The transcription factor Flo8 mediates CO_2 sensing in the human fungal pathogen *Candida albicans*

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ABSTRACT **Physiological levels of CO_2 have a profound impact on prominent biological attributes of the major fungal pathogen of humans, *Candida albicans*. Elevated CO_2 induces filamentous growth and promotes white-to-opaque switching. However, the underlying molecular mechanisms of CO_2 sensing in *C. albicans* are insufficiently understood. Here we identify the transcription factor Flo8 as a key regulator of CO_2-induced morphogenesis in *C. albicans* by screening a gene null mutant library. We show that Flo8 is required for CO_2-induced white-to-opaque switching, as well as for filamentous growth. Ectopic expression of *FLO8* hypersensitizes *C. albicans* cells to the elevated CO_2 levels. Furthermore, we demonstrate that CO_2 signaling in *C. albicans* involves two pathways: the already reported cAMP/protein kinase A and another major one that is unidentified. The two pathways converge on the transcription factor Flo8, which is the master regulator of CO_2 sensing in *C. albicans* and plays a critical role in regulation of white-to-opaque switching and filamentous growth. Our findings provide new insights into the understanding of CO_2 sensing in pathogenic fungi that have important implications for higher organisms.**

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INTRODUCTION

To improve fitness, organisms must have the ability to sense and adapt to their natural environments. Carbon dioxide (CO_2) is a key signal for most living organisms. Not only is this molecule a product of cellular respiration, but it is also involved in the regulation of many biological processes (Hetherington and Raven, 2005; Bahn and Muhlschlegel, 2006; Sharabi *et al.*, 2009b). For instance, blood-feeding mosquitoes use environmental CO_2 as a cue for prey-seeking behavior (Gillies and Wilkes, 1969), whereas fruit flies detect CO_2 as an alarmone to elicit avoidance behavior (Turner and Ray, 2009). In animals, CO_2/bicarbonate activates sperm maturation and motility (Esposito *et al.*, 2004). Recent studies suggest that elevated CO_2 levels modulate lifespan in *Caenorhabditis elegans* and *Drosophila melanogaster* (Sharabi *et al.*, 2009a; Poon *et al.*, 2010). The CO_2 concentration in humans (4.5–30.0%) is >100 times higher than that in air (0.03%; Levitt and Bond, 1970), and it is now well established that CO_2 regulates the expression of virulence determinants such as filamentation or capsule biosynthesis in fungal pathogens of humans (Mardon *et al.*, 1969; Granger *et al.*, 1985; Bahn *et al.*, 2005; Bahn and Muhlschlegel, 2006; Klengel *et al.*, 2005; Huang *et al.*, 2006).

We previously showed that a high level of CO_2 also promotes *Candida albicans* white-to-opaque switching, thus facilitating mating (Huang *et al.*, 2009). White and opaque cells differ in many features, including colony and cellular appearances, mating competence, and virulence (Anderson and Soll, 1987; Kvaal *et al.*, 1997, 1999; Miller and Johnson, 2002; Soll *et al.*, 2003; Soll, 2004). To mate, *C. albicans* must undergo homozygosis at the mating type–like locus *MTL* and then switch from the white to the opaque form (Miller and Johnson, 2002).

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Abbreviations used: cAMP, cyclic adenosine monophosphate; GlcNAc, *N*-acetylglucosamine; MTL, mating type–like locus; PKA, protein kinase A.

A White to opaque switching frequencies (%) (Lee's + glucose medium)

Strain	air(0.03% CO_2) Swit. freq. (%)	Op colonies (%)	Mixed colonies (%)	Air +5% CO_2 Swit. freq. (%)	Op colonies (%)	Mixed colonies (%)
WT(GH1013)	0.9±0.2	0.7±0.4	0.5±0.7	84.1±6.2	10.6±0.6	73.3±6.9
cyr1/cyr1	0.3±0.4	<0.3	0.3±0.4	53.8±5.9	8.5±0.8	45.3±6.7
WT(WUM5A)	3.2±1.5	2.5±0.5	0.7±1.0	83.3±5.5	4.2±0.5	79.2±5.0
pde2/pde2	97.5±1.3	9.2±1.4	88.3±2.7	100.0±0.0	30.4±5.4	69.7±5.4

B Responses to CO_2 and GlcNAc in *pde2/pde2* mutant

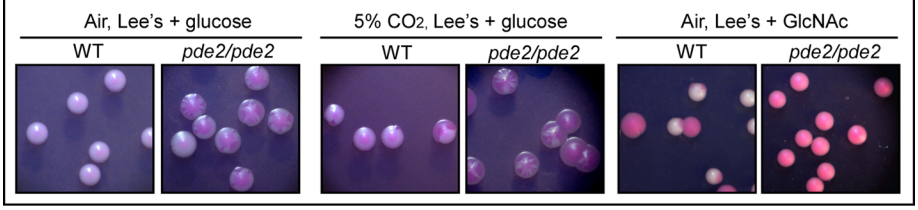

FIGURE 1: Effect of activation and inactivation of cAMP signaling on CO_2-induced white-to-opaque switching. (A) Switching frequencies (Swit. freq.) of the *cyr1/cyr1* (inactivation of cAMP signaling), *pde2/pde2* (activation of cAMP signaling) mutants, and their corresponding reference strains (WT) in air and 5% CO_2. The cells were grown on Lee's plus glucose agar plates with phloxin B at 25°C for 7 d. <, no colonies containing cells of alternative phenotype were observed. (B) Examples of colonies formed by the WT and *pde2/pde2* mutant on glucose agar in air, glucose agar in 5% CO_2, and GlcNAc agar in air. The cells were grown on agar plates with phloxine B at 25°C for 7 d.

CO_2-induced filamentation in *C. albicans* involves the fungal adenylyl cyclase Cyr1 (also named Cdc35; Klengel *et al.*, 2005; Hall *et al.*, 2010); however, CO_2-induced promotion of white-to-opaque switching is independent of this enzyme, as we recently showed (Huang *et al.*, 2009). In fact, the *CYR1*-null mutant showed white-to-opaque switching frequencies that were similar to those of the wild-type control when exposed to high levels of CO_2 (20%; Huang *et al.*, 2009). Recently we found that the Ras1-Cyr1 cAMP/protein kinase A (PKA) pathway is the major pathway regulating *N*-acetylglucosamine (GlcNAc)–induced white-to-opaque switching (Huang *et al.*, 2010). Of interest, CO_2 and GlcNAc showed a synergistic effect in promoting opaque cell formation (Huang *et al.*, 2010), suggesting that the major pathways involved in CO_2- and GlcNAc-induced white-to-opaque switching are different.

In spite of the importance of CO_2 in morphogenesis and pathogenesis, it is unclear how CO_2 is sensed and how the signal is transduced in *C. albicans*. In this study, we identify the transcription factor Flo8 as a master regulator of CO_2-induced morphogenesis in *C. albicans* by screening a null mutant library for nearly 200 genes with combined analysis of white-to-opaque switching and filamentous growth. Flo8, a LisH domain–containing protein, has been reported to be essential for filamentous growth and virulence in *C. albicans* (Cao *et al.*, 2006). Here we find that Flo8 is required for CO_2-induced white-to-opaque switching, as well as for filamentous growth, via different downstream regulators. Further experiments demonstrate that Wor1 and Wor2 function downstream of Flo8 and are essential for CO_2-induced white-to-opaque switching. Efg1 and Hgc1 are required for CO_2-induced filamentous growth. We also provide evidence indicating that the adenylyl cyclase Cyr1 is not essential for the activating function of Flo8, suggesting the presence of a major, Cyr1-independent, CO_2-sensing pathway.

