous” growth transition (Huang, 2012). Integration of these two systems could lead to increases in phenotypic plasticity and thus considerably diversify the niches that this pathogen colonizes.

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Supporting Information Additional supporting information may be found in the online version of this article.

White and opaque cell types of *C. albicans* display a number of distinct features including colony and cellular appearances and gene expression profiles (Anderson and Soll, 1987; Tuch *et al.*, 2010; Huang, 2012). White cells are relatively round and small and form white colonies, while opaque cells are bean-shaped and form gray or “opaque” colonies on nutrient-agar surfaces; the cell wall surface of opaque cells is pimples (Anderson and Soll, 1987). White and opaque cells also differ in mating competency and virulence (Huang, 2012). In order to mate, white cells must first switch to opaque, and only opaque cells have been reported to mate efficiently (Miller and Johnson, 2002).

Numerous studies indicate that genetic and epigenetic regulation of gene expression and environmental cues control white-opaque switching in *C. albicans* (Huang, 2012). A variety of overlapping environmental cues and genes regulate white-opaque switching as well as filamentous growth (Huang, 2012). The ability to undergo filamentation is a defining feature of virulence in *C. albicans* (Whiteway and Bachewich, 2007; Lo *et al.*, 1997); filamentous cells are more invasive and better at tissue penetration than yeast-form cells. The central pathway controlling this process is the cAMP-signaling pathway (Biswas *et al.*, 2007; Whiteway and Bachewich, 2007; Huang, 2012). Inactivation of the cAMP pathway by deletion of *CYR1*, encoding the only adenylyl cyclase in *C. albicans*, blocks filamentous growth under a variety of conditions (Rocha *et al.*, 2001). Consistently, the downstream transcriptional regulators of the cAMP pathway, Flo8 and Efg1, are required for filamentous development and virulence (Lo *et al.*, 1997; Cao *et al.*, 2006; Huang, 2012). Hgc1, a G1 cyclin-related protein, is enriched in filamentous cells and regulates hyphal morphogenesis; *HGC1* is transcriptionally regulated by the cAMP pathway (Zheng and Wang, 2004). The *HGC1* transcript is not detectable in the *cyr1/cyr1*, *efg1/efg1* or *flo8/flo8* mutants under filament-inducing conditions (Zheng and Wang, 2004; Cao *et al.*, 2006). pH signaling and MAPK pathways are also involved in the regulation of hyphal development (Biswas *et al.*, 2007; Huang, 2012). Acidic pH conditions inhibit filamentous growth, while neutral and basic pH conditions favor filamentation (Biswas *et al.*, 2007; Huang, 2012). The general transcriptional repressors Tup1, Nrg1 and Rfg1 are repressors of the yeast-to-filament transition in *C. albicans* (Braun and Johnson, 1997; Braun *et al.*, 2001; Kadosh and Johnson, 2001; Murad *et al.*, 2001; Huang, 2012). Deletion of *TUP1*, *NRG1* or *RFG1* leads to constitutive filamentous growth under a number of different culture conditions. Tup1 also plays a role in white-opaque switching and the regulation of phase-specific gene expression (Zhao *et al.*, 2002; Park and Morschhauser, 2005a). Filamentous cells formed by the *MTL α tup1/tup1* mutant are mating-competent, indicating that they are functionally opaque (Park and Morschhauser, 2005a). Nrg1 is a zinc-finger transcription factor that is typically associated with Tup1 (Huang, 2012). Rfg1 is an HMG-domain protein that transcriptionally regulates filamentous genes via Tup1-dependent or independent pathways (Kadosh and Johnson, 2005).

Both *MTL* homozygous and *MTL* heterozygous white cells can undergo filamentation under standard filament-inducing laboratory conditions. Opaque cells, on the other hand, have not been known to form filaments under these conditions (Anderson *et al.*, 1989), although they have been found to undergo filamentation when grown in a perfusion chamber or on a monolayer of human epithelial skin cells (Anderson *et al.*, 1989). Therefore, once the *MTL* homozygous cells differentiate into the specialized opaque type, they lose the ability to filament under standard laboratory culture conditions and acquire a number of opaque-specific attributes, such as the ability to mate and colonize the skin (Huang, 2012). Although several studies have focused on the mechanisms of filamentous growth regulation in *MTL* heterozygous cells (or white cells) of *C. albicans* (see Whiteway and Bachewich, 2007 for a review), little is known about the molecular mechanisms involved in the regulation of filament development in opaque cells.

Recently, Si *et al.* (2013) have found that the cAMP signaling pathway and some general transcriptional regulators including Nrg1, Ume6 and Rfg1 are involved in the regulation of opaque cell filamentation under several cultural conditions (Si *et al.*, 2013). These regulators may play a general activating or repressing role in the regulation of filamentation both in white cells and in opaque cells. In this study, we have identified Bcr1 as a unique and key regulator of opaque cell filamentation.

Although opaque cells can not undergo filamentation under standard filament-inducing laboratory conditions (Anderson *et al.*, 1989; Si *et al.*, 2013), Si *et al.* (2013) have reported that SOR medium (synthetic complete dextrose medium supplemented with 1 M sorbitol) and MIN medium (synthetic medium lacking amino acids) can efficiently induce opaque cell filamentation (Si *et al.*, 2013). The growth of *C. albicans* cells with the latter two media may represent two “stressful” conditions (high osmotic pressure for the SOR medium and starvation stress for the MIN medium, respectively). In this study, we have identified two pathways that control filament development in the opaque cells of *C. albicans* under standard laboratory inducing conditions: a positive and a negative pathway. The positive pathway is the cAMP-signaling pathway, in which the adenylyl cyclase Cyr1, the transcription factors Flo8, Efg1, and the G1 cyclin-related protein Hgc1, are required for opaque cell filamentation. These findings are consistent with the recently published paper (Si *et al.*, 2013). We have discovered the negative pathway mediated by the Bcr1 transcription factor and its downstream regulators, including Nrg1, Cup9 and Czf1. We find that the central regulator, Bcr1, is an opaque-phase-specific repressor of filamentation in *C. albicans* under the conditions tested.

Results

Identification of Bcr1, Cup9 and Czf1 as opaque cell filamentation regulators by screening a library of transcription factor deletion mutants

Opaque cells of *C. albicans* are unable to form hyphae under most standard laboratory conditions (Anderson *et al.*, 1989 and our unpublished data). To identify the regulators of opaque cell filamentation, we screened a library containing about 160 transcription factor gene deletion mutants, which were homozygous at the *MTL* locus (Du *et al.*, 2012a). The white and opaque cells of the mutants were plated onto Lee’s glucose medium plates and incubated at 25°C in air for 5 days. The WT control and most mutants of the library formed typical “white” (white and smooth) and/or “opaque” (red, rough and flat) colonies on Lee’s glucose medium containing the red dye phloxine B. The white colonies of two strains (*cup9/cup9* and *rfg1/rfg1*) were wrinkled. The opaque colonies of three mutants showed obvious wrinkled (*czf1/czf1*) or fuzzy (*bcr1/bcr1* and *cup9/cup9*) appearances (Fig. 1A). The three mutants could also undergo filamentation in liquid cultures at 25°C (Fig. 1B). Of the three mutants identified, the opaque cells of the *bcr1/bcr1* mutant were able to undergo the most robust filamentation (hereafter, we will refer to these cells as filamentous opaque cells), while its white cells remained in yeast form under the same culture condition, suggesting that Bcr1 is an opaque-specific regulator of filamentation. The white colonies of the *cup9/cup9* mutant were wrinkled, while the opaque colonies showed filamentous growth at their periphery and top regions. The cellular morphology verified that both white and opaque cells were able to undergo filamentation, indicating that Cup9 regulates filamentous growth in opaque and white cells. The opaque colonies of the *czf1/czf1* mutant formed slightly wrinkled colonies by day 5, and became more wrinkled and fuzzy at day 10. Aerial hyphae were also observed in the opaque colonies of the *czf1/czf1* mutant. The white colonies remained smooth and contained only yeast form cells after incubation for 5 to 10 days (Fig. 1A). These results suggest that Czf1 is a relatively weak regulator of opaque cell filamentation dependent on culture time period or cell aging.

Spontaneous switching between the white and opaque cell types is an important feature of the white-opaque transition in *C. albicans*. Indeed, both the white and filamentous opaque types of the *bcr1/bcr1*, *cup9/cup9* and *czf1/czf1* mutants were capable of switching back and forth at 25°C. The white-to-opaque switching frequencies of these mutants are shown in Table S1.

