Roles of the Transcription Factors Sfl2 and Efg1 in White-Opaque Switching in α/α Strains of Candida albicans

Yang-Nim Park, a Kayla Conway, a Thomas P. Conway, a Karla J. Daniels, a David R. Soll a

ABSTRACT Candida albicans remains the most pervasive fungal pathogen colonizing humans. The majority of isolates from hosts are heterozygous at the mating type locus (MTLα/α), and a third of these have recently been shown to be capable of switching to the opaque phenotype. Here we have investigated the roles of two transcription factors (TFs) Sfl2 and Efg1, in repressing switching in α/α strains. Deleting either gene results in the capacity of α/α cells to switch to opaque en masse under facilitating environmental conditions, which include N-acetylglucosamine (GlcNAc) as the carbon source, physiological temperature (37°C), and high CO2 (5%). These conditions are similar to those in the host. Our results further reveal that while glucose is a repressor of sfl2Δ and efg1Δ switching, GlcNAc is an inducer. Finally, we show that when GlcNAc is the carbon source, and the temperature is low (25°C), the efg1Δ mutants, but not the sfl2Δ mutants, form a tiny, elongate cell, which differentiates into an opaque cell when transferred to conditions optimal for α/α switching. These results demonstrate that at least two TFs, Sfl2 and Efg1, repress switching in α/α cells and that α/α strains with either an sfl2Δ or efg1Δ mutation can switch en masse but only under physiological conditions. The role of opaque α/α cells in commensalism and pathogenesis must, therefore, be investigated.

KEYWORDS Candida albicans, Efg1, Sfl2, white-opaque switching

Candida albicans remains the most pervasive opportunistic fungal pathogen colonizing humans (1–3). The majority of natural strains are heterozygous (α/α) at the mating type locus (4–7) and cannot mate (8, 9). To mate, these cells must undergo homoyzogy at the mating type locus (4, 8–11) and then switch from the yeast phase “white” phenotype to the unique “opaque” phase phenotype (10, 11). Thus, a paradigm was established that only MTL-homozygous strains (α/α and α/α) could switch to opaque and that the opaque phase cell served as the mating-competent phenotype. This paradigm was altered in 2013 when Xie et al. (7) found that approximately one third of a collection of α/α strains isolated from hosts underwent white-opaque switching, forming mating-incompetent opaque cells, albeit at low frequencies, when cultured on medium with N-acetylglucosamine (GlcNAc) at 25°C in 5% CO2 (7). These results suggested that in a significant minority of colonized hosts, the α/α strain forms opaque cells that play a role in colonization. Here, we have investigated the roles of two transcription factors, Sfl2 and Efg1, in α/α switching, by using deletion mutants generated in two α/α strains that did not switch. SFL2 is upregulated in cells during growth at 37°C, but not at lower temperatures, and is involved in EFG1-dependent regulation of hypha formation (12, 13). Efg1 has also been shown to be a repressor of
white-opaque switching in *C. albicans* (14–16). Xie et al. (7) previously provided evidence that Efg1 was a repressor of switching in a/α strains. Here, parent a/α strains and *sfl2Δ, efg1Δ*, and *sfl2Δ efg1Δ* mutants, were tested for switching under eight sets of conditions that included all combinatorial permutations of sugar source (glucose versus GlcNAc), temperature (25°C versus 37°C), and CO2 level (0.04% [air] versus 5%).

Our results indicate the following. (i) a/α *sfl2Δ* and *efg1Δ* mutants and the *sfl2Δ efg1Δ* double mutant undergoes mass conversion (>90%) from white to opaque on GlcNAc-containing agar at 37°C in 5% CO2. (ii) The effects of environmental conditions on white-to-opaque switching by the *sfl2Δ* and *efg1Δ* mutants are similar. (iii) *WOR1, OP4*, and a number of other genes associated with switching are upregulated in opaque cells of both mutants. (iv) The stability of the opaque phenotype of the two mutants differ at 25°C. (v) Glucose is an inhibitor and GlcNAc is an inducer of switching. (vi) *efg1Δ* cells, but not a/α *sfl2Δ* cells, form tiny, elongate cells on GlcNAc agar at 25°C. (vii) These tiny, elongate cells, when incubated under optimal conditions for switching, grow directly into opaque cells. The observations that approximately a third of natural a/α *C. albicans* strains switch and that switching en masse by a/α mutants of repressor genes requires all three physiological conditions suggest that the opaque phenotype may be expressed in a third of *C. albicans* infections. Further investigation of a/α switching and the role of a/α opaque cells in pathogenesis is therefore warranted.