RESULTS

Roles of adenylyl cyclase and high-affinity cyclical AMP phosphodiesterase in CO_2-regulated white-to-opaque switching

The cells of the *cyr1/cyr1* mutant have no detectable cAMP levels (Rocha *et al.*, 2001), whereas the cells of the mutant of *PDE2* gene, encoding the high-affinity cyclical AMP phosphodiesterase gene in *C. albicans*, show constitutively high levels of cAMP (Jung and Stateva, 2003). These two mutants respectively represent the inactivated and activated states of cAMP signaling. The switching frequencies can be easily quantified because of the bistable feature of the white-to-opaque transition (Slutsky *et al.*, 1987; Huang *et al.*, 2006). Therefore the CO_2-induced responses can be indicated by the increased fold of switching frequencies even in the mutants with varied basal levels of switching ability.

To evaluate the role of cAMP signaling in CO_2 sensing, we first tested the responses to 5% CO_2 in the *cyr1/cyr1* mutant by using white-to-opaque phenotypic switching assay. Here the switching frequency was defined as the percentage of the colonies containing cells of alternative phenotype. The switching frequencies are shown in Figure 1A. Consistent with our previous study (Huang *et al.*, 2009), the white-to-opaque switching frequency of the *cyr1/cyr1* mutant in 5% CO_2 (53.8 ± 5.9%) was 179 times higher than in air (0.3 ± 0.4%), although it was lower than for the wild-type (WT) control in 5% CO_2 (84.1 ± 6.2%). According to our previous study, the frequencies of the *cyr1/cyr1* mutant in 1 and 20% CO_2 were 0.3 ± 0.3 and 97.6 ± 0.8%, respectively (Huang *et al.*, 2009). These results suggest that cAMP is not required for high levels of CO_2-induced opaque cell formation but is required for low levels of CO_2-induced efficient switch. To further evaluate the roles of cAMP signaling in CO_2-induced response, we examined the switching ability of the *pde2/pde2* mutant, in which the cAMP level was constitutively high (Jung and Stateva, 2003). We found that the white-to-opaque switching frequency of the *pde2/pde2* mutant was almost 100% both in air and in 5% CO_2. Noticeably, although 5% CO_2 increased the homogeneous opaque colony formation in the *pde2/pde2* mutant, the percentage of mixed or sectored colonies remained very high (69.7 ± 5.4%; Figure 1, A and B). Consistent with our previous results, we found that the *pde2/pde2* mutant formed 100% homogeneous opaque colonies on nutrient agar containing GlcNAc, which has been proved to stimulate opaque cell formation primarily via the cAMP pathway (Huang *et al.*, 2010; Figure 1B). These results indicate that constitutive activation of cAMP signal by deletion of *PDE2* gene hypersensitizes *C. albicans* cells to GlcNAc but not to the elevated levels of CO_2. On the basis of the data presented here and our previous reports (Huang *et al.*, 2009, 2010), we suggest that whereas the cAMP signaling plays a critical role in white-to-opaque switching, it is not the major pathway for high CO_2 concentration–induced white-to-opaque switching in *C. albicans*.

Screen for the CO_2-sensing regulators

We were interested to know what pathways or key regulators predominantly control CO_2-regulated *C. albicans* morphogenesis. To

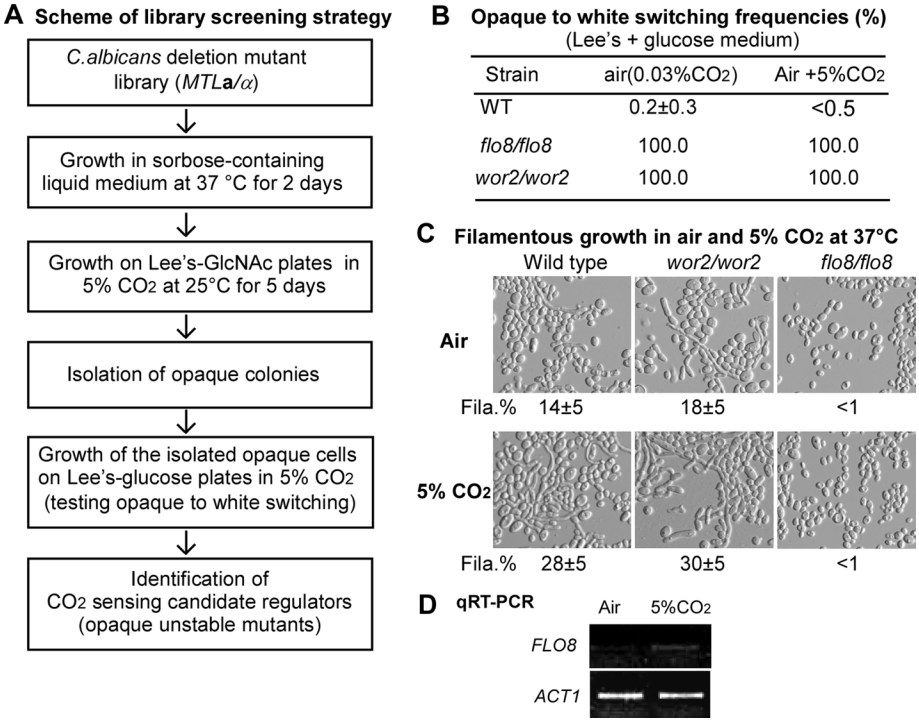

FIGURE 2: A library screening identified the transcription factor Flo8 as a master regulator of CO_2-induced morphogenesis in *C. albicans*. (A) Scheme of library screening strategy. To revive the library strains from glycerol stock at −80°C, the cells were first grown on YPD plates for 2 d at 30°C. (B) Opaque-to-white switching frequencies (given as percentages) of the *flo8/flo8* and *wor2/wor2* mutants on Lee's plus glucose agar plates in air and in 5% CO_2. The cells were grown on agar plates with phloxin B at 25°C for 6 d. The *flo8/flo8* and *wor2/wor2* mutants, isolated from the screening in A, were *MTL***a**/a at the mating type–like locus. The wild-type strain (*MTL***a**/a) was used as the control. <, no colonies containing cells of alternative phenotype were observed. (C) Filamentous growth of the *flo8/flo8* and *wor2/wor2* mutants (*MTL***a**/α) on Lee's plus glucose agar plates in air and in 5% CO_2. Two thousand cells of each strain in 3 μl of double-distilled H_2O were dropped and grown on agar plates at 37°C for 2 d. The wild-type strain (166 in Table S1, *MTL***a**/α) was used as the control. Fila.%, percentage of filamentous cells. (D) Semiquantitative reverse transcription (RT)-PCR. Two thousand cells of a wild-type strain were dropped onto Lee's glucose plates and cultured in air and in 5% CO_2 for 48 h at 37°C. Total RNA was extracted and used for quantitative RT-PCR (25 cycles).

this end, we screened a library containing 197 null mutants for transcription factors, kinases, and other proteins by using the white-to-opaque switching assay.