Bcr1 is a key opaque-specific regulator of filamentation

Of the mutants identified in our screen, the *bcr1/bcr1* mutant was able to undergo the most robust opaque cell filamentation. Therefore, we further extensively investigated the role of Bcr1 in this process. The *bcr1/bcr1* mutant formed two types of colonies: typical “white” and “fuzzy” red filamentous opaque colonies (Fig. 2A). The cellular morphology indicated that the WT white colonies contained white yeast cells, while WT opaque colonies formed typical elongated opaque cells. The white colonies of the *bcr1/bcr1* mutant also contained only white yeast cells, while the red “fuzzy” colonies were composed of highly filamentous cells (Fig. 2A). One of the defining features of opaque cells is their uniquely pimped cell surface (Anderson and Soll, 1987). To confirm that the cell identity of the “fuzzy” colonies was opaque, the cell surface was examined by scanning electron microscopy (SEM). As shown in Fig. S1, the surface of some, but not all, filamentous cells of the *bcr1/bcr1* mutant contained obvious pimples as did that of the WT opaque, while the white cells of both the WT and *bcr1/bcr1* mutant were smooth.

To confirm that deletion of *BCR1* in *C. albicans* resulted in robust filamentation of opaque cells, a fragment containing the *BCR1* gene was introduced back into the genome and integrated into the original *BCR1* locus. We found that either white or opaque cells of the *bcr1/bcr1*+*BCR1*p-*BCR1* reconstituted strain showed similar colony and cellular phenotypes to those of the WT (Fig. 2A). The phenotypes of filamentation in opaque cells were verified in an *MTLa/a* strain of an independent *bcr1/bcr1* mutant (CJN702) (Nobile and Mitchell, 2005). To verify that opaque cell filamentation of the *bcr1/bcr1* mutant is not specific to Lee’s medium, we also performed filamentation experiments in YPD, Spider, and synthetic complete defined (SCD) medium. As shown in Fig. S2, opaque cells of the *bcr1/bcr1* mutant were able to undergo obvious filamentation in all three media, while white cells remained in the yeast form. These results demonstrate that Bcr1 functions as an opaque-specific repressor of filamentous growth in *C. albicans*.

The *bcr1/bcr1* mutant filamentous opaque cells undergo invasive growth

Invasive growth of *C. albicans* is important for invading and colonizing host tissues (Biswas *et al.*, 2007; Huang, 2012). To investigate whether the *bcr1/bcr1* mutant could undergo invasive growth, white and opaque cells of the WT, *bcr1/bcr1* mutant and *bcr1/bcr1*+*BCR1*p-*BCR1* reconstituted strain were spotted onto Lee’s glucose medium plates. After 3 days of growth at 25°C, the plates were imaged before and after being washed with water. As shown in Fig. 2B, only filamentous opaque cells of the *bcr1/bcr1* mutant were able to undergo strong invasive growth. White cells of the three strains and opaque cells of the WT and *BCR1* complemented strain were almost completely washed away.

We next tested whether the filamentous cells of the *bcr1/bcr1* mutant contain a combination of filamentous and opaque features. Northern blot analysis showed that both opaque-enriched and filamentous-enriched genes (*OP4*, *WOR1*, *HWPI* and *ECE1*) were exclusively expressed in filamentous cells of the *bcr1/bcr1* mutant (Fig. 2C) (Birse *et al.*, 1993; Morrow *et al.*, 1993; Srikantha *et al.*, 1995; Staab *et al.*, 1999; Huang *et al.*, 2006; Srikantha *et al.*, 2006; Zordan *et al.*, 2006).

Czf1 and Cup9 are opaque cell filamentation regulators downstream of Bcr1

CUP9 is downregulated in response to hyphal growth inducers (Nantel *et al.*, 2002; Garcia-Sanchez *et al.*, 2005). Deletion of *CUP9* resulted in filamentation in white cells as well as in opaque cells (Fig. 1), suggesting that Cup9 is a general repressor of filamentation. The surface of filamentous opaque cells of the mutant was obviously pimpled, while the surface of white cells was smooth (Fig. S1). We found that Bcr1 binds to the promoter of *CUP9* both in white and opaque cells (Fig. 3).

Czf1 is a transcriptional regulator of white-opaque switching and hyphal development under certain conditions, such as embedded cultures (Brown *et al.*, 1999; Zordan *et al.*, 2007). Deletion of *CZF1* had a moderate effect on the induction of opaque cell filamentation, but not on white cell filamentation (Fig. 1). ChIP-PCR experiments demonstrated that Bcr1 binds to the promoter of *CZF1* both in white and in opaque cells, although the binding signal in white cell was relatively weak (Fig. 3). Consistently, Bcr1 also binds to the promoter of *CZF1* in *MTL* heterozygous cells (Nobile *et al.*, 2012). This suggests that Czf1 is downstream of Bcr1 in the regulation of opaque cell filamentation.

Filamentous opaque cells of the *bcr1/bcr1* and *cup9/cup9* mutants are mating competent

Mating competency is one of the most important characteristics of opaque cells, which mate much more efficiently than white cells (Miller and Johnson, 2002). To further confirm that the filamentous cells of the *bcr1/bcr1* and *cup9/cup9* mutants were functionally opaque, a quantitative mating assay was performed. WT opaque cells (*MTLa/a*) were used as the mating tester. We found that the mating efficiency of filamentous opaque cells was over 1,000 fold higher than that of the white cell counterparts of the *bcr1/bcr1* and *cup9/cup9* mutants (Fig. 4 and Table S2). As expected, the mating efficiency of opaque cells was also 1,000 times higher than that of white cells of the WT and reconstituted *bcr1/bcr1*+BCR1p-BCR1 reference strains (Fig. 4). Taken together, this data strongly suggests that the filamentous opaque cells are mating-competent.

High temperature (37°C) induces filamentous opaque cells of the *bcr1/bcr1* and *cup9/cup9* mutants to switch to the white phase

Opaque cells are sensitive to temperature changes. In vitro experiments have shown that high temperature (37°C) induces a mass conversion from the opaque to white phase in WT strains (Anderson and Soll, 1987) (Fig. 5). To investigate whether the filamentous opaque phenotypes of the *bcr1/bcr1* and *cup9/cup9* mutants are unstable at high temperature, the cells were plated onto nutrient agar plates at 37°C for a series of time periods (0 to 5 days), and then transferred to 25°C for an additional incubation (5 to 0 days). The colony and cellular morphology was examined after a total culture time of 5 days at both temperatures. As expected, when cultured at 25°C, the majority of colonies of either the WT or the two mutants were opaque or filamentous opaque. Most colonies of the WT became white after incubation at 37°C for 2 days and 25°C for 3 days (Fig. 5), suggesting that 37°C induced a mass conversion to white. At this culture condition, most colonies of the *bcr1/bcr1* and *cup9/cup9* mutant strains become white, white-sectoring, or mixed. Extended treatments (3 and 5 days) at 37°C led to a gradual increase in white or white filamentous growth (Fig. 5). The *bcr1/bcr1* mutant cultures of 2 or 3 days treatment at 37°C formed mostly white colonies, containing a majority of yeast cells and a minority of filaments. After incubating at 37°C for 5 days, the *bcr1/bcr1* mutant formed wrinkled filamentous colonies. It is clear that high temperature induced an opaque to white (yeast form) switch followed by filamentation of the switched white cells in the WT and *bcr1/bcr1* mutant (Fig. 5). Filamentous white and opaque colonies of the *bcr1/bcr1* and *cup9/cup9* mutants were morphologically distinct. Filamentous white colonies of the WT, *bcr1/bcr1* and *cup9/cup9* mutants were wrinkled, while opaque filamentous colonies of the *bcr1/bcr1* and *cup9/cup9* mutants were fuzzy. To

confirm their cell phase, mating competencies of the filamentous white and opaque cells were tested (data not shown). This data also suggests that, similar to the WT opaque cells, high temperature induced the switch from filamentous opaque cells to white (or filamentous white) phase in the *bcr1/bcr1* and *cup9/cup9* mutants (Fig. 5). These results provide additional evidence that the filamentous opaque cells of the mutants are functionally opaque.

Nrg1 regulates both white and opaque cell filamentation

We did not identify Nrg1 as a regulator of opaque cell filamentation in our library screen because the *nrg1/nrg1* mutant was able to undergo hyper-filamentation under the culture condition of our screen, which interfered with our ability to assess the transition between white and opaque cell phases. We have found that Bcr1 binds to the promoter of *NRG1* in white and opaque cells of an *MTL* homozygous strain (Fig. 3). Consistently, Nobile *et al.* also recently reported that Bcr1 binds to the promoter of *NRG1* in *MTLa/a* cells (Nobile *et al.*, 2012). We, therefore, predicted that Nrg1 may also be involved in the regulation of opaque cell filamentation. To test this hypothesis, we deleted *NRG1* in GH1013, a characterized *MTLa/a* strain (Huang *et al.*, 2009). The *nrg1/nrg1* mutant spontaneously switched between two cell types of filamentous colonies (white and opaque) on Lee's glucose medium plates (pH 6.8, Fig. 6). Similar to the *bcr1/bcr1* and *cup9/cup9* mutants, the surface of opaque cells of the *nrg1/nrg1* mutant was also pimpled, while the surface of its respective white cells was smooth (Fig. S1). A quantitative mating assay further confirms that the filamentous opaque cells of the *nrg1/nrg1* mutants are functionally opaque (Table S2). These results indicate that Nrg1 is also involved in opaque cell filamentation.