**RESULTS**

The two parent a/α strains do not undergo white-to-opaque switching. Exploring the roles of *SFL2* and Efg1 in repressing switching by mutant analysis required parental a/α strains that did not switch. We selected two wild-type (wt) a/α strains, SC5314 (17) and P37039 (18), which in preliminary studies did not switch from white to opaque under the conditions employed by Xie et al. (7). The two strains were tested for switching under eight sets of conditions, which included all combinatorial permutations of three environmental parameters, carbon source (1.25% glucose versus 2% GlcNAc), temperature (25°C versus 37°C), and CO2 level (air [0.04% CO2] versus 5% CO2). The frequency of white-to-opaque switching was assessed at the colony level on supplemented Lee’s agar (19) containing either 1.25% glucose or 2% GlcNAc as the carbon source. “Opaque colonies” were assessed as those fully opaque or with one or more opaque sectors. Data are means ± standard deviations from three or more independent experiments. Neither of the two a/α parent strains switched from white to opaque under any of the eight sets of conditions (Fig. 1A and Table 1). Regardless of colony morphologies under the various sets of conditions, no opaque cells were observed microscopically (Fig. 2A and B, respectively).

White-to-opaque switching by a/α *sfl2Δ* mutants. Both alleles of *SFL2* were deleted in each of the two wild-type a/α strains to generate SC5314sfl2Δ and P37039sfl2Δ mutants (see Table S1 in the supplemental material). With glucose as the carbon source, neither SC5314sfl2Δ nor P37039sfl2Δ switched to opaque under any of the four sets of conditions (25°C, air; 25°C, 5% CO2; 37°C, air; 37°C, 5% CO2) (Table 1 and Fig. 1A), as was the case for the parental strains (Table 1). With GlcNAc as the carbon source, both sfl2Δ mutants SC5314sfl2Δ and P37039sfl2Δ switched at low to negligible frequencies at 25°C in air (0.1 and 0%, respectively) and at low frequencies at 25°C in 5% CO2 (4.9 and 1.9%, respectively) (Table 1 and Fig. 1A). On GlcNAc-containing agar at 37°C in air, the two sfl2Δ mutants also switched at low to negligible frequencies, but at 37°C in 5% CO2 both switched from white to opaque en masse (94% and 100%, respectively) (Fig. 1A and Table 1). The opaque colonies and the opaque sectors contained cells with signature opaque morphologies (20, 21) (Fig. 2A and B). White colonies, regardless of colony morphology (round versus irregular with filamentous edge), contained typical round white cells (Fig. 2A and B), or typical white cells and hyphae. The surfaces of mature opaque cells selectively stained in a punctate fashion with the opaque cell-specific, antipimple polyclonal antibody (22) (Fig. 3C and D, respectively). PCR analysis revealed that opaque cells remained a/α (Fig. 2C). These
results indicate that mass conversion of the sfl2Δ mutants from white to opaque occurs only on GlcNAc-containing agar at 37°C in 5% CO2.

**a/α sfl2Δ opaque cells do not mate.** Xie and coworkers (7) found that opaque cells formed by natural a/α strains were incapable of mating. To test whether opaque a/α sfl2Δ cells mated, we generated an a/α SC5314sfl2Δ derivative, SC5314sfl2Δ-Hr and an a/α P37039sfl2Δ derivative, P37039sfl2Δ-Hr, both resistant to hygromycin B (Table S1). We also generated the MTL-homozygous mating partners P37005-Sr (a/a) and WO-1-Sr (a/α), both resistant to nourseothricin. A hygromycin B-resistant a/a strain, P37005-Hr (a/a), was also generated to measure the mating competency of the MTL-homozygous mating partners (Table 2). Combined resistance indicated mating. Control crosses of opaque a/a and a/α cells resulted in frequencies of mating of 4 × 10⁻³ and 6.7 × 10⁻³ after 2 and 4 days of incubation, respectively (Table 2). Crosses of opaque a/α and a/a cells resulted in frequencies of 2.8 × 10⁻⁷ to <8.7 × 10⁻⁹ (Table 2). Crosses of opaque a/α and a/α cells resulted in frequencies of 3.5 × 10⁻⁷ to <9.5 × 10⁻⁹ (Table 2). The frequencies of hygromycin B/nourseothricin-resistant offspring resulting from the a/α sfl2Δ × a/a or a/α sfl2Δ × a/α opaque cell crosses were more than 5 orders of magnitude lower than that of a/a × a/α opaque cell crosses, indicating that MTL-heterozygous sfl2Δ opaque cells did not acquire the capacity to mate.