Most of the mutants we used were heterozygous at the *MTL* locus and not switching competent. The *MTL* locus is on chromosome 5, which can be reduced to monosomy in sorbose-containing medium and then become homozygous at the *MTL* locus through chromosome duplication (Janbon *et al.*, 1998; Magee and Magee, 2000). To convert the mutants to *MTL*-homozygous cells, we first grew the mutant cells in liquid YPS medium (1% yeast extract, 2% peptone, and 10% sorbose) at 37°C for 48 h. The reported method for identification of *MTL*-homozygous isolates is laborious and inefficient (Magee and Magee, 2000). Genomic DNA has to be extracted from each isolate and then be used for PCR of *MTL***a** and *MTL*α genes. Given the synergistic effect of GlcNAc and CO_2 on induction of opaque cell formation (Huang *et al.*, 2010), we hypothesized that most *MTL*-homozygous cells resulting from sorbose treatment would grow as opaque colonies on GlcNAc-containing medium in 5% CO_2. This method was proved to be very efficient. We found that most of the mutants (95.5%) formed opaque colonies on GlcNAc medium in 5% CO_2. Four examples are shown in Supplemental Figure S1A. To confirm that the opaque isolates were homozygous at *MTL*, we randomly selected 10 mutants for *MTL* locus PCR. They were all *MTL***a** or α homozygous (Supplemental Figure S1B, lanes 2–11). An *MTL***a**/α reference strain served as control for PCR (Supplemental Figure S1B, lane 1). A few strains of the library failed to form opaque colonies either because they failed to grow in YPS or GlcNAc media or underwent constitutive filamentous growth (such as *sfl1/sfl1* and *tup1/tup1*mutants). The *wor1/wor1* mutant, which is locked in white phase under all conditions (Huang *et al.*, 2006), served as the negative control.

CO_2 not only induces opaque cell formation, but it also stabilizes the opaque phenotype (Huang *et al.*, 2009). The induction of white-to-opaque switching requires relatively high levels of CO_2, and the efficiencies vary greatly in different natural strains and mutants, whereas the maintenance of the opaque phenotype needs very low levels of CO_2 (<1%; Huang *et al.*, 2009, and unpublished data). We hypothesized that the opaque cells of the CO_2-sensing candidate gene mutants should be unstable even when cultured in high levels of CO_2 in the absence of GlcNAc (since GlcNAc stabilized the opaque phenotype via a pathway different from that of CO_2). We therefore replated the opaque cells of the mutants initially grown on GlcNAc medium onto Lee's medium plus glucose plates and incubated in 5% CO_2. The opaque-to-white switching frequencies of the majority were <4% (Supplemental Table S1). We noted that the mutants of orf19.3809, orf19.1032, and 19.2088 showed the switching frequencies of 4.1, 9.4, and 4.2%, respectively. Surprisingly, the *wor2/wor2* and *flo8/flo8* mutants completely converted to white phase (frequency, 100%; Supplemental Table S1). To confirm these results, we performed the opaque-to-white switching experiments in the *wor2/wor2* and *flo8/flo8* mutants by using a standard method (Huang *et al.*, 2009). The switching frequencies of the two mutants were 100% in either air or 5% CO_2 on glucose medium, whereas the frequency of the WT control was <1% under both culture conditions (Figure 2B). These data indicated that Wor2 and Flo8 might be key candidates of CO_2-sensing regulators.

The transcription factor Flo8 carries a conserved LisH domain, which is found in many eukaryotic proteins with a wide range of functions. In *C. albicans*, Flo8 is essential for virulence and controls filamentous growth under a wide variety of culture conditions, including Lee's medium, serum, and GlcNAc induction (Cao *et al.*, 2006). Wor2 is a zinc finger transcription factor, which is essential for maintenance of the opaque state in glucose medium (Zordan *et al.*, 2007). To ensure the roles of Flo8 and Wor2 in the CO_2-sensing process, we examined whether these two transcription factors were required for CO_2-induced filamentous growth on Lee's plus glucose plates. The *flo8/flo8* mutant failed to undergo filamentous growth both in air and in 5% CO_2, whereas the *wor2/wor2* mutant and the WT control formed filamentous colonies under both conditions (unpublished data). In addition, CO_2 induced more wrinkled morphologies in the

A White to opaque switching frequencies (%)

Medium	Strain	air(0.03% CO_2) Swit. freq. (%)	Op colonies (%)	Mixed colonies (%)	Air +5% CO_2 Swit. freq. (%)	Op colonies (%)	Mixed colonies (%)
Lee's + glucose	WT	11.4±2.7	2.0±0.2	9.5±2.5	86.1±0.4	15.9±1.7	70.2±1.3
	flo8/flo8	<0.3	<0.3	<0.3	<0.4	<0.4	<0.4
Lee's + GlcNAc	WT	84.1±4.7	81.6±4.8	2.5±0.9	100.0±0.0	100.0±0.0	<0.1
	flo8/flo8	83.8±6.0	81.9±3.9	2.0±2.2	100.0±0.0	100.0±0.0	<0.2

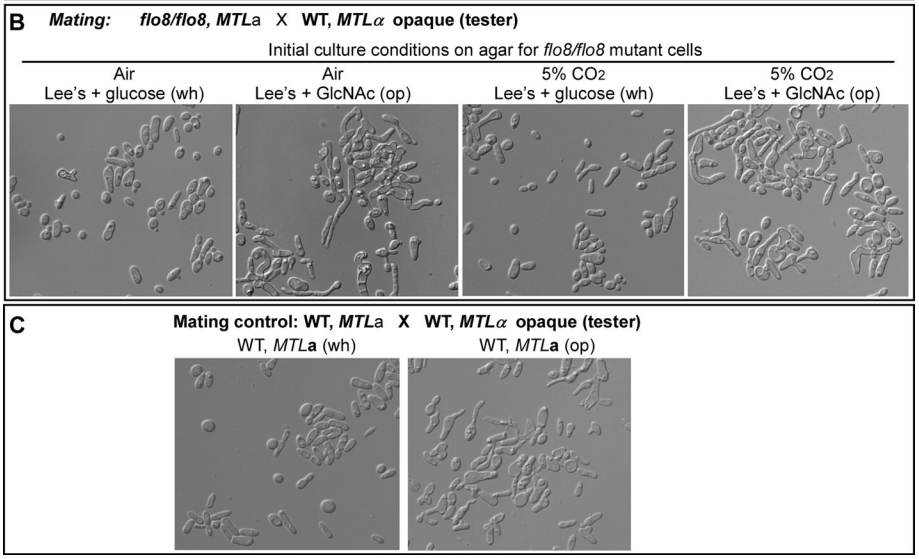

FIGURE 3: White-to-opaque switching of the WT and *flo8/flo8* mutant in air and in 5% CO_2. (A) White-to-opaque switching frequencies. The cells were grown on agar plates with phloxin B at 25°C for 6 d. The strains used were *MTL***a**/**a** at the mating type–like locus. For switching on GlcNAc agar, aged cells (5 d old in liquid Lee's glucose medium) were used for plating. <, no colonies containing cells of alternative phenotype were observed. (B) Mating of the *flo8/flo8* mutant. The mating experiments were performed in liquid glucose medium at 25°C for 24 h. The *flo8/flo8* cells were collected from typical colonies on agar plates cultured under different conditions as indicated. The tester was GH1349, an *MTL*α/α strain, in the opaque state. (C) Mating of the wild type in white and opaque states. This experiment was used as a control experiment to B. Opaque and white cells of the wild-type *MTL***a**/**a** strain grown on glucose agar were used for this experiment. The tester was GH1349 in the opaque state.

last two strains. The cellular phenotypes are shown in Figure 2C. The ratios of filamentous cells of the WT and *wor2/wor2* mutant increased from 14 ± 5 to 28 ± 5% and from 18 ± 5 to 30 ± 5%, respectively, whereas the majority of *flo8/flo8* mutant cells exhibited no filamentous growth. These results suggest that Wor2 is not required for CO_2-mediated filamentous growth but is required for white-to-opaque switching. Semiquantitative reverse transcription PCR demonstrated that 5% CO_2 obviously increased *FLO8* expression in a WT strain, suggesting that the expression of *FLO8* is regulated by the concentration of environmental CO_2 (Figure 2D).