Regulation of white and opaque cell filamentation by Bcr1, Cup9, Nrg1, Czf1 and Rfg1 under pH 4.0 to 8.0 at 25°C

With the exception of Bcr1, the transcription factors Cup9, Nrg1, Czf1 and Rfg1 have been well characterized in the regulation of white cell filamentation (Nantel *et al.*, 2002; Garcia-Sanchez *et al.*, 2005; Biswas *et al.*, 2007; Whiteway and Bachewich, 2007; Huang, 2012). It has been shown that Bcr1 directly binds to the promoter regions of *CUP9*, *NRG1*, *CZF1* and *RFG1* genes in *MTLa/a* cells of *C. albicans* (Nobile *et al.*, 2012). Chromatin immunoprecipitation PCR (ChIP-PCR) confirmed that Bcr1 also binds to the promoter regions of *CUP9*, *NRG1* and *CZF1* in *MTL* homozygous strains (Fig. 3). pH is a critical environmental cue regulating white cell filamentation in *C. albicans* (Davis, 2003; Biswas *et al.*, 2007). We, therefore, tested the filamentous growth ability of white and opaque cells of the *bcr1/bcr1*, *cup9/cup9*, *nrg1/nrg1*, *czf1/czf1* and *rfg1/rfg1* mutants under different pH conditions. As expected, both white and opaque cells of the WT control did not form hyphae under all pH conditions tested (Fig. 6). White cells of the *bcr1/bcr1* mutant also did not undergo filamentation, while opaque cells of the *bcr1/bcr1* mutant were able to undergo robust filamentation under all pH conditions (pH 4.0 to 8.0). White cells of the *cup9/cup9* mutant formed filamentous colonies at pH 6.8 and 8.0, but not at acidic pHs (4.0 to 6.0). Although the opaque colonies of the *cup9/cup9* mutant contained less filamentous cells at acidic pHs (4.0 to 6.0) compared to pH 6.8 and 8.0, acidic pH did not completely block filamentation in opaque cells. White cells of the *nrg1/nrg1* mutant developed obvious filamentous colonies at pH 6.0 to 8.0, but not at pH 4.0 and 5.0. Similar to the *bcr1/bcr1* mutant, opaque cells of the *nrg1/nrg1* mutant formed robust filamentous colonies at pH 4.0 to 8.0. White cells of the *czf1/czf1* mutant only grew as yeast form, while opaque cells of the *czf1/czf1* mutant were able to undergo moderate filamentation at all pH conditions. Deletion of *RFG1* induced filamentous development of white cells, but had no obvious effect on opaque cell filamentation. The colony and cellular morphologies of the mutants are shown in Fig. 6 and Fig. S3, respectively. The robustness of filamentation of all the mutants tested at pH 4.0 to 8.0 is summarized in Table 1. Taken together, these results demonstrate that

opaque cell filamentation is less dependent on the environmental pH signal than white cell filamentation.

To verify the cell identity of white and opaque cells of the mutants tested in the pH experiments (Fig. 6 and Fig. S3), the mating competency was tested under these conditions (data not shown). Opaque or filamentous opaque cells of all the mutants were mating-competent, while white or filamentous white cells of all the mutants were mating-incompetent (data not shown).

cAMP signaling is required for Bcr1-controlled filamentation of opaque cells

cAMP signaling regulates not only filamentous growth but also white-opaque switching in *C. albicans* (Rocha *et al.*, 2001; Huang *et al.*, 2010). Deletion of *CYR1*, encoding the only adenylyl cyclase in *C. albicans*, completely blocks filamentous growth under a variety of culture conditions (Rocha *et al.*, 2001). Inactivation of the cAMP pathway leads to dramatically reduced white to opaque switch frequencies in GlcNAc containing medium, while activation of this pathway by deletion of *PDE2*, encoding a high affinity cyclic nucleotide phosphodiesterase, results in hypersensitivity to GlcNAc in *C. albicans* (Huang *et al.*, 2010). We next investigated whether cAMP signaling is required for filamentation in opaque cells. Opaque cells of the WT and *cyr1/cyr1* mutant did not undergo filamentous growth (Fig. 7A), suggesting that deletion of *CYR1* does not increase filamentation. A *cyr1/cyr1 bcr1/bcr1* double mutant was then generated. We found that, compared with the *bcr1/bcr1* single mutant, filamentation of opaque cells of the *cyr1/cyr1 bcr1/bcr1* double mutant was reduced (Fig. 7A). Supplementation of dibutyryl-cAMP (dbcAMP) to the *cyr1/cyr1 bcr1/bcr1* double mutant increased filamentation of opaque cells. However, equal amounts of dbcAMP added to the *cyr1/cyr1* opaque culture had no obvious effects on filamentous growth (Fig. 7B). These results indicate that cAMP signaling plays a critical role in Bcr1-controlled filamentation in opaque cells.

Role of Efg1, Flo8 and Hgc1 in opaque cell filamentation of the *bcr1/bcr1* mutant

The cAMP signaling downstream regulators, Efg1 and Flo8, function coordinately to control filamentous growth in *C. albicans* (Noffz *et al.*, 2008; Cao *et al.*, 2006; Du *et al.*, 2012a). They also play a central role in the regulation of white-opaque switching (Huang, 2012). To test their roles in filamentation of opaque cells, we generated the *efg1/efg1 bcr1/bcr1* and *flo8/flo8 bcr1/bcr1* double mutants in *C. albicans*. Compared with the *bcr1/bcr1* single mutant, the ability to undergo filamentous growth in the *efg1/efg1 bcr1/bcr1* double mutant opaque cells was dramatically reduced (Fig. 8A), although extended culturing (for more than 7 days) induced weak filamentation. Since deletion of *FLO8* “locks” *C. albicans* in the white phase in Lee’s glucose medium, and since the mutant of *flo8/flo8* can only form opaque colonies in Lee’s GlcNAc medium (Du *et al.*, 2012a), we grew the *flo8/flo8* mutant and *flo8/flo8 bcr1/bcr1* double mutant in both media. As shown in Fig. 8B, neither the *flo8/flo8* single mutant nor the *flo8/flo8 bcr1/bcr1* double mutant was able to filament on Lee’s glucose medium. On Lee’s GlcNAc medium plates, the *flo8/flo8* single mutant formed opaque colonies containing only yeast form cells, while the *flo8/flo8 bcr1/bcr1* double mutant formed weakly wrinkled colonies (Fig. 8B). Although the filamentation ability of the *flo8/flo8 bcr1/bcr1* double mutant was weaker than that of the *bcr1/bcr1* single mutant, cellular morphology confirmed the existence of typical opaque and filamentous cells in the wrinkled colonies (Fig. 8B). These results suggest that Efg1 and Flo8 contribute to filamentation of opaque cells, yet inactivation of these two transcription factors does not completely block Bcr1-controlled filamentous growth.

Hgc1 is transcriptionally regulated by Efg1 and Flo8 and is downstream of the cAMP-signaling pathway (Zheng and Wang, 2004; Cao *et al.*, 2006). We, therefore, generated an

hgc1/hgc1 bcr1/bcr1 double mutant. While opaque cells of the *bcr1/bcr1* single mutant were able to undergo robust filamentation, hyphal development of the *hgc1/hgc1 bcr1/bcr1* double mutant opaque cells was completely blocked (Fig. 8A), suggesting that Hgc1 is essential for opaque cell filamentation induced by inactivation of Bcr1.

Overexpression of white cell hyphal-inducing gene HGC1, BRG1, and TEC1, promotes opaque cell filamentation

Hgc1, Flo8, Efg1, Tec1 and Brg1 are well characterized regulators of white cell filamentation (Homann *et al.*, 2009; Du *et al.*, 2012b; Huang, 2012). Tec1, Brg1 and Bcr1 are members of the master transcriptional network of biofilm formation and bind to the promoters of each other (Nobile *et al.*, 2012). To identify activators of opaque cell filamentation, we overexpressed *HGC1*, *FLO8*, *EFG1*, *TEC1* and *BRG1* in white and opaque cells of a WT strain (GH1013, a derivative of SC5314) using a tetracycline-controlled promoter (TETp) (Park and Morschhauser, 2005b; Du *et al.*, 2012a; Du *et al.*, 2012b). The activity of the TET-On promoter of the plasmid pNIM1 is considerably lower in opaque cells because the Tet-On system is based on expression of *cartTA* from the *ADHI* promoter, which is less active in opaque cells than in white cells. To increase the ectopic expression levels of these genes, a copy of the *cartTA* gene was first integrated into the *OP4* (an opaque cell enriched gene) locus of GH1013 (WT+OP4p-*cartTA*). To test whether this engineered overexpression system works, we examined the expression of GFP protein with fluorescence microscopy assays. Under the inducing condition, visible GFP fluorescence could only be observed in a few white cells, but not opaque cells, of the WT+pNIM1 strain. However, the expression level of the GFP reporter in opaque cells of the WT+OP4p-*cartTA*+pNIM1 strain was significantly higher than that in opaque cells of the WT+pNIM1 strain under the inducing condition (Fig. 9A). Cells of the overexpression strains were grown on Lee's glucose plates with or without 40 $\mu\text{g ml}^{-1}$ doxycycline at 25°C. 40 $\mu\text{g ml}^{-1}$ doxycycline was used for inducing ectopic expression because high concentrations (>50 $\mu\text{g ml}^{-1}$) of this chemical could have an inhibitory effect on filamentation (Park and Morschhauser, 2005b). As shown in Fig. 9B, overexpression of *HGC1*, *TEC1*, or *BRG1* in opaque cells induced filamentous growth, while overexpression of *FLO8* had no obvious effect. Overexpression of the white cell enriched gene *EFG1* indeed induced filamentation, but we observed that *EFG1* first induced an opaque-to-white conversion and resulted in filamentation of the converted white cells (data not shown).