**Dependence of a/α sfl2Δ switching on WOR1.** For MTL-hemizygous or MTL-homozygous cells, switching depends on the expression of WOR1 (also referred to as

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**FIG 1** White-to-opaque switching by sfl2Δ, efg1Δ, and sfl2Δ efg1Δ mutants generated in the a/α wild-type strains SC5314 and P37039 on glucose or GlcNAc agar under four sets of environmental conditions. The sets of conditions are presented at the top of the figure. (A) sfl2Δ mutants; (B) efg1Δ mutants; (C) sfl2Δ efg1Δ mutants. The frequencies of uniformly opaque or opaque-sectored colonies are presented as solid colored bars. Colonies containing a mixture of either yeast, tiny elongate and opaque cells (25°C), or yeast and opaque cells (37°C) are presented as white bars outlined in color. The error bars represent standard deviations for data from at least three experiments. The quantitative data for these bar graphs are presented in Table 1.
TABLE 1 White-to-opaque switching frequencies of the a/α wild-type SC5314 and P37039 strains and their sfl2Δ, efg1Δ, and sfl2Δ efg1Δ mutants*

| Strain         | Carbon source | 25°C, air | 25°C, 5% CO₂ | 37°C, air | 37°C, 5% CO₂ |
|---------------|--------------|-----------|--------------|-----------|--------------|
|               | Total col. no. | Frequency (%) | Total col. no. | Frequency (%) | Total col. no. | Frequency (%) | Total col. no. | Frequency (%) |
| SC5314        | Glucose      | 1,278     | 0            | 1,298      | 0            | 1,287        | 0            | 1,441        |
|               | GlcNAc       | 1,458     | 0            | 1,251      | 0            | 1,135        | 0            | 1,401        |
| SC5314        | Glucose      | 3,797     | 0            | 2,033      | 0            | 3,015        | 0            | 2,740        |
| sfl2Δ         | GlcNAc       | 2,101     | 0.1 ± 0.2    | 3,701      | 4.9 ± 5.7    | 3,298        | 0.0 ± 0.1    | 2,544        |
| SC5314        | Glucose      | 2,986     | 0            | 1,696      | 0            | 2,166        | 0            | 2,828        |
| efg1Δ         | GlcNAc       | 1,567     | 3.1 ± 3.3d   | 1,467      | 3.3 ± 2.6e   | 2,235        | 3.4 ± 6.8f   | 2,341        |
| SC5314        | Glucose      | 3,527     | 0            | 2,214      | 0            | 2,825        | 0            | 2,826        |
| sfl2Δefg1Δ    | GlcNAc       | 2,209     | 4.5 ± 2.4d   | 2,511      | 5.6 ± 4.0e   | 2,698        | 0f          | 2,678        |
| P37039        | Glucose      | 1,464     | 0            | 1,201      | 0            | 1,299        | 0            | 1,315        |
|               | GlcNAc       | 1,330     | 0            | 1,178      | 0            | 1,156        | 0            | 1,370        |
| P37039        | Glucose      | 2,618     | 0            | 1,734      | 0            | 2,038        | 0            | 2,128        |
| sfl2Δ         | GlcNAc       | 1,969     | 0            | 2,837      | 1.9 ± 3.6    | 1,763        | 0            | 2,243        |
| P37039        | Glucose      | 2,315     | 0            | 1,158      | 0            | 1,299        | 0            | 1,315        |
| efg1Δ         | GlcNAc       | 1,354     | 0f          | 1,962      | 1.0 ± 1.0e   | 1,480        | 0.2 ± 0.3f   | 1,849        |
| P37039        | Glucose      | 2,195     | 0            | 1,167      | 0            | 1,615        | 0            | 1,739        |
| sfl2Δefg1Δ    | GlcNAc       | 1,141     | 0.3 ± 0.4d   | 2,421      | 18.3 ± 7.5e  | 1,242        | 1.9 ± 2.4f   | 1,578        |

*Total colony numbers and white-to-opaque switching frequencies of a/α wild-type (wt) SC5314 and P37039 strains and their sfl2Δ, efg1Δ, and sfl2Δ efg1Δ mutants grown under four different conditions on two different carbon sources. For the efg1Δ mutants, the formation of “white” colonies containing tiny, elongate cells or mixtures of tiny, elongate and opaque cells is noted by footnotes d to f.

The frequencies are presented as the means ± standard deviations for three or more separate experiments.

Colonies assessed as “white” were composed of a majority of tiny, elongate cells and a small minority of round yeast cells (Fig. 5B).

Colonies assessed as “white” were composed of a mixture of tiny, elongate cells and opaque cells, varying in proportions (Fig. 5B).

Colonies assessed as “white” were composed of a majority of opaque cells and very minor proportions of white yeast phase cells (Fig. 5B).

TO59) (23–25). Xie et al. (7) also showed by mutational analyses that a/α switching depended on WOR1. To test whether white-to-opaque switching in a/α sfl2Δ mutants required WOR1, we deleted WOR1, generating strains SC5314sfl2Δwor1Δ and P37039sfl2Δwor1Δ (Table S1). No switching was observed in either of the a/α sfl2Δ wor1Δ mutants under any of the eight sets of conditions (Table S3). These results demonstrate that white-to-opaque switching by the a/α sfl2Δ mutants requires WOR1, just as switching by MTL-hemizygous and MTL-homozygous strains requires WOR1 (23–25).