As mentioned, the opaque phase of orf19.3809, orf19.1032, and 19.2088 mutants was relatively unstable in 5% CO_2, but we found that their white-to-opaque switching frequencies were remarkably increased when cultured under the same condition (unpublished data). These results suggest that the three genes are not essential for CO_2-induced responses and can be excluded as CO_2-sensing regulators.

Flo8 is required for CO_2-induced white-to-opaque switching and efficient mating in glucose medium

To investigate the role of Flo8 in the white-to-opaque switching process, we plated the cells of *flo8/flo8* mutant and the WT onto glucose medium and GlcNAc medium. No opaque or opaque-sectored colonies of the *flo8/flo8* mutant were observed on glucose-medium plates both in air (switching frequency, <0.3%) and in 5% CO_2 (switching frequency, <0.4%), whereas the switching frequencies of the WT were 11.4 ± 2.7% in air and 86.1 ± 0.4% in 5% CO_2 (Figure 3A). On GlcNAc medium in air, the switching frequencies of the *flo8/flo8* mutant and the WT were 83.8 ± 6.0 and 84.1 ± 4.7%, respectively. When cultured on GlcNAc medium in 5% CO_2, both the mutant and the WT formed 100% opaque colonies (Figure 3A). These results demonstrate that Flo8 is essential for CO_2-induced opaque cell formation but is not required for GlcNAc-stimulated switching, which has been shown to play a role mainly via the Ras1-cAMP pathway (Huang *et al.*, 2010).

Only opaque cells can undergo efficient mating in *C. albicans* (Miller and Johnson, 2002). To further confirm that the *flo8/flo8* mutant (*MTL***a**/**a**) was capable of forming bona fide opaque cells on GlcNAc medium, we tested the mating ability of the representative colonies collected from glucose-medium plates and GlcNAc-medium plates. The mating responses were indicated by the formation of "shmoos" and "conjugation tubes." As shown in Figure 3B, the *flo8/flo8* mutant cells from GlcNAc-medium plates cultured both in air and in 5% CO_2 could undergo efficient mating with an *MTL*α opaque tester, whereas the cells from glucose medium showed poor mating ability under both culture conditions. The mating control experiments of the WT white and opaque cells are shown in Figure 3C. As expected, the WT opaque, but not white, underwent efficient mating. These data demonstrate that the *flo8/flo8* mutant has the basal level of switching ability and formed bona fide opaque colonies on GlcNAc medium plates.

Ectopic expression of *FLO8* hypersensitizes *C. albicans* cells to elevated CO_2 levels

We showed that deletion of *FLO8* blocked CO_2-induced opaque cell formation and filamentous development. Given that Flo8 is the key CO_2-sensing regulator, we reasoned that overexpression of *FLO8* in a wild-type strain should increase the sensitivity to elevated CO_2 levels. To test this hypothesis, we first examined the white-to-opaque switching frequencies of the strains WT+vector and WT+TETp-FLO8 in air and in 1% CO_2. Consistent with our previous study, the WT+vector control showed low switching frequencies (<5%) in air either under the noninducing condition (without doxycycline) or under the inducing condition (with 50 μg/ml doxycycline). At 1% CO_2, the switching frequencies of the WT+vector control were increased to 48.7 ± 6.8 and 39.2 ± 9.1% in the absence and presence of 50 μg/ml doxycycline, respectively. Under the noninducing condition, the switching frequency of the WT+TETp-FLO8 overexpression strain was similar as that of the WT+vector control either in air or in 1% CO_2. Under the inducing condition, the switching frequency of the strain WT+TETp-FLO8 was increased to 100%

A White to opaque switching frequencies (%)

Air (0.03% CO_2)	0 μg/ml Dox			50 μg/ml Dox		
Strain	Swit. freq. (%)	Op colonies (%)	Mixed colonies (%)	Swit. freq. (%)	Op colonies (%)	Mixed colonies (%)
WT+vector	1.1±0.1	<0.6	1.1±0.1	4.3±0.1	<0.5	4.3±0.1
WT+TETp-FLO8	5.1±0.7	<0.5	5.1±0.7	100.0±0.0	2.4±0.4	97.6±0.4
Air +1% CO_2						
	0 μg/ml Dox			50 μg/ml Dox		
Strain	Swit. freq. (%)	Op colonies (%)	Mixed colonies (%)	Swit. freq. (%)	Op colonies (%)	Mixed colonies (%)
WT+vector	48.7±6.8	2.8±1.0	45.9±5.8	39.2±9.1	2.5±1.0	36.7±8.1
WT+TETp-FLO8	53.3±3.9	2.7±1.2	50.6±5.1	100.0±0.0	100.0±0.0	<0.3