Deletion of TEC1, BRG1 and CPH1 in the bcr1/bcr1 mutant does not affect opaque cell filamentation

Since overexpression of *TEC1* and *BRG1* in opaque cells induced filamentous growth, we subsequently tested whether *TEC1* and *BRG1* are required for filamentation in the *bcr1/bcr1* mutant. As shown in Fig. 8A, the double mutants of *tec1/tec1 bcr1/bcr1* and *brg1/brg1 bcr1/bcr1* underwent robust filamentation, suggesting that deletion of these two genes has no obviously effect on Bcr1-regulated filamentous growth. Consistently, Si *et al.* also found that Tec1 is not required for environmental cues induced opaque cell filamentation (Si *et al.*, 2013). We also found that deletion of *CPH1* in the *bcr1/bcr1* mutant did not affect opaque cell filamentation (Fig. 8A).

Deletion of TEC1, BRG1, ROB1, EFG1 and NDT80 does not induce opaque cell filamentation

The transcription factors Bcr1, Tec1, Brg1, Rob1, Efg1 and Ndt80 comprise a master circuit that controls biofilm formation and form an elaborate, interconnected transcriptional network in *C. albicans* (Nobile *et al.*, 2012). We, therefore, examined whether Tec1, Brg1, Rob1, Efg1 and Ndt80 were also involved in repressing opaque cell filamentation. As shown in Fig. S4 and Fig. 8A, deletion of *TEC1*, *BRG1*, *ROB1*, *EFG1* or *NDT80* did not induce

filamentous growth of opaque cells, suggesting that a different transcriptional network is involved in the regulation of opaque cell filamentation.

Discussion

Eukaryotic cells become morphologically and functionally specialized through cell differentiation. *C. albicans* can undergo a phenotypic switch between white and opaque cell types, which are functionally specialized in the processes of filamentation, mating and infection of different host tissues (Lohse and Johnson, 2009; Soll, 2009). White cells can undergo filamentation, whereas opaque cells appear to lose the capacity to grow as a filamentous form under most inducing conditions ((Anderson *et al.*, 1989; Si *et al.*, 2013); and our unpublished data). White-opaque switching is a bistable and heritable epigenetic event, where both cell types are maintained for many generations (Slutsky *et al.*, 1987). However, the yeast-filamentous growth transition is apparently non-heritable and highly environment-dependent (Huang, 2012). Despite many shared features of the white-opaque switch and the yeast-filamentous growth transition (Huang, 2012), they have traditionally been thought to exist as two separate phenotypic switching systems. Previous studies demonstrate that opaque cells indeed form filaments when grown on the surface of a perfusion chamber or on a monolayer of human epithelial skin cells (Anderson *et al.*, 1989), suggesting that a cryptic program of filamentous growth exists in opaque cells. In this study, we report that both cAMP-signaling and Bcr1-mediated pathways are involved in the regulation of filamentation of opaque cells in *C. albicans*. Numerous studies have linked filamentous growth with pathogenesis in *MTL* heterozygous strains of *C. albicans* (Huang, 2012; Whiteway and Bachewich, 2007). One hypothesis is that the combination of the ability to filament with the ability to mate as an opaque cell (in addition to other opaque cell features), may be adaptive by permitting *C. albicans* to persist in the human host, providing advantages over other phenotypes in specific host niches.

In this study, we have identified Bcr1 as a central regulator of opaque cell filamentation. We find that Bcr1-regulated opaque cell filamentation is dependent on the cAMP signaling pathway. These findings provide new insights into a longstanding question of why opaque cells have historically been unable to undergo filamentation under standard hyphal-inducing conditions. A recent study by Si *et al.* reports that distinctive pathways are involved in filamentous growth in white and opaque cells (Si *et al.*, 2013). While our study and the Si *et al.* study both focus on a similar topic, they have diverse and complementary features. For example, most culture media used in our study are standard laboratory media (e.g. Lee's medium and YPD), whereas SOR (with 1 M sorbitol) and MIN (without amino acids) media were used for opaque cell filamentation in the Si paper (Si *et al.*, 2013). Different culture conditions may represent different natural niches for *C. albicans* in vivo.

Bcr1, the central regulator of opaque cell filamentation

Bcr1 is the first transcription factor that has been reported to be required for biofilm development in vitro and in vivo, but is not required for filamentous growth in *MTL* heterozygous *C. albicans* isolates (Nobile and Mitchell, 2005). Bcr1 regulates the expression of a subset of cell surface genes including *HWPI*, *ECE1*, *RBT5* and *ALS3* (Nobile *et al.*, 2006), which are also enriched during filamentation (Huang, 2012). The expression of *BCR1* is partly dependent on the hyphal regulator Tec1 and is repressed by the general transcription repressor Tup1 (Kadosh and Johnson, 2005; Nobile and Mitchell, 2005), although Bcr1 also transcriptionally regulates *TEC1* through an interwoven network during biofilm formation (Zordan *et al.*, 2007; Nobile *et al.*, 2012). Recently, Fanning *et al.* reported that Bcr1 divergently controls the expression of many genes during the process of biofilm formation in vitro and in vivo (Fanning *et al.*, 2012). Moreover, Bcr1 and Wor1 bind to each other's promoters (Zordan *et al.*, 2007; Nobile *et al.*, 2012). These findings are consistent

with the idea that Bcr1 is linked to filamentation and white-opaque switching and can act differentially in “fine-tuning” the regulation of gene expression under different circumstances. In the present study, we have discovered a novel role for Bcr1 in the regulation of opaque filamentous growth. Disruption of *BCR1* in opaque cells leads to hyper-filamentous and hyper-invasive growth, demonstrating that Bcr1 is a powerful repressor of opaque cell filamentation. We showed that the physiological temperature of the human host (37°C) induces opaque to white switching followed by filamentation after a prolonged incubation period (Fig. 5). However, the latter filaments are present both in the WT and in the *bcr1/bcr1* mutant. Thus, Bcr1 does not repress filamentous growth in white cells under the condition tested. Nobile *et al.* has also found that white cells of the *bcr1/bcr1* mutant and the WT control produced hyphae to comparable extents under a variety of culture conditions (Nobile *et al.*, 2012). However, we cannot exclude the possibility that Bcr1 might play a role in white cell filamentation under some un-discovered condition, such as a specific host niche.

Filamentous opaque cells of the *bcr1/bcr1* mutant are mating competent, confirming that their cell identity is opaque. Notably, the mating efficiency of the *bcr1/bcr1* mutant opaque cell type is about 10 times higher than that of the WT opaque cell type. One potential explanation for this observation is that the larger length of the filamentous opaque cells of the *bcr1/bcr1* mutant may increase the opportunity for interactions between mating partners by providing a larger surface area. Another explanation is that filamentous opaque cells may more easily form elongated mating projections, a prerequisite of mating, since they are already in an elongated form.

Role of previously characterized hyphal growth regulators (Czf1, Cup9, Nrg1, Rfg1, Tup1, Brg1, and Tec1) in opaque cell filamentation

The transcription factors Czf1, Cup9 and Nrg1 are also involved in the regulation of opaque cell filamentation (Table 1, Fig. 1, 6 and Fig. S3). Bcr1 directly binds to the promoter regions of these genes in *MTL* heterozygous as well *MTL* homozygous cells (Nobile *et al.*, 2012 and our unpublished data). Deletion of *CZF1* has only a moderate inducing effect on the development of filaments in aged opaque cells (Fig. 1 and Fig. 6). Consistent with this, Zordan *et al.* has reported that opaque colonies of the *czf1/czf1* mutant are rougher than those of WT strains (Zordan *et al.*, 2007). Cup9 and Nrg1 are involved in opaque cell filamentation as well as white cell filamentation, indicating that they are not opaque cell specific regulators of filamentous growth, but general transcriptional repressors. We have also identified a white cell specific regulator, Rfg1, an HMG-domain-containing DNA binding protein acting in Tup1-dependent and -independent pathways (Kadosh and Johnson, 2001). Deletion of *RFG1* affects white cell filamentation but not opaque cell filamentation (Fig. 6 and Fig. S3). This result is consistent with the recent study reporting that Rfg1 represses filamentation in white cells but not in opaque cells, and further demonstrates that Rfg1 functions as a positive regulator of opaque cell filamentation (Si *et al.*, 2013). Environmental pH plays an important role in the regulation of hyphal development in *MTL* heterozygous and *MTL* homozygous white cells (Davis, 2003; Biswas *et al.*, 2007) (Fig. 6 and Fig. S3). However, alterations in pH appear to have no obvious effect on opaque cell filamentation in the *bcr1/bcr1*, *nrg1/nrg1* and *czf1/czf1* mutants.