Gene expression in sfl2Δ opaque cells. Expression of WOR1 and 21 additional genes, including several associated with switching of MTL-hemizygous strains was compared between wild-type a/α SC5314 and sfl2Δ cells grown on GlcNAc-containing agar at 37°C in 5% CO₂. Under these conditions, sfl2Δ cell populations exhibited the opaque cell morphology, and more than 95% formed opaque colonies, while wild-type SC5314 cell populations formed colonies containing hyphae (Fig. 2A). Expression of the 21 genes was assessed by qRT-PCR. Relative expression in the sfl2Δ mutant was computed as the fold increase or decrease relative to the level of expression in parental SC5314 cells grown under the same conditions. As is evident in Fig. 4A, the level of WOR1 expression in opaque cells of the a/α SC5314sfl2Δ strain was 320-fold greater than in a/α SC5314 wild-type cells. Expression of the transcription factors (TFs) encoded by WOR2, WOR3, WOR4, CZF1, and AHR1, all implicated in a network of interacting TFs (26–29), were respectively upregulated approximately 70-fold, 48-fold, 20-fold, 11-fold, and 11-fold, respectively, in the mutant (Fig. 4A). The SFL1 gene, which has been suggested to play the opposite role to that of SFL2 in hypha formation (13, 30), was upregulated 18-fold (Fig. 4A). The gene most dramatically upregulated in the sfl2Δ mutant was OP4 (520-fold), the first gene to be identified as opaque specific (31). Three other genes implicated in switching, SSN6 (32), TUP1 (33), and CDR3 (34), were also upregulated as well as the genes CAG1, NRG1, BRG1, RFG1, and ZCF21 (Fig. 4A). NDT80 was downregulated in opaque cells of the sfl2Δ mutant (Fig. 4A). Expression of MTLa1
and MTLα2, which encode the α1-α2 corepressor of both switching and mating (4, 10, 11), were relatively unaffected by the white-to-opaque switch (Fig. 4A).

Opaque stability of a/α sfl2Δ mutants. The stability of the opaque phenotype (i.e., switching from opaque back to white) of the sfl2Δ mutants was then tested under the eight sets of conditions. When opaque cells of the mutants SC5314sfl2Δ and P37039sfl2Δ were plated on glucose-containing agar, there was mass conversion (95 to 100%) to white under all four sets of conditions (Fig. 5A and Table 3). On GlcNAc-containing agar under all four sets of conditions, switching from opaque to white of both sfl2Δ mutants occurred, but at reduced frequencies and with nuances. First, in both mutant strains, switching from opaque to white on GlcNAc agar was lowest at 37°C in 5% CO2 (Fig. 5A and Table 3), the same set of conditions that supported the highest frequency of white-to-opaque switching (Fig. 1A and Table 1). Second, under the remaining three sets of conditions on GlcNAc agar (25°C, air; 25°C, 5% CO2; 37°C, air), the frequencies of opaque to white switching were higher than at 37°C in 5% CO2.
GlcnAc, 37°C, 5% CO2

A. SC5314  B. P37039  

C. SC5314sfl2Δ  D. P37039sfl2Δ  

E. SC5314efg1Δ  F. P37039efg1Δ  

G. SC5314efg1Δsfl2Δ  H. P37039efg1Δsfl2Δ  

I. SC5314efg1Δ  J. P37039efg1Δ  

**FIG 3** Immunostaining of opaque-cell-specific pimples. Cells were immunostained with antipimple antiserum and an Alexa Fluor 488-tagged secondary anti-rabbit IgG antibody. (A and B) White cells of the parental a/α strains SC5314 (A) and P37039 (B). Bar in panel A, 2 μm. (C and D) Opaque cells of the sfl2Δ mutants. (E and F) Opaque cells of the efg1Δ mutants. (G and H) Opaque cells of the efg1Δ sfl2Δ mutants. (I and J) Tiny, elongate cells of efg1Δ mutants. Opaque and white cells were obtained from opaque and white colonies, respectively, and grown on GlcnAc agar at 37°C in 5% CO2. Tiny elongate cells were obtained from GlcnAc agar cultures grown at 25°C in air. The latter cells were suspended in medium, the larger opaque cells were allowed to settle, and the supernatant, enriched for tiny cells, was imaged.

(Fig. 5A and Table 3). Together, these results indicate that GlcnAc not only supports switching but also stabilizes the opaque phenotype of a/α sfl2Δ cells and that 5% CO2 and high temperature (37°C), in combination, enhance GlcnAc stabilization. Glucose, on the other hand, induces mass conversion to white.