B Examples of colony and cellular pictures in A

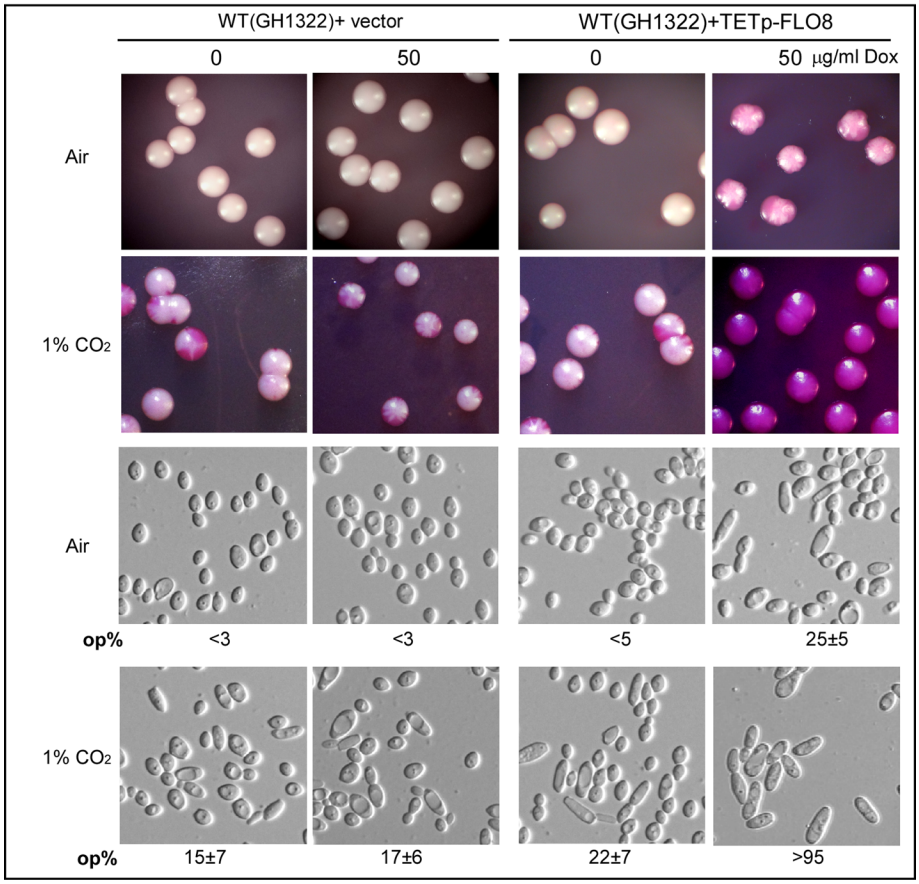

FIGURE 4: Overexpression of *FLO8* stimulates white-to-opaque switching in 1% CO_2. (A) White-to-opaque switching frequencies in air and in 1% CO_2. The cells were grown on glucose agar with phloxin B at 25°C for 6 d. The WT strain used was WUM5A, an *MTL*α/α strain derived from the clinically isolated strain WO-1. <, no colonies containing cells of alternative phenotype were observed. (B) Examples of colony and cellular phenotypes in A. Cells collected from a representative plate of each strain were mixed and imaged. Op%, percentage of opaque cells.

both in air and in 1% CO_2. Remarkably, when cultured in air under the inducing condition, the strain WT+TETp-FLO8 mainly formed highly opaque-sectored colonies (97.6 ± 0.4%), whereas it formed 100% homogeneous opaque colonies in 1% CO_2 (Figure 4, A and B). The cellular phenotypes of a representative plate of each strain and the percentages of opaque cells are also shown in Figure 4B. Although the switching frequencies of the strain WT+TETp-FLO8 under inducing condition both in air and in 1% CO_2 were 100%, the percentage of opaque cells of a representative plate in CO_2 (>95%) was much higher than that in air (20 ± 5%). These results suggest that overexpression of *FLO8* leads to the hypersensitivity of *C. albicans* cells to elevated CO_2 levels.

To further prove that overexpression of *FLO8* contributes to the increase of sensitivity to elevated CO_2 levels in *C. albicans*, we introduced the plasmid pNIM-FLO8 (TETp-FLO8) into a natural *MTL***a**/α strain SC5314 and examined the effect of overexpressing *FLO8* on filamentous development. The WT (SC5314) carrying the empty vector or TETp-FLO8 underwent robust filamentous growth at 37°C either in air or in elevated levels of CO_2, which would skew the examination. We did the experiments at 25°C, a temperature not conducive to filamentous growth, which allowed us to examine the effect of overexpressing *FLO8*. The WT+vector control formed only smooth colonies containing yeast cells in air and in 5% CO_2 under either noninducing condition or inducing condition. The WT+TETp-FLO8 strain also developed smooth colonies in air under both inducing and noninducing conditions. In 5% CO_2, whereas the WT+TETp-FLO8 strain primarily formed smooth colonies under noninducing condition, it indeed grew as rough and wrinkled colonies containing both yeast and filamentous cells under inducing condition (Figure 5). The cellular morphology confirmed that the colonies formed by the WT+TETp-FLO8 strain under inducing condition in 5% CO_2 contained filamentous cells (Figure 5). These data indicate that overexpression of *FLO8* also promotes CO_2-induced filamentous growth besides opaque cell formation.

Flo8-promoted filamentous growth partially bypasses cAMP signaling

cAMP signaling has been reported to be required for CO_2-induced filamentous growth in *C. albicans* (Klengel *et al.*, 2005). *C. albicans* Flo8 is a *Saccharomyces cerevisiae* Flo8 homologue, which is a target of the cAMP/PKA pathway required for pseudohyphal development (Liu *et al.*, 1996; Pan and Heitman, 1999; Cao *et al.*, 2006). By physical interaction with Efg1, a transcription factor regulated by the catalytic subunit of cAMP-dependent protein kinase Tpk2 (Bockmuhl and Ernst, 2001), *C. albicans* Flo8 has been indicated to function downstream of the cAMP/PKA pathway and regulate hyphal development (Cao *et al.*, 2006). To test whether Flo8-mediated CO_2 sensing is dependent on the cAMP signaling, we overexpressed *FLO8* in WT and a *cyr1/cyr1* mutant and examined the effect of CO_2 on filamentous growth. Because the filamentous growth ability of the *cyr1/cyr1* mutant is very poor, we did this experiment at 37°C for 5 d. Compared to the WT+vector control, the WT+TETp-FLO8 strain underwent more robust filamentous growth under inducing condition either in air or in 5% CO_2 (unpublished data). As

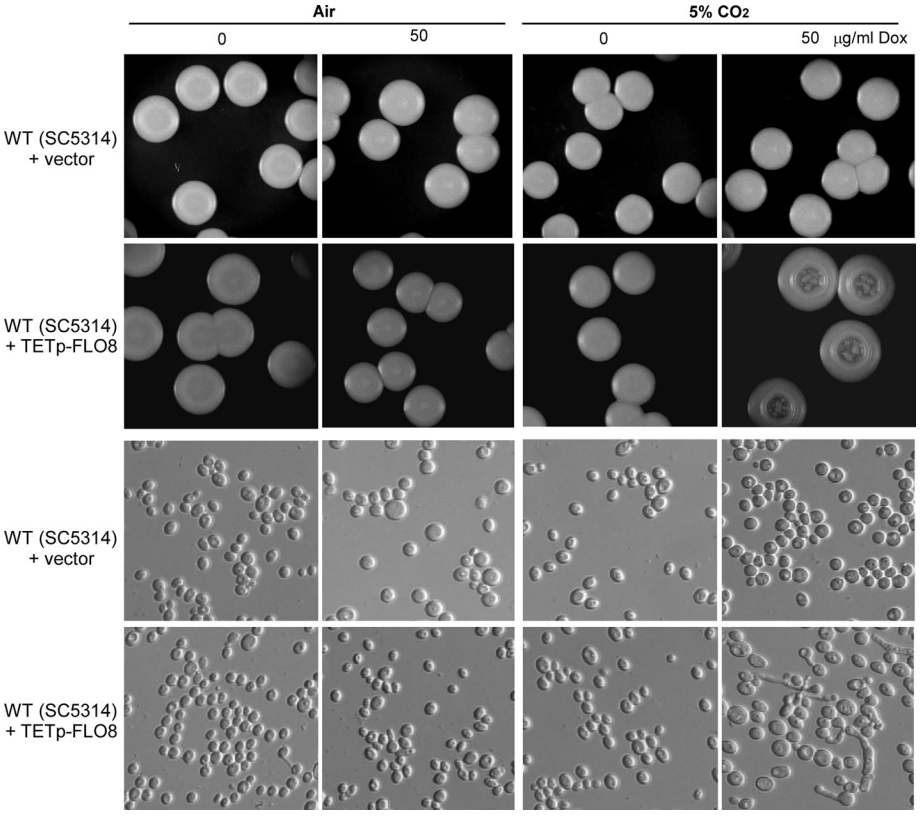

FIGURE 5: Overexpression of *FLO8* induces filamentous growth at 25°C in 5% CO_2. The cells were grown on Lee's plus glucose agar in air and in 5% CO_2 at 25°C for 5 d. The WT strain was SC5314, a clinically isolated *MTL*a/α strain. The cellular phenotypes of a representative colony under each culture condition are also shown.