Bcr1 directly binds to the promoter regions of *NRG1* and *CUP9* and may control their transcription ((Nobile *et al.*, 2012) and Fig. 3). The major machinery of opaque cell filamentation, including the conserved and central cAMP-signaling pathway, the negative regulators of filamentation (such as Tup1, Nrg1, Czf1 and Cup9) and the cell wall components, are also used in the regulation of white cell filamentation. A slight modification

of these pathways via Bcr1 may have permitted the reprogramming of a new biological process.

The *MTL* homozygous mutant of the repressor *TUP1* can switch between four different colony types (Zhao *et al.*, 2002; Park and Morschhauser, 2005a). The regulation of filamentous growth by Tup1 is more general in *C. albicans* since Tup1 is a global and highly pleiotropic transcriptional regulator. Disruption of *TUP1* in *MTL* heterozygous strains also leads to hyper-filamentous growth under all conditions tested (Braun and Johnson, 1997). The Tup1 regulator is highly conserved and functions similarly in other yeasts. Tup1 and its associated transcription factor Nrg1 may also repress opaque cell filamentation via a pathway independent of Bcr1. Bcr1, Tec1, and Brg1, coordinate to regulate biofilm formation in *C. albicans* (Nobile *et al.*, 2012). Of note, both *TEC1* and *BRG1* play a critical role in the regulation of white cell filamentation (Du *et al.*, 2012b). Although deletion of *TEC1* and *BRG1* had no effect on the induction of opaque cell filamentation, overexpression of *TEC1* and *BRG1* obviously induced filamentous growth in opaque cells (Fig. 9). The regulatory pathways of opaque cell filamentation are shown in Fig. 10A.

The cAMP signaling is required for opaque cell filamentation

The adenylyl cyclase Cyr1 occupies the central position of the regulatory network of filamentation in *MTL* heterozygous isolates of *C. albicans* (Huang, 2012). We set out to explore the role of cAMP signaling in filamentous growth of opaque cells. Inactivating cAMP signaling by disruption of *CYR1* results in a significant decrease in filamentation in the *bcr1/bcr1* mutant opaque cells. Of note, deletion of *CYR1* results in the lack of the basal level of cAMP, which may affect a range of cellular functions including cell morphogenesis and growth rate. Therefore, to some extent, the defect of filamentation in the *bcr1/bcr1 cyr1/cyr1* double mutant may also be a secondary effect.

Consistent with the phenotype observed in the *bcr1/bcr1 cyr1/cyr1* double mutant, two major transcription regulators downstream of cAMP signaling, Efg1 and Flo8, are also required for hyper-filamentation of the *bcr1/bcr1* mutant. Filamentous-specific G1 cyclin-related protein Hgc1 is transcriptionally regulated by the cAMP pathway and the Efg1 and Flo8 transcription regulators (Zheng and Wang, 2004; Cao *et al.*, 2006). *HGC1* is essential for opaque cell filamentation in the *bcr1/bcr1* mutant (Fig. 8A). Deletion of *EFG1* or *FLO8* partially suppresses filamentous growth, while deletion of *HGC1* completely blocks opaque cell filamentation in the *bcr1/bcr1* mutant. The transcriptional level of *HGC1* in opaque cells of the *bcr1/bcr1* mutant is 4 fold higher than that in opaque cells of the WT control (data not shown). Consistently, overexpression of *HGC1* promotes filamentous development in opaque cells. These results suggest that the regulation of Bcr1-controlled filamentous growth in opaque cells is regulated by the major cAMP pathway and a minor unidentified pathway, which converge on Hgc1.

It is possible that the specialization of Bcr1 and cAMP signaling in the gene regulatory network of filamentation in opaque cells may have been gained after the evolution of white-opaque switching in *C. albicans*. Filamentous enriched genes *HWPI* and *ECE1* are expressed in filamentous opaque cells of the *bcr1/bcr1* mutant (Fig. 2C), suggesting that downstream functional components of filamentation of white cells are harnessed by the mutant. The central regulatory pathway of filamentous growth in white cells, cAMP signaling and its downstream regulators Efg1, Flo8 and Hgc1 are also utilized by opaque cells. Disruption of a single gene, *BCR1*, is able to reprogram the regulatory circuitry of filamentation, emphasizing the importance of Bcr1 in opaque cells. Environmental cues could signal opaque cells to undergo filamentation via the positive pathway of cAMP signaling as well as the Bcr1-mediated negative transcriptional circuit.

Opaque cell filamentation may increase phenotypic plasticity of *C. albicans*

Evolution increases phenotypic plasticity in living organisms, where new phenotypes may arise as an adaptation to changing environmental conditions or via integration of existing regulatory networks through genetic drift. For the human fungal pathogen *C. albicans*, the traditionally separate white-opaque and yeast-filamentous circuits have been regarded as separate systems (Fig. 10B). Integration of the opaque and filamentous circuits into a new filamentous opaque network provides an additional level of phenotypic regulation, which results in a complex multiple-component switching system (Fig. 10B). Compared to the former systems, the latter has increased phenotypic flexibility, which is important for the pathogen to better adapt to fluctuations in the host environment. Moreover, our study of reprogramming typical opaque cells to filamentous opaque cells by disruption of *BCR1* provides an example of a simple cell differentiation system in *C. albicans* that may serve as a model for the study of other developmental processes.

Experimental procedures

Culture conditions, strains and plasmids

YPD (1% yeast extract, 2% peptone, 2% glucose) and modified Lee's glucose medium were used for routine culture of *C. albicans* cells (Huang *et al.*, 2010). Lee's medium is a chemically defined medium, which was developed based on the aminopeptidases of *C. albicans* (Lee *et al.*, 1975). 0.07 grams per liter of L-arginine was added to the modified Lee's medium. Modified Lee's medium with 1.25 % (w v⁻¹) glucose as a carbon source is referred to as Lee's glucose medium. Modified Lee's medium with GlcNAc 1.25 % (w v⁻¹) as a carbon source is referred to as Lee's GlcNAc medium. YPmal medium (1% yeast extract, 2% peptone, 2% maltose) was used for FLP-mediated excision of the *SAT1*/flipper cassette.

The *MTLa/α* strains of *C. albicans* were converted to *MTL* homozygotes by selection in YPS (1% yeast extract, 2% peptone and 10% sorbose) medium as previously described (Du *et al.*, 2012a). To verify the phenotypes of *C. albicans* *MTL* homozygotes, the *a* or *α* locus was also deleted in some *MTLa/α* strains by targeted disruption with the plasmid pSFS2a-MTLKO to generate *MTLa/Δ* or *MTLΔ/α* isolates (Thyagarajan and Soll, unpublished). To generate the *bcr1/bcr1+BCR1p-BCR1* reconstituted strain, the same strategy was used as we reported for the construction of a *BRG1* reconstituted strain (Du *et al.*, 2012b). The construct contained two fragments (one 3.1 kb fragment with the sequence of *BCR1* ORF, 5'-UTR and 3'-UTR; the other 0.5 kb fragment contained the 3'-UTR of *BCR1*). The construct was integrated into the original *BCR1* locus. The strains used in this study are listed in supplemental material Table S3.

To construct the *BCR1* knockout plasmid pSFS2a-*BCR1*KO, two *BCR1* flanking fragments were inserted into the *Apal/XhoI* and *SacII/SacI* sites of the plasmid pSFS2A (Reuss *et al.*, 2004).

The *cyr1/cyr1* mutant, GH1109, was used to generate the *cyr1/cyr1 bcr1/bcr1* double mutant. To delete the first copy of *BCR1*, GH1109 was transformed with a fragment of the *Apal/SacI* linearized pSFS2a-*BCR1*KO, containing a *SAT1*/flipper cassette. The resulting strain *bcr1/bcr1 BCR1/bcr1::SAT1-FLIP* was then grown in YPmal medium for FLP-mediated excision of the *SAT1*/flipper cassette generating the strain *cyr1/cyr1 BCR1/bcr1::FRT*. To delete the second copy of *BCR1*, the strain *cyr1/cyr1 BCR1/bcr1::FRT* was transformed with the fusion PCR product of the *URA3* gene flanked by the *BCR1* gene 5' and 3' fragments. The plasmid pGEM-*URA3* was used as a PCR template (Wilson *et al.*, 1999). The fusion PCR assay was performed as previously reported (Hernday *et al.*, 2010).