**White-to-opaque switching by a/α efg1Δ mutants.** It has been shown that the transcription factor Sfl2 depends upon Efg1 for the activation of hypha formation (13, 30) and in coordination with Efg1 regulates the TCA cycle in response to high CO2 (35). Sfl2 has also been shown to bind to several of the same gene promoters (30). To assess the role of Efg1 in a/α switching, we generated the a/α efg1Δ mutants, SC5314efg1Δ and P37039efg1Δ (Table S1), and analyzed switching in the two mutants under the same eight sets of conditions. As was the case for both a/α sfl2Δ mutants, neither of the a/α efg1Δ mutants underwent white-to-opaque switching on glucose agar under the four permutations of temperature and CO2 (Table 1 and Fig. 1B). Cells in efg1Δ white colonies formed on glucose agar under the four sets of conditions and exhibited the standard round to ovoid white yeast phase phenotype (20, 21). However, on GlcnAc agar, there were fundamental differences in cell phenotype between the a/α efg1Δ and a/α sfl2Δ mutants. On GlcnAc agar at 25°C in air or 5% CO2, white cells of both a/α efg1Δ mutants underwent low to negligible levels of switching to opaque (0 to 5%), as
assessed by colony phenotype (Table 1 and Fig. 1B). The few opaque colonies that formed contained cells exhibiting the signature opaque cell phenotype (Fig. 5B). These opaque cells stained selectively for pimples, with the antipimple polyclonal antibody (Fig. 3E and F). However, on GlcNAc agar at 25°C in air, morphologically white colonies contained a majority of a tiny, elongate phenotype and a minority of yeast phase cells.

**TABLE 2** Opaque cells of α/α sfl2Δ mutants do not mate

| Strains crossed | Mating period (no. of days) | Mating frequency |
|-----------------|-----------------------------|------------------|
| P37005-Hr (α/α) × WO-1-Sr (α/α) | 2 | (4.0 ± 0.2) × 10⁻³ |
|  | 4 | (6.7 ± 0.4) × 10⁻³ |
| P37005-Sr (α/α) × SC5314sfl2Δ-Hr (α/α) | 2 | (<1.3 ± 0.1) × 10⁻⁸ |
|  | 4 | (2.8 ± 3.5) × 10⁻⁷ |
| P37005-Sr (α/α) × P37039sfl2Δ-Hr (α/α) | 2 | (<1.3 ± 0.2) × 10⁻⁸ |
|  | 4 | (<8.7 ± 1.6) × 10⁻⁹ |
| WO-1-Sr (α/α) × SC5314sfl2Δ-Hr (α/α) | 2 | (<1.1 ± 0.1) × 10⁻⁸ |
|  | 4 | (3.5 ± 2.3) × 10⁻⁷ |
| WO-1-Sr (α/α) × P37039sfl2Δ-Hr (α/α) | 2 | (<1.1 ± 0.1) × 10⁻⁸ |
|  | 4 | (<9.5 ± 2.0) × 10⁻⁹ |

*Mating was tested on GlcNAc agar at 25°C in air. Mating was assessed as the frequencies of offspring in the population resistant to both hygromycin B and nourseothricin. Descriptions of the strains generated for mating are presented in Table S1 in the supplemental material. At the end of the strain names, Hr and Sr stand for hygromycin B resistant and nourseothricin resistant, respectively.

**b** All cells tested were in the opaque phase.

**c** Frequencies are presented as means ± standard deviations.

assessed by colony phenotype (Table 1 and Fig. 1B). The few opaque colonies that formed contained cells exhibiting the signature opaque cell phenotype (Fig. 5B). These opaque cells stained selectively for pimples, with the antipimple polyclonal antibody (Fig. 3E and F). However, on GlcNAc agar at 25°C in air, morphologically white colonies contained a majority of a tiny, elongate phenotype and a minority of yeast phase cells.

**FIG 4** Expression levels of genes associated with switching, in opaque phase cells of α/α sfl2Δ and efg1Δ mutants. The qRT-PCR result for each gene is presented as the relative expression level of cells in mutant colonies versus cells of the parental wild-type α/α SC5314 colonies grown on GlcNAc agar at 37°C in 5% CO₂. While the mutant colonies were opaque, the wild-type colonies were irregular and contained hyphae. (A) SC5314sfl2Δ; (B) SC5314efg1Δ. Error bars represent standard deviations from three repeats for two separate experiments. wt, wild type; ND, not detectable. Statistical significance compared to the wild type was determined using Student’s t test analysis and indicated as follows: gray asterisk, 0.01 < P < 0.05; black asterisk, 1 × 10⁻¹³ < P < 0.001.
and on GlcNAc agar at 25°C in 5% CO₂, white colonies contained mixtures of tiny, elongate cells and opaque cells (Fig. 6B). On GlcNAc agar at 37°C in air, a/α efg1Δ cells in white colonies contained a minority of yeast phase cells and a majority of opaque cells (Fig. 6B). On GlcNAc agar at 37°C in 5% CO₂, the conditions that supported mass conversion to opaque in a/α sfl2Δ mutants (Fig. 1A and Table 1), cells of the two a/α efg1Δ mutants switched en masse (100%) to opaque (Table 1 and Fig. 1B) and exhibited the signature opaque cell phenotype (Fig. 6B). Opaque cells of a/α efg1Δ mutant strains, in which mCherry was placed under the control of the opaque-specific OP4 promoter, were highly fluorescent (Fig. 6C).