expected, the *cyr1/cyr1*+vector strain grew as smooth yeast colonies under all conditions. Although the *cyr1/cyr1*+TETp-FLO8 overexpression strain grew as yeast colonies under noninducing condition, it formed obviously wrinkled filamentous colonies under inducing condition both in air and in 5% CO_2 (Figure 6). Of note, under inducing conditions, the filamentous growth of the overexpression mutant was more evident in 5% CO_2 than in air. The cellular morphology showed that overexpression of *FLO8* indeed induced filamentous growth (including elongated cells, hyphae, and pseudohyphae) in the *cyr1/cyr1* mutant (Figure 6). To verify the effect of overexpressing *FLO8* in CO_2, we did the experiments by using a different method. To avoid secondary effects, we dropped 3 μl of double-distilled H_2O containing 2000 cells of the strains on Lee's plus glucose and cultured them for 2 d at 37 °C (rather than 5 d). The cellular phenotypes and the percentage of filamentous cells of the solid cultures are shown in Supplemental Figure S2. The ratio of filamentous cells of the *FLO8* overexpression strains in 5% CO_2 was obviously higher than that in air. The filamentous growth of the *cyr1/cyr1*+TETp-FLO8 strain in air might represent the effect induced by low concentration of CO_2 in air or the metabolically produced CO_2 inside the colony. These results suggest that Flo8 mediates CO_2-induced filamentous growth and at least partially bypasses Cyr1 or the cAMP signal.

Roles of Efg1 and Hgc1

The opaque phase of the *efg1/efg1* and *hgc1/hgc1* mutants were stable in air as well as in 5% CO_2, suggesting that Efg1 and Hgc1were not involved in CO_2-induced opaque cell formation. Cao *et al.* (2006) reported that Flo8 physically interacts with Efg1, and the two proteins play a similar role in regulation of filamentous growth. Hgc1 is a G1 cyclin-related protein and is required for hyphal growth (Zheng and Wang, 2004). The expression of *HGC1* is regulated by Flo8. Northern blot and microarray analysis indicated that deletion of *FLO8* blocked expression of *HGC1* in *C. albicans* (Cao *et al.*, 2006). To test whether these two genes were involved in CO_2-induced filamentous growth, we cultured the *efg1/efg1* and *hgc1/hgc1* mutants on Lee's glucose medium in air and in 5% CO_2. The WT and the *flo8/flo8* mutant were used as positive and negative controls, respectively. In contrast to the WT reference strain, the *flo8/flo8*, *efg1/efg1*, and *hgc1/hgc1* mutants could not undergo invasive (Supplemental Figure S3A) and filamentous (Supplemental Figure S3B) growth either in air or in 5% CO_2. Overexpression of *EFG1* in the *flo8/flo8* mutant had no effect on filamentous growth either in air or in 5% CO_2 (unpublished data), suggesting that Flo8 was required for the function of the Flo8–Efg1 complex. However, overexpression of *HGC1* in the *flo8/flo8* mutant induced filamentous growth both in air and in 5% CO_2 (Supplemental Figure S4), suggesting that Hgc1 is downstream of Flo8.

Roles of Wor1 and Wor2

Although Wor1 and Wor2 were not required for CO_2-induced filamentous growth, they were required for the white-to-opaque transition. The CO_2 signal must be finally passed to the master regulator Wor1 to induce opaque cell formation. Overexpression of *WOR1* in the *flo8/flo8* mutant promoted white-to-opaque switching in air or in 5% CO_2 (unpublished data), suggesting that Wor1 functioned downstream of Flo8. Overexpression of *WOR2* in the *flo8/flo8* mutant had no notable effect on switching (unpublished data), suggesting that Flo8 and Wor2 might function in different pathways.

DISCUSSION

CO_2 is a ubiquitous molecule and an important environmental cue to all living organisms. The concentration of CO_2 in the human host is much higher than that in the ambient atmosphere (Levitt and Bond, 1970). To better survive and colonize in the host, *C. albicans* must be capable of sensing and responding to ever-changing CO_2 levels. In this study, we set out to explore the molecular mechanisms of CO_2 sensing and how the CO_2 signaling is transduced in *C. albicans*. Taking advantage of the unstable feature of *C. albicans* chromosome 5 in sorbose-containing medium and the synergistic effect of GlcNAc and CO_2 on induction of opaque cell formation (Janbon *et al.*, 1998; Huang *et al.*, 2010), we developed a simple and efficient method to convert *MTL*-heterozygous into *MTL*-homozygous strains. This method is very useful for studies of white-to-opaque switching and mating, since the majority of gene mutants are made in the *MTL***a**/α background strain SC5314 and its derivatives. By screening the null-mutant library homozygous for *MTL*, we identified the transcription factor Flo8 as a key regulator of CO_2-induced morphogenesis in this fungal pathogen. Flo8 was

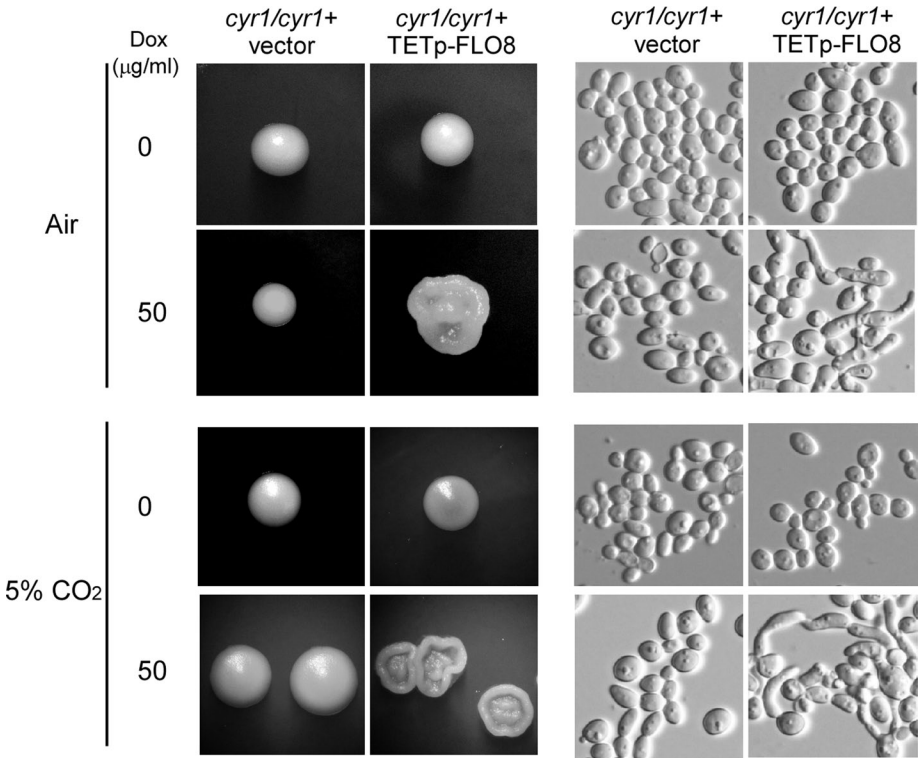

FIGURE 6: Upstream pathways of Flo8 in CO_2 sensing and morphogenesis regulation. The adenylyl cyclase Cyr1 (cAMP) was not required for Flo8-induced filamentous growth. The cells were grown on Lee's plus glucose agar with or without 50 μg/ml doxycycline. The plates were incubated in air or in 5% CO_2 at 37°C for 5 d before acquiring the images. The cellular phenotypes of a representative colony under each culture condition are also shown.

initially characterized as a critical filamentous growth regulator (Cao *et al.*, 2006). Here we showed that Flo8 governs white-to-opaque switching, as well as filamentous development, in response to elevated levels of CO_2 via distinct downstream regulators. Hyphal-specific regulators Efg1 and Hgc1 mediate CO_2-induced filamentous growth, and opaque-specific regulators Wor1 and Wor2 are required for CO_2-induced white-opaque switching.