The *flo8/flo8* mutant, GH1296, was used to generate the *flo8/flo8 bcr1/bcr1* double mutant. To delete the first copy of *BCR1*, GH1296 was transformed with a fragment of the *ApaI/SacI* linearized pSFS2a-BCR1KO. The resulting strain *flo8/flo8 BCR1/bcr1::SAT1-FLP* was grown in YPmal medium for FLP-mediated excision of the *SAT1/flipper* cassette generating the strain *flo8/flo8 BCR1/bcr1::FRT*, and then grown on 5-fluoroorotic acid (5-FOA) containing medium for counter-selection of *ura3⁻* isolates. Similarly as in the generation of the *cyr1/cyr1 bcr1/bcr1* double mutant, the second copy of *BCR1* was deleted using the fusion PCR method. The plasmid pGEM-URA3 was used as a PCR template (Wilson *et al.*, 1999).

The *efg1/efg1* mutant, MMY620, and the *cph1/cph1* mutant, JKC18a, were used to generate the *efg1/efg1 bcr1/bcr1* double mutant. JKC18a (*MTLa/a*) was derived from JKC18 by growing the strain in sorbose-containing medium (Liu *et al.*, 1994). The two copies of *BCR1* were disrupted in the *efg1/efg1* mutant and the *cph1/cph1* mutant with the same method used in generating the *flo8/flo8 bcr1/bcr1* double mutant.

The *hgc1/hgc1* mutant, GH1281, was used to generate the *hgc1/hgc1 bcr1/bcr1* double mutant. To delete the first copy of *BCR1*, GH1281 was transformed with a fragment of the *ApaI/SacI* linearized pSFS2a-BCR1KO, containing a *SAT1/flipper* cassette. To delete the second copy of *BCR1*, the strain *hgc1/hgc1 BCR1/bcr1::FLP* was transformed with the fusion PCR product of the *URA3* gene flanked by *BCR1* gene 5' and 3' fragments. The plasmid pGEM-URA3 was used as a PCR template. All primers used are listed in Table S4.

TF22 (*brg1/brg1* mutant, *MTLa/a*) of the transcription factor mutant library was first converted to an *MTLΔ/a* strain by targeted disruption with the plasmid pSFS2a-MTLKO to generate *MTLΔ/a* isolate (TF22a). TF22a was then grown in YPmal medium for FLP-mediated excision of the *SAT1/flipper* cassette to generate the strain TF22a.1. To delete the first copy of *BCR1* in TF22a.1, a fragment of the *ApaI/SacI* linearized pSFS2a-BCR1KO was used for disruption. To delete the second copy of *BCR1*, cells were transformed with the fusion PCR products of the *ARG4* gene and two fragments (of the 5' and 3' terminals) of the *BCR1* gene. Genomic DNA of SC5314 and the plasmid pRS-ARG4 was used as initial PCR templates.

A similar strategy to the deletion of *BCR1* in TF22a.1 was used to generate the *tec1/tec1 bcr1/bcr1* double mutant. The two alleles of *BCR1* were subsequently disrupted in the *tec1/tec1* mutant TF115a with the *ApaI/SacI* linearized pSFS2a-BCR1KO plasmid and the fusion PCR products of the *ARG4* gene and two fragments (of the 5' and 3' terminals) of the *BCR1* gene.

At least three independent isolates of each double mutant were used for the phenotypic transition analysis in order to exclude any effects induced by non-specific mutations (e.g. 5-FOA induced spontaneous mutations).

The strain E5.3I-3 of the *czf1/czf1* mutant was used in Fig. 1, 6 and S3. The phenotype of the *czf1/czf1* mutant was verified in the strain WCZF1M4B, a *czf1/czf1* mutant generated in a different background strain, WO-1 (Ramirez-Zavala *et al.*, 2008).

Northern blot analysis

Northern blot analysis was performed according to our previous studies (Huang *et al.*, 2009). Purified PCR products were used to make probes. Primers used for the PCR reactions are listed in Table S4.

Chromatin Immunoprecipitation PCR (ChIP-PCR)

The myc-tagged strain CJN1787 (*MTLa/a BCR1/BCR1-Myc*) was converted to an *MTLa/Δ* strain by deletion of the α locus with the plasmid pSFS2a-MTLKO. The resulting strain was named as CJN1787a (*MTLa/Δ::SAT1 BCR1/BCR1-Myc*). To test whether Bcr1 binds to the candidate targets, white and opaque cells of the strain CJN1787a were lysed and the supernatant was subjected to sonication to obtain sheared chromatin averaging 500 to 1000 bp in length. The ChIP assay was conducted as described previously (Hernday *et al.*, 2010; Nobile *et al.*, 2012). Anti-Myc polyclonal antibody (sc-789) (Santa Cruz Biotechnology, Inc.) was used. Immunoprecipitates were recovered and DNA was then extracted for PCR amplification.

Microscopy

Cells collected from liquid cultures or from nutrient agar plates as indicated in the text were used for morphological analysis. Differential interference contrast (DIC) optics was used for standard cell morphology detection. To discriminate hyphae and pseudohyphae, cells were dyed with Calcofluor white, which stains septa and chitin in the cell wall. Fluorescence microscopy was used to detect septa. Calcofluor white staining was performed as described previously (Wang *et al.*, 2011). Scanning electron microscopy (SEM) was performed as previously described (Du *et al.*, 2012b).

Mating assay

Mating experiments were conducted on Lee's glucose plates as previously reported (Miller and Johnson, 2002; Huang *et al.*, 2009). Briefly, the experimental cell samples were collected from the colonies of the right phase phenotype. The tester opaque cells were collected from a liquid culture in middle exponential phase. 1×10^6 of *MTLa/a* cells and 1×10^6 of *MTLa/α* cells were mixed in Lee's glucose medium and spotted onto Lee's glucose medium plates. After 4 days of growth at 25°C, the mating mixture was resuspended and plated onto selectable plates (depleted of uridine, or Arginine, or uridine/Arginine). Colonies grown on the three types of plates were counted and mating efficiencies were calculated.

Filamentous and invasive growth assays

YPD, spider and SCD medium were used in Fig. S2. Spider medium was made according to Liu *et al.* (1994) (Liu *et al.*, 1994). SCD medium (pH6.8) was buffered with K_2HPO_4 . For Fig. 8B, the *flo8/flo8* and *flo8/flo8 bcr1/bcr1* mutants were cultured on both Lee's glucose and Lee's GlcNAc media.

To examine the ability of invasive growth, 3 μ l of liquid medium containing about 6×10^4 cells was spotted onto the Lee's glucose medium plates and incubated for 3 days at 25 °C. The plates were imaged before and after washing with H₂O.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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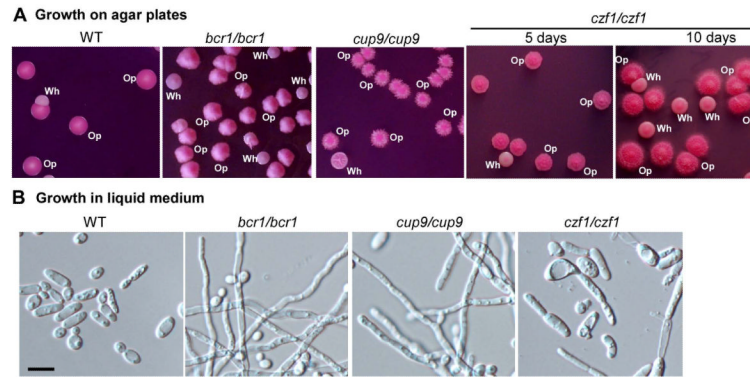


Fig. 1. Identification of Bcr1, Cup9 and Czf1 as opaque cell filamentation regulators by screening a library of transcription factor deletion mutants. A. Colony morphology of the WT (SN250 α), *bcr1/bcr1*, *cup9/cup9* and *czf1/czf1* mutants. Cells were plated onto Lee's glucose medium plates and cultured at 25°C for 5 days. Incubation of the *czf1/czf1* mutant was extended to 10 days. B. Cellular images of the growth cultures in liquid Lee's glucose medium at 25°C. Opaque colonies were inoculated into liquid medium. Cells were grown to the mid-exponential phase and imaged. Scale bar, 10 μ m.