**Tiny, elongate cell phenotype.** We tested whether tiny, elongate cells of strains SC5314efg1ΔmCh and P37039efg1ΔmCh, in which mCherry was under the regulation of the promoter of the opaque-specific gene OP4 (31) (Table S1), were fluorescent. While opaque cells of these strains fluoresced red (Fig. 6C), the tiny, elongate cells exhibited negligible fluorescence (Fig. 6D). Tiny, elongate cells were also tested for staining by the polyclonal antipimple antibody (22). The antibody did not stain tiny, elongate cells (Fig. 3I and J, respectively). Because tiny, elongate cells were mixed with opaque cells in efg1Δ cultures grown under conditions suboptimal for mass conversion to opaque, we tested whether the tiny, elongate cells, when transferred to GlcNAc medium at 37°C in 5% CO₂, formed opaque cells. More than 10 tiny, elongate cells dispersed under

**FIG 5** Opaque-to-white switching, a measure of opaque cell stability of the a/α SC5314 and a/α P37039 mutants under the eight sets of environmental conditions. (A to C) sfl2Δ mutants (A), efg1Δ mutants (B), and sfl2Δ efg1Δ mutants (C). Statistical significance was determined using Student’s t test analysis. Switching frequencies that are statistically significantly different between cells grown on glucose agar versus on GlcNAc agar under the four sets of conditions are indicated with asterisks, while switching frequencies that are significantly different for cells grown at 25°C in air, at 25°C in 5% CO₂, and at 37°C in air versus at 37°C in 5% CO₂ are indicated by solid circles. A gray asterisk or solid gray circle indicates 0.001 < P < 0.05. A black asterisk or solid black circle indicates 1 × 10⁻³⁵ < P < 0.001.
agaroose overlayers were incubated microscopically for 30 h. The individual tiny, elongate cells grew into vacuolated opaque cells, which then continued to multiply in the opaque phase to form opaque microcolonies (Fig. 6E).

**Gene expression in efg1Δ opaque cells.** The same set of genes analyzed in a/a SC5314Δsfl2Δ opaque cells by qRT-PCR were analyzed in a/a SC5314Δefg1Δ opaque cells formed on GlcNAc agar at 37°C in 5% CO₂ (Fig. 4B). Opaque cells of both mutants exhibited dramatic increases in WOR1 expression (322- and 135-fold, respectively) and OP4 expression (525- and 1,197-fold, respectively) (Fig. 4A and B, respectively). Opaque cells of both sfl2Δ and efg1Δ mutants also exhibited increases in WOR2, WOR3, WOR4, SFL1, SSN6, CDR3, and ZCF21 (Fig. 4A and B). Opaque cells of the efg1Δ mutant did not exhibit the increases in the expression of AHR1, TUP1, CAG1, NRG1, BRG1, and RFG1 observed in the sfl2Δ mutant (Fig. 4A). On the other hand, expression of NDT80 increased, and expression of CZF1 and UME6 decreased in opaque cells of the efg1Δ mutant (Fig. 4B), unlike expression in the sfl2Δ mutant (Fig. 4A).

**Opaque stability of the efg1Δ mutants.** The efg1Δ mutants were tested for opaque cell stability (i.e., opaque-to-white switching) under the eight sets of conditions. On glucose agar at 25°C in air or 5% CO₂, opaque cells of both efg1Δ mutants were relatively stable (Fig. 5B and Table 3). This differed markedly from opaque cells of the two sfl2Δ mutants, which switched en masse (95 to 100%) (Fig. 5A and Table 3). On glucose agar at 37°C in air or 5% CO₂, opaque cells of both efg1Δ mutants underwent mass conversion to white (i.e., were completely unstable) (Fig. 5B and Table 3), similar to those for opaque cells of the sfl2Δ mutants (Fig. 5A and Table 3). Opaque cells of the efg1Δ mutants were moderately unstable under all four sets of conditions, switching from opaque to white at frequencies between 0.2 and 11% (Fig. 5B and Table 3). For both strains, opaque stability under the four conditions on GlcNAc agar, was on average greater for the efg1Δ mutants than for the sfl2Δ mutants.

**White-to-opaque switching by a/a sfl2Δ efg1Δ mutants.** To assess whether the effects of the mutations of SFL2 and EFG1 are synergistic or whether either is dominant under one or more of the eight sets of conditions, the double a/a mutants SC5314Δsfl2Δefg1Δ and P37039Δsfl2Δefg1Δ (Table S1) were tested under the eight sets of conditions. The absence of switching on glucose agar under all four permutations of temperature and atmosphere was the same as that for the individual sfl2Δ and efg1Δ mutants (Table 1 and Fig. 1A to C). The patterns of switching under the four sets of conditions on GlcNAc agar, including the formation of tiny, elongate cells, was similar to that of the individual efg1Δ mutants (Table 1 and Fig. 1C). Mass conversion to opaque on GlcNAc agar at 37°C in 5% CO₂ was similar to that of both of the single