We showed that the transcription factor Flo8 plays a central role in CO_2 sensing and morphogenesis in *C. albicans*. First, deletion of the *FLO8* gene specifically blocked white-to-opaque switching induced by CO_2 but had no effect on GlcNAc induction. These data suggest that Flo8 is not a general regulator required for opaque cell formation under all circumstances but is a component specifically essential for CO_2 induction. Second, overexpression of the *FLO8* gene sensitized the cells to the elevated levels of CO_2 and induced mass conversion of white to opaque. Of interest, we also observed an increase of the white-to-opaque switching frequency of the *FLO8* overexpression strain in air. This hypersensitivity to CO_2 could be an effect resulting from accumulated metabolic CO_2 inside colonies. In a recent study, Hall *et al*. demonstrated that there is an accumulation of metabolic CO_2 inside a fungal biomass, and the CO_2 released by metabolism is sufficient to induce the growth of the *nce103/nce103* mutant, which cannot grow under circumstances of low CO_2 levels as in the ambient atmosphere (Klengel *et al.*, 2005; Huang *et al.*, 2009; Hall *et al.*, 2010). When the colonies initially formed, the quantity of CO_2 released by the cells was very low and diffused into the air. Therefore the cells of the WT+TETp-FLO8 colonies initially maintained in white form in air even under the inducing condition. With the growth of the colonies, metabolic CO_2 accumulated inside the colonies and then signaled the cells of the WT+TETp-FLO8 strain to switch to opaque, which resulted in the highly opaque–sectored phenotype observed later. Third, the effect of deletion and overexpression of *FLO8* on filamentous growth further confirmed that Flo8 plays a critical role in CO_2 sensing. Fourth, the cAMP/PKA pathway, which primarily mediates GlcNAc induction in white-to-opaque switching and filamentous growth, plays a minor role in CO_2 sensing (this study; Cassone *et al.*, 1985; Huang *et al.*, 2010). We propose that there must exist a distinct unidentified pathway predominantly responsible for CO_2 sensing and regulation of Flo8 in *C. albicans* (Figure 7). Of interest, the existence of a cAMP-independent, CO_2-sensing pathway in *C. albicans* was also recently reported (Cottier *et al.*, 2012). In this study, the authors show that the transcriptional regulator Rca1 induces the expression of the carbonic anhydrase Nce103 in response to low environmental CO_2 concentration independent of the cAMP/PKA pathway. Given that Flo8 and Rca1 are both transcriptional regulators under the control of a yet-uncharacterized CO_2-sensing pathway, one could hypothesize the existence of a common pathway resulting in the control of different outcomes like the white-to-opaque switch and control of *NCE103* expression.

According to our results in this study and previous reports (Huang *et al.*, 2009, 2010), several lines of evidence further demonstrate that the unidentified pathway is independent of cAMP signaling. First, deletion of the adenylyl cyclase gene *CYR1* blocks cAMP synthesis but not cell growth (Rocha *et al.*, 2001), suggesting that cAMP is not essential for the survival of *C. albicans*. However, deletion of *NCE103*, a gene encoding the carbonic anhydrase catalyzing the conversion of CO_2 into HCO_3^-, blocks cell growth in air (Rocha *et al.*, 2001; Huang *et al.*, 2009), suggesting that CO_2 is an essential molecule for *C. albicans* growth. Therefore at least some biological processes (e.g., cell growth) in which CO_2 is involved are independent of cAMP signaling in *C. albicans*. Second, Cyr1 is not required for CO_2-regulated white-to-opaque switching and stabilization of the opaque phenotype (Huang *et al.*, 2009; Supplemental Table S1 and Figure 1). Third, constitutive activation of the cAMP signal by deletion of the high-affinity cAMP phosphodiesterase gene *PDE2* did not sensitize the cells to the elevated levels of CO_2, whereas the mutant indeed became hypersensitive to the cAMP pathway activator GlcNAc (Figure 1; Huang *et al.*, 2010).

Previous studies indicated that Flo8 functions downstream of the cAMP/PKA pathway in both *C. albicans* and *S. cerevisiae* (Pan and Heitman, 1999; Cao *et al.*, 2006). Klengel *et al.* (2005) showed that CO_2 directly activates the adenylyl cyclase and then stimulates hyphal growth in *C. albicans*. By sequence analysis, we found three putative PKA phosphorylation sites at the C-terminal of *C. albicans* Flo8: KKES (513–516), KRKS (603–606), and KKES (649–652). However, the experiment on the ectopic expression of *FLO8* in the *cyr1/cyr1* mutant proves that Flo8 regulates CO_2-induced morphogenesis at least partially bypassing cAMP signaling (Figure 6). It is also notable that the filamentous growth of the *cyr1/cyr1*+TETp-FLO8 strain was not as robust as that of the WT+TETp-FLO8 strain. This might be

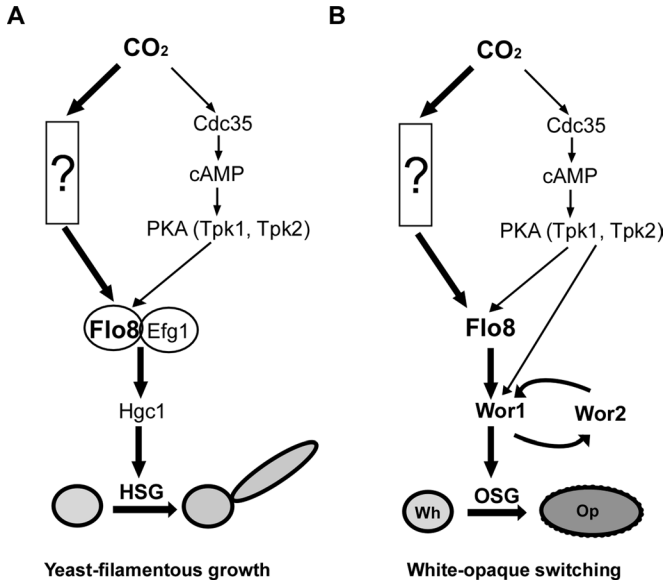

FIGURE 7: Models of CO_2 mediated morphogenesis in *C. albicans*. The unidentified pathway represents the major pathway involved in CO_2-induced responses. The cAMP signaling and the unidentified pathway converge on the key regulator Flo8 in the regulation of white-to-opaque switching, whereas the two pathways converge on the Flo8–Efg1 complex in the regulation of filamentous growth. HSG, hyphal-specific genes; OSG, opaque-specific genes. (A) A model of CO_2-induced filamentation. (B) A model of CO_2-induced white-to-opaque switching. Wor1 contains a conserved potential PKA phosphorylation site and has been proven to function downstream of the cAMP pathway.

attributed to the slow cell growth or partially to the regulation of cAMP signaling. On the basis of these and other results (Klengel *et al.*, 2005; Huang *et al.*, 2009, 2010; Hall *et al.*, 2010), we propose a model in which both the major unidentified pathway and cAMP signaling are involved in CO_2-sensing regulation. The two pathways converge on Flo8 or the Flo8–Efg1 complex, which then plays a key role in morphogenesis (Figure 7). The two pathways may function together and fine tune the CO_2 response under different circumstances.