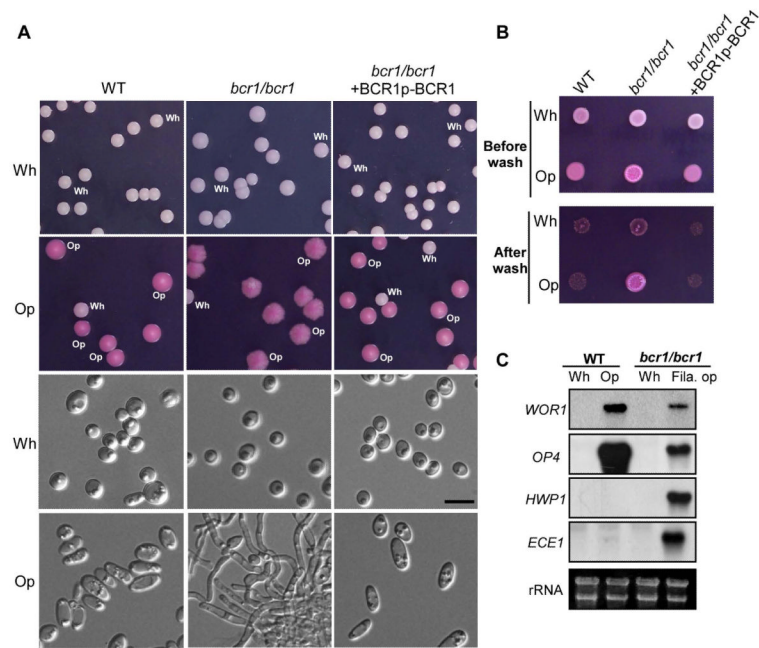


Fig. 2. Deletion of *BCR1* results in filamentous and invasive growth of opaque cells. A. Colony and cellular images of the WT (SN250 α), *bcr1/bcr1* mutant and *bcr1/bcr1*+BCR1p-BCR1 reconstituted strain. Wh, white phase; op, opaque phase. Scale bar, 10 μ m. B. Invasive growth of the WT (SN250 α), *bcr1/bcr1* mutant and *bcr1/bcr1*+BCR1p-BCR1 reconstituted strain. White and opaque cells ($\sim 6 \times 10^4$) were spotted on Lee's glucose medium plates and incubated at 25°C for 3 days. C. Expression of opaque (*WOR1* and *OP4*) or filamentous (*HWP1* and *ECE1*) enriched genes in the *bcr1/bcr1* mutant analyzed by Northern blot.

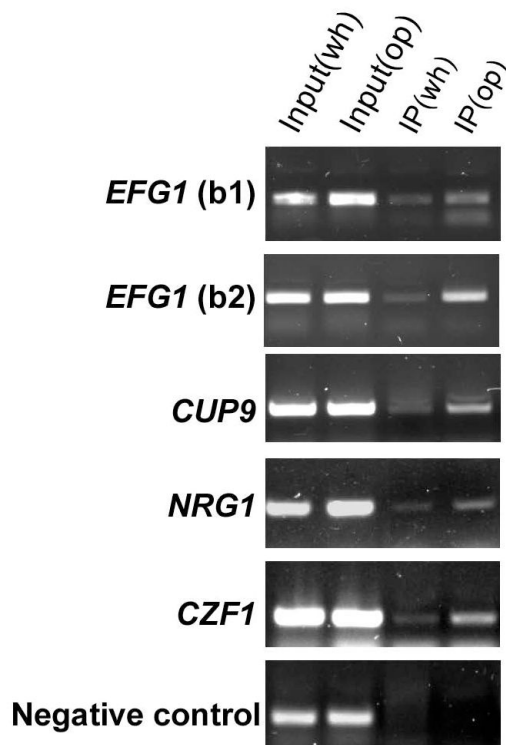
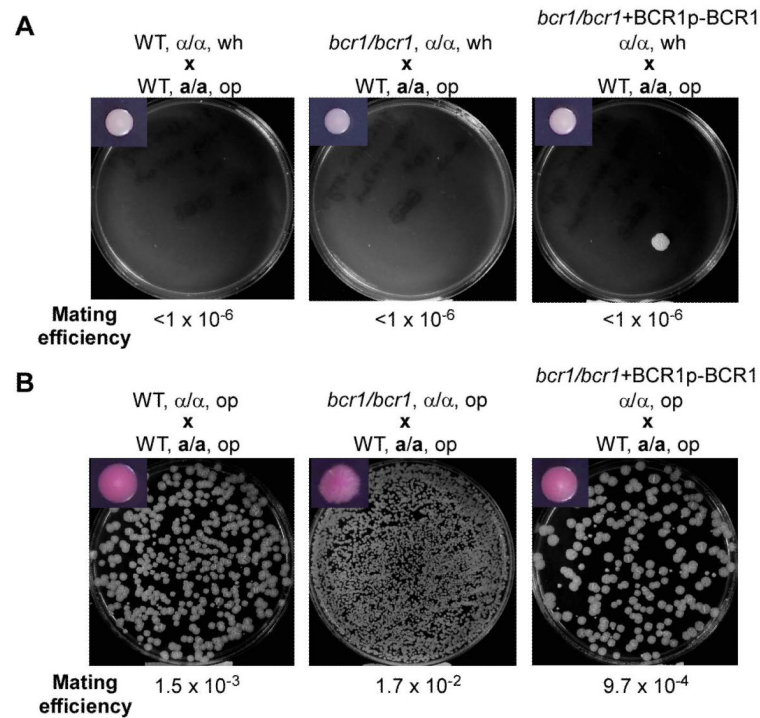


Fig. 3. ChIP-PCRs of the promoter regions of Bcr1-regulated genes. *EFG1*(b1), Bcr1 binding site 1 (–7188 to –7422); *EFG1*(b2), Bcr1 binding site 2 (–2220 to –1886). PCR products were detected after 27 cycles. The primers were designed according to the Bcr1 binding sites reported by Nobile *et al.*, 2012. The ChIP-PCR of the *HGC1* promoter, which was known not to be bound by Bcr1 (Nobile *et al.*, 2012), served as the negative control. White and opaque cells of the strain CJN1787a (*MTLa/α Δ::SAT1 BCR1/BCR1-Myc*) were used for the experiments.

**Fig. 4.**

Quantitative mating assay indicates that filamentous opaque cells of the *bcr1/bcr1* mutant are mating-competent. *MTLa/a* and *MTLa/a* cells in white or opaque phase were mixed and cultured on Lee's glucose plates for 4 days at 25°C. About 1×10^6 cells of each mating mixture were resuspended and plated onto selectable SCD plates. A representative selectable plate (depleted with uridine and arginine) of each cross was imaged. The mating efficiencies were calculated and shown below the images. A colony image indicating the phase of experimental cells used for mating was also shown (top left corner). A. Mating between the WT opaque tester (GH1012, *MTLa/a*, *ura3*⁻) and white cells of the WT (*MTLa/a*, *arg4*⁻), *bcr1/bcr1* mutant (*MTLa/a*, *arg4*⁻) and *bcr1/bcr1*+BCR1p-BCR1 reconstituted strain (*MTLa/a*, *arg4*⁻). B. Mating between the WT opaque tester (GH1012, *MTLa/a*, *ura3*⁻) and opaque cells of the WT (*MTLa/a*, *arg4*⁻), *bcr1/bcr1* mutant (*MTLa/a*, *arg4*⁻) and *bcr1/bcr1*+BCR1p-BCR1 reconstituted strain (*MTLa/a*, *arg4*⁻).

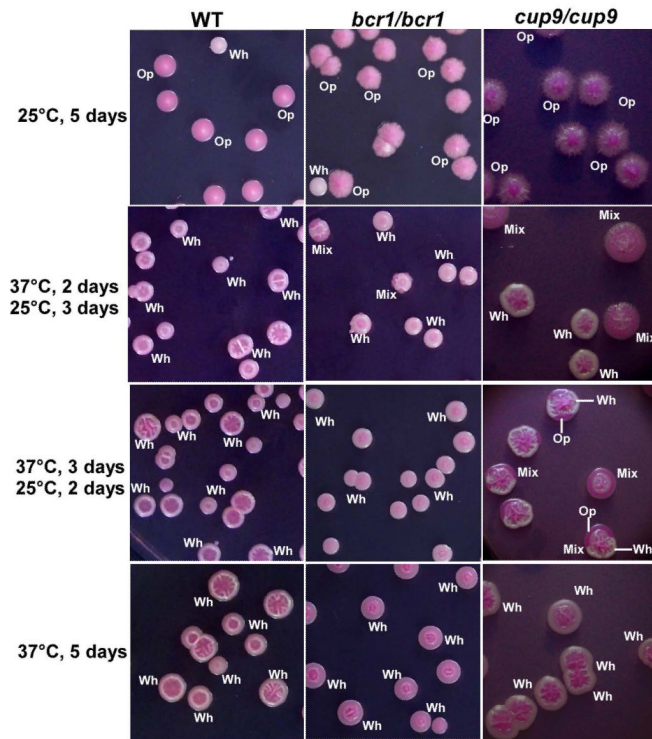


Fig. 5. High temperature (37°C) induces filamentous opaque cells switching to white phase. Opaque cells of the WT (SN250α), *bcr1/bcr1* and *cup9/cup9* mutants were plated onto Lee's glucose plates and cultured at the indicated temperatures for 5 days. Colony morphology of the WT (SN250α), *bcr1/bcr1* and *cup9/cup9* mutants is shown. Wh, white colonies; Op, opaque colonies; Mix, white and opaque mixed or sectorial colonies.