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**Table 3** Opaque-to-white switching, a measure of opaque phase stability, of a/a sfl2Δ, efg1Δ, and sfl2Δ efg1Δ mutants

| Strain | Carbon source | 25°C, air | 25°C, 5% CO₂ | 37°C, air | 37°C, 5% CO₂ |
|--------|---------------|-----------|-------------|-----------|-------------|
|        | Total col. no. | Frequency (%) | Total col. no. | Frequency (%) | Total col. no. | Frequency (%) | Total col. no. | Frequency (%) |
| SC5314 | Glucose       | 1,458      | 95.2 ± 5.6 | 1,407      | 100         | 1,511       | 100         | 1,504       | 100         |
| sfl2Δ  | GlcNAc        | 1,522      | 11.2 ± 4.8 | 1,466      | 5.6 ± 4.0   | 1,560       | 13.2 ± 7.5  | 1,882       | 2.3 ± 1.3   |
| SC5314 | Glucose       | 648        | 4.4 ± 8.1 | 700        | 0.9 ± 1.0   | 743         | 100         | 659         | 100         |
| efg1Δ  | GlcNAc        | 792        | 3.3 ± 4.3 | 774        | 3.9 ± 8.8   | 786         | 3.4 ± 7.7   | 964         | 0.2 ± 0.4   |
| SC5314 | Glucose       | 753        | 2.6 ± 3.3 | 741        | 0.0 ± 0.0   | 819         | 100         | 864         | 100         |
| sfl2Δefg1Δ | GlcNAc | 847        | 3.4 ± 3.1 | 922        | 7.7 ± 6.9   | 843         | 2.4 ± 5.2   | 1,092       | 2.6 ± 7.2   |
| P37039 | Glucose       | 660        | 100       | 677        | 100         | 703         | 100         | 679         | 100         |
| sfl2Δ  | GlcNAc        | 737        | 49.6 ± 40.4 | 729     | 27.0 ± 20.2 | 681         | 49.3 ± 40.8 | 1,042       | 1.9 ± 2.1   |
| P37039 | Glucose       | 696        | 9.5 ± 13.7 | 675        | 0.5 ± 0.8   | 519         | 99.5 ± 1.1  | 448         | 100         |
| efg1Δ  | GlcNAc        | 678        | 8.0 ± 13.3 | 693        | 7.5 ± 13.0  | 697         | 8.4 ± 13.4  | 944         | 11.4 ± 16.4 |
| P37039 | Glucose       | 632        | 6.4 ± 7.0 | 651        | 1.3 ± 2.0   | 724         | 100.0       | 743         | 100         |
| sfl2Δefg1Δ | GlcNAc | 727        | 2.7 ± 5.1 | 727        | 2.8 ± 5.1   | 724         | 1.6 ± 3.0   | 868         | 0.3 ± 0.8   |

*pSwitching was assessed under the eight sets of conditions, including all permutations of carbon source (glucose versus GlcNAc), temperature (25°C versus 37°C), and CO₂ level (air [0.04% CO₂] versus 5% CO₂).

*Total colony number (Total col. no.) represents the total number of colonies pooled for the experiments.

*The frequencies of opaque to white switching are presented as the mean % ± standard deviations for ≥ 3 independent experiments.
mutants (Fig. 1C). These results indicate that in the double mutant, the efg1Δ mutation overrides the sfl2Δ mutation.

**Opaque stability of the a/α sfl2Δ efg1Δ mutants.** The stability of opaque cells of the a/α sfl2Δ efg1Δ mutants on both glucose and GlcNAc agar under the four sets of environmental conditions mimicked that of the efg1Δ mutants, rather than that of the sfl2Δ mutants (Fig. 5 and Table 3). These results indicate that just as in the case of the white-to-opaque transition, the efg1Δ mutation overrides the sfl2Δ mutation in the opaque-to-white transition of the sfl2Δ efg1Δ mutants.

**Complementation of the sfl2Δ and efg1Δ mutants.** To test whether the switching phenotypes on GlcNAc agar exhibited by the two sfl2Δ mutants and the two efg1Δ mutants were due to the targeted mutated genes, all were complemented with the wild-type gene. Complementation was targeted to the native locus. Switching data of
the complemented strains are provided in Table 4 for GlcNAc agar, 37°C, 5% CO₂, and for all eight sets of conditions in Table S5. The addition of one copy of SFL2 to each of the two sfl2Δ mutants (SC5314sfl2Δ and P37039sfl2Δ) resulted in a reduction, but not in the elimination, of switching, the latter representing the parental wild-type phenotype (Table 4 and Table S5). The reduction observed in the sfl2Δ mutants receiving one copy of SFL2 was similar to that of the heterozygous deletion derivative (Table 4 and Table S5). However, additions of two copies of SFL2 to the native allelic loci resulted in the complete elimination of white-to-opaque switching on GlcNAc agar at 25°C or 37°C in 5% CO₂ (Table 4 and Table S5). These results demonstrate that the switching phenotypes of the two sfl2Δ mutants and the two efg1Δ mutants analyzed in detail were the result of the deletion of the targeted genes.