Another host environmental cue, GlcNAc, also induces both filamentous growth and opaque cell formation predominantly via the cAMP/PKA pathway (Huang *et al.*, 2010). CO_2 and GlcNAc have a synergistic effect on induction of opaque formation (Huang *et al.*, 2010). In this study, we proved that Flo8 is required for CO_2-regulated white-to-opaque switching but not for GlcNAc-regulated switching. These data suggest that CO_2 and GlcNAc activate opaque cell formation via distinct major pathways. CO_2 activates Flo8 primarily via an unidentified pathway, whereas GlcNAc directly stimulates Wor1 by phosphorylation through the cAMP/PKA pathway (Huang *et al.*, 2010).

Both Efg1 and Hgc1 are important filamentous growth regulators. Efg1 coordinates with Flo8 by physical interaction to regulate filamentous growth (Cao *et al.*, 2006). Deletion of *EFG1* blocks CO_2-induced filamentous growth, suggesting that Efg1 is essential for the function of the Efg1–Flo8 complex. Recently Nobile *et al.* (2012) found that Efg1 binds to the promoter region of the *FLO8* gene in *C. albicans*, suggesting that Efg1 could play a role in the regulation of *FLO8* expression at the transcriptional level. Transcriptional regulation of *HGC1* indicates that Hgc1 functions downstream of the Flo8–Efg1 complex. Induction of filamentous growth by ectopic expression of *HGC1* in the *flo8/flo8* mutant confirms this hypothesis.

In this study, we also isolated the *wor2/wor2* mutant, which shows the unstable opaque phenotype at high levels of CO_2. We excluded the transcription factor Wor2 as a central CO_2-sensing regulator because the *wor2/wor2* mutant indeed responded to the elevated levels of CO_2 when tested in a filamentous growth experiment (Figure 2C). Deletion of the master regulator *WOR1* gene locks *C. albicans* in white phase either in air or in 5% CO_2. Overexpression of *WOR1* in the *flo8/flo8* mutant promotes opaque cell formation (unpublished data). Although Wor1 and Wor2 are not directly involved in CO_2 sensing in *C. albicans*, they may function downstream of the central regulator Flo8 to specifically control CO_2-induced white-to-opaque switching. Because we do not have direct evidence showing that CO_2 binds to the transcription factor Flo8, it is also possible that Flo8 responds to other changes induced by high levels of CO_2 in the cell (such as reduced pH levels and changes in metabolic profiles).

On the basis of our results and previous reports (Cao *et al.*, 2006), we propose that CO_2 activates Flo8 via the cAMP/PKA and another, unidentified pathway (Figure 7). In the context of different external environments and internal genetic backgrounds, the activated Flo8 then delivers a signal to a distinct regulatory gene circuits to induce filamentous growth or white-to-opaque switching. Our study provides an example in which the same environmental factor regulates two different phenotypic switching systems via the same upstream pathways and distinct downstream regulatory circuits.

Conclusion

We set out to elucidate the molecular mechanisms of CO_2 sensing by using the pathogenic fungus *C. albicans* as a model. Taking advantage of the bistable feature of white-to-opaque transition and the fact that CO_2 and GlcNAc regulate this biological process via distinct pathways, we identified the transcription factor Flo8 as a master regulator of CO_2 sensing in *C. albicans*. Flo8 controls CO_2-induced white-to-opaque switching, as well as yeast filamentous growth transition, via distinct downstream factors. Further analysis suggests that there are two pathways involved in CO_2-induced morphogenesis: the major, unidentified pathway and the minor, cAMP/PKA pathway. CO_2 signal is transduced via the two pathways, which converge on Flo8. As mentioned, Flo8 is a transcription factor containing a conserved LisH domain that has been found in a wide variety of eukaryotic organisms, ranging from yeast to human (Reiner *et al.*, 1993; Neer *et al.*, 1994; Cao *et al.*, 2006). Therefore our findings will provide new insights into the understanding of CO_2 sensing in pathogenic fungi, as well as in higher organisms.

MATERIALS AND METHODS

Strains and plasmids

The strains used in this study are listed in Supplemental Tables S1 and S2. The original *MTL***a**/α strains (001–167) listed in Supplemental Table S2 were the transcription factor (TF)–knockout set generated by Homann *et al.* (2009) and obtained from the Fungal Genetics Stock Center (University of Missouri, Kansas City, Kansas City, MO). The *MTL***a**/α strains, including the TF set and others collected from the *Candida* research community, were converted to *MTL*-homozygous strains as described in the text (Figure 2A). The strains were first grown in liquid YPS medium in a test tube at 37°C for 48 h. The cells were diluted and replated onto Lee's GlcNAc plates for further growth in 5% CO_2. After incubation for 5 d, opaque colonies were isolated for further experiments.

The *FLO8* open reading frame was amplified by PCR from SC5314 genomic DNA and digested with *Bam*HI and *Sal*I. The primers for PCR were FLO8F (5-TCATTTGTCGACATGGTTCCCAACACAACTAAAC-3) and FLO8R (5-TCATTTGGATCCTAATCGCCATTTTCAATTGGATC-3). The GFP fragment of the plasmid pNIM1 was replaced with the digested PCR product of *FLO8*, generating the overexpression plasmid pNIM-FLO8 (TETp-FLO8; Park and Morschhauser, 2005).

White-to-opaque switching assay

White-to-opaque switching experiments were performed as previously described (Huang *et al*., 2009, 2010), with slight modification. Briefly, the original white or opaque colonies were first plated or streaked on agar contain Lee's glucose or Lee's GlcNAc medium (Lee *et al*., 1975; Huang *et al*., 2010). Modified Lee's medium with 1.25% (wt/vol) glucose as carbon source is referred to as Lee's glucose. Modified Lee's medium with GlcNAc 1.25% (wt/vol) as carbon source is referred to as Lee's GlcNAc medium. The homogeneous white or opaque colonies were then picked and replated onto glucose- or GlcNAc-medium plates. After incubation for 5–7 d in air or CO_2 at 25°C, the colonies exhibiting different phenotypes were counted, and the cellular morphologies of several representative colonies were examined to ensure their phenotypes.

Mating assay

The mating experiments in Figure 3 were performed in Lee's glucose liquid medium. To obtain opaque colonies, we first grew the tester *MTL*α/α strain (GH1349) in 5% CO_2. The isolated opaque colonies were streaked on Lee's glucose plates and incubated for 5 d. The experimental cell samples of the *flo8/flo8* mutant (*MTL***a**/**a**) were collected from the same set of plates presented in Figure 3A. The experimental white and opaque cell samples of the WT were collected from Lee's glucose plates. A total of 1×10^7 of *MTL***a**/**a** cells and 1×10^7 of *MTL*a/α cells were mixed in 100 µl of Lee's glucose medium in a 1.5-ml centrifuge tube. Before acquiring the cellular images, the tubes containing **a** and α cell mixture were incubated at 25°C for 24 h with gentle shaking.

Filamentous growth assay

The cells were grown on YPD (1% yeast extract, 2% peptone, and 2% glucose) agar for 1–2 d before plating onto Lee's glucose agar for filamentous development. The plates were incubated at 25 or 37°C for 5–7 d as indicated in the text.

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