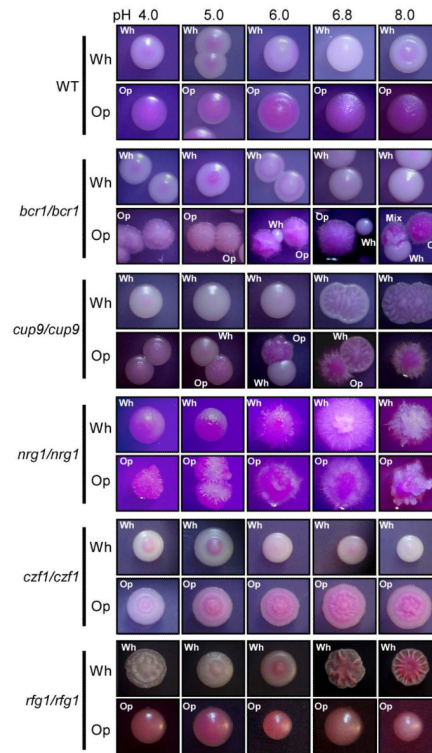


Fig. 6. Regulation of white and opaque cell filamentation by Bcr1, Cup9, Nrg1, Czf1 and Rfg1. White and opaque cells were plated onto Lee's glucose medium with different pH (4.0 to 8.0). The colony and cellular images of the WT (SN250 α), *bcr1/bcr1*, *cup9/cup9*, *nrg1/nrg1* and *rfg1/rfg1* were taken after incubation at 25°C for 5 days. The *czf1/czf1* mutant was incubated at 25°C for 8 days and imaged. Wh, white colonies; Op, opaque colonies. Cellular morphology of the mutants is shown in Fig. S3.

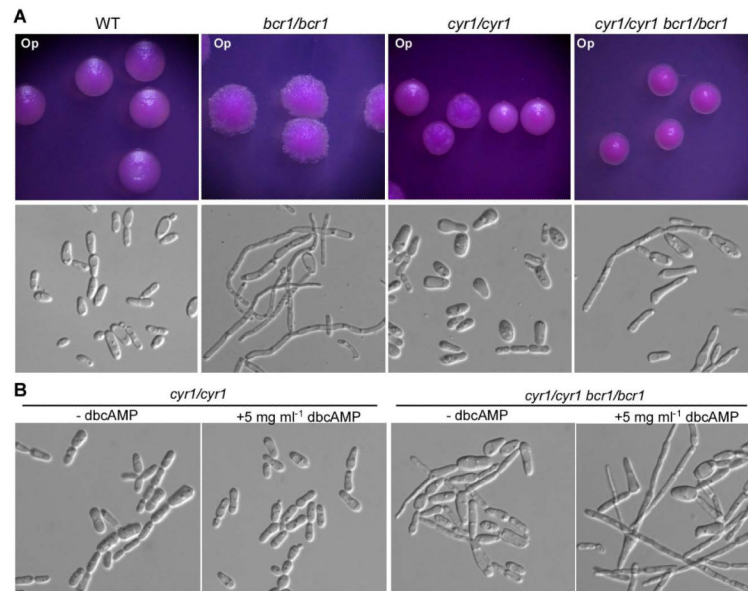


Fig. 7. cAMP signal plays a major role in Bcr1-regulated filamentation of opaque cells. A. Opaque cells of the *cyr1/cyr1 bcr1/bcr1* double mutant showed reduced filamentation. Cells were cultured on Lee's glucose medium plates. B. Dibutyryl-cAMP (dbcAMP) recovered the robust filamentation ability of opaque cells of the *bcr1/bcr1 cyr1/cyr1* double mutant. The liquid cultures of the *cyr1/cyr1* mutant and *bcr1/bcr1 cyr1/cyr1* double mutant in the presence and absence of dbcAMP or not were incubated at 25°C for 36 hours with shaking.

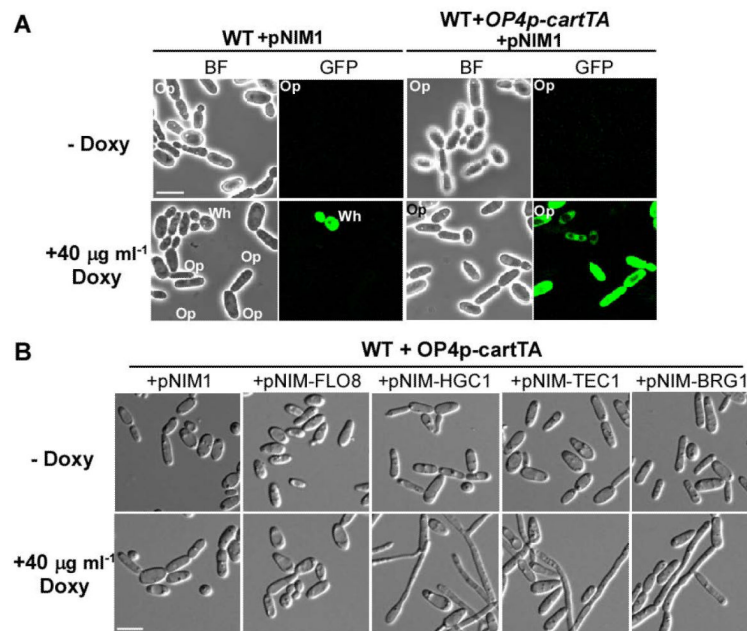


Fig. 9. Roles of overexpression of *FLO8*, *HGC1*, *TEC1*, and *BRG1* genes in opaque cell filamentation. WT, GH1013, an *MTLa/a* strain of SC5314 background. A. Expression of GFP protein in the WT+pNIM1 and WT+OP4p-cartTA+pNIM1 strains. To generate the WT+OP4p-cartTA+pNIM1 strain, a copy of the *cartTA* gene was integrated into the *OP4* (an opaque cell enriched gene) locus of GH1013. B. Cellular images of overexpression strains grown in inducing (with doxycycline) or non-inducing (without doxycycline) liquid Lee's glucose medium plates at 25°C for 36 hours. 40 µg ml⁻¹ doxycycline was added to the inducing medium. Scale bar, 10µm.

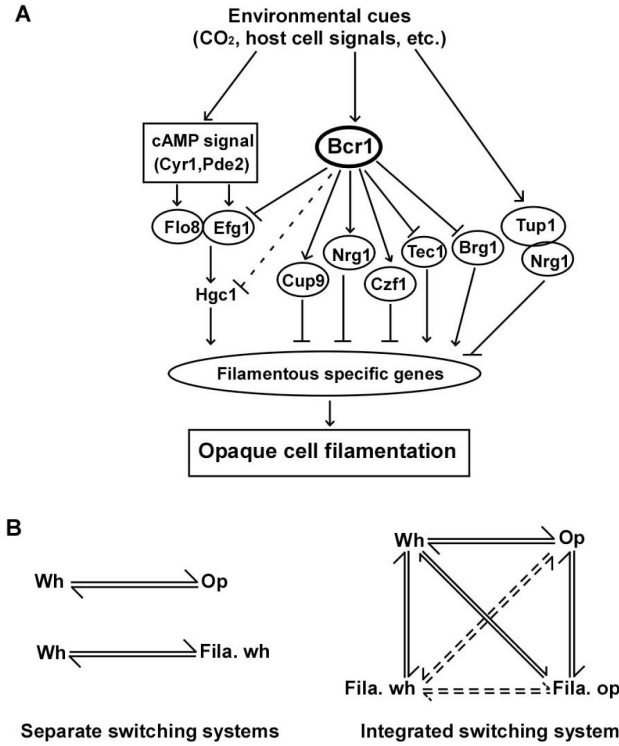


Fig. 10. Model of the regulation of opaque cell filamentation in *C. albicans*. A. Pathways involved in the regulation of opaque cell filamentation. Environmental cues signal the positive cAMP pathway and the negative pathway mediated by the key regulator Bcr1 to control the expression of filamentous specific genes. B. Models of separate and integrated phenotypic switching systems. Integration of the white-opaque switching and yeast-filamentous transition systems increases the complexity and plasticity of phenotypic switching in *C. albicans*. Solid lines represent confirmed phenotypic switches. Dashed lines represent unconfirmed switches. Wh, white cell phase; Op, opaque cell phase; Fila. wh, filamentous white cell phase; Fila. op, filamentous opaque cell phase.

Table 1

pH regulates white and opaque cell filamentation.

Strain	Cell phase	pH				
		4.0	5.0	6.0	6.8	8.0
WT	Wh	-	-	-	-	-
	Op	-	-	-	-	-
<i>bcr1/bcr1</i>	Wh	-	-	-	-	-
	Op	+++++	+++++	+++++	+++++	+++++
<i>cup9/cup9</i>	Wh	-	-	-	+++	++++
	Op	++	++	+++	++++	++++
<i>nrg1/nrg1</i>	Wh	+	++	++++	+++++	+++++
	Op	++++	+++++	+++++	+++++	+++++
<i>czf1/czf1</i>	Wh	-	-	-	-	-
	Op	++	++	++	++	++
<i>rfg1/rfg1</i>	Wh	+++	++	+	++++	++++
	Op	-	-	-	-	-

Cells were grown on Lee's glucose plates of pH4.0 to 8.0 as indicated for 5 days at 25°C. The robustness of filamentation was evaluated by examining the percentage and length of filamentous cells in a representative colony. Wh, white cells; op, opaque cells. "-" indicated no filamentous cells were observed. The number of "+" indicated the robustness of filamentation.