**Inhibition versus induction by the sugar sources.** The preceding results led us to the hypothesis that glucose may be a repressor and GlcNAc an inducer of white-opaque switching in the mutants. We tested whether glucose was a repressor by decreasing the concentration from 1.2%, the standard concentration in supplemented Lee’s medium (19, 36), to 0.1%, close to the average concentration in the gut (37), and then analyzing switching from white to opaque at 37°C in 5% CO₂. If glucose acted as a repressor, the decrease in glucose concentration would result in an increase in the frequency of white-to-opaque switching. Neither the parent a/α strains nor the two derivative sfl2Δ mutants underwent switching on 0.1% glucose agar at 37°C in 5% CO₂ (Fig. 7A and D). However, both of the efg1Δ mutants and both of the sfl2Δ efg1Δ mutants, which did not switch in 1.25% glucose (Fig. 1 and Table 1), underwent mass conversion from white to opaque (100%) on 0.1% glucose agar (Fig. 7A and D). The opaque colonies formed by the a/α efg1Δ and efg1Δ sfl2Δ mutants on 0.1% glucose agar contained cells with signature opaque morphologies. Therefore, mass conversion from white to opaque by the a/α efg1Δ and sfl2Δ efg1Δ mutants was due in this case to the reduction of glucose concentration, suggesting that the standard concentration of glucose (1.25%) used in vitro represses switching of the a/α efg1Δ mutants, but not the a/α sfl2Δ mutants.
For our previous analyses on GlcNAc agar, a concentration of 2% was routinely employed. We therefore first tested whether reducing the concentration of GlcNAc by half affected switching. As previously demonstrated here (Fig. 1 and Table 1), a GlcNAc concentration of 2% at 37°C in 5% CO2 did not support white-to-opaque switching of the two parental a/α/H9251 strains but supported mass conversion (95% to 100%) from white to opaque of all three mutants (sfl2Δ, efg1Δ, and sfl2Δ efg1Δ mutants) (Fig. 7B and E). A 1% concentration of GlcNAc, like 2%, also resulted in mass conversion of the efg1Δ and sfl2Δ efg1Δ mutants (Fig. 7B and E). However, for both sfl2Δ mutants, 1% GlcNAc resulted in a reduced frequency of switching (48% and 78%, respectively) (Fig. 7B and E, respectively). These results did not exclude the possibility that the efg1Δ and sfl2Δ derivatives underwent mass conversion as a default response in the absence of glucose inhibition. However, the decrease in switching by both sfl2Δ mutants in 1% GlcNAc supported an inductive role.

If glucose is an inhibitor and GlcNAc is an inducer of white-to-opaque switching in a/α strains that carry an sfl2Δ or efg1Δ mutation, then when the two sugars are present in combination, the effect of one may be dominant over that of the other. We therefore tested the following combinations of glucose and GlcNAc at 37°C in 5% CO2: 2%:0%, 1%:1%, and 2%:2%. For all combinations, white-to-opaque switching did not occur in the wild-type a/α strains or their sfl2Δ derivatives. The results indicated that for sfl2Δ mutants, glucose repression overrode GlcNAc induction. However, for the efg1Δ derivatives, the results were more complex. At glucose-to-GlcNAc ratios of 2%:1%, the morphologically "white" efg1Δ and sfl2Δ efg1Δ colonies contained a majority of yeast phase cells, but a minority of opaque phase cells as well (dotted bars in Fig. 7C and F; Fig. S1). At ratios of 1%:1% and 2%:2%, morphologically "white" colonies contained a majority of opaque cells and a minority of white cells (dashed bars in Fig. 7C and F; Fig. S1). None of the morphologically "white" colonies formed by the efg1Δ mutant on the three sugar mixtures contained tiny, elongate cells, since the formation of this phenotype is repressed by high temperature (37°C). These results indicate that when glucose is the sole carbon source, it acts as a repressor of white-to-opaque switching.
by sfl2Δ and efg1Δ mutant cells, when GlcNAc is the sole carbon source, it functions as an inducer, and when in combination, GlcNAc induction of white to opaque partially overrides glucose repression of switching.

**DISCUSSION**

To assess by mutational analyses the roles of Sfl2 and Efg1 in repressing white-opaque switching in \( a/\alpha \) strains of *Candida albicans* under different sets of environmental conditions, two parental \( a/\alpha \) strains were selected that did not undergo switching under any of the eight sets of environmental conditions, which included all permutations of carbon source, temperature, and CO\(_2\) level. Xie et al. (7) found that approximately a third of natural \( a/\alpha \) strains switched from white to opaque on GlcNAc agar at 25°C in 5% CO\(_2\). Here, we have shown that with 1.25% glucose as the carbon source, neither the wild type nor the three tested mutants of both strains, the sfl2Δ, efg1Δ, and sfl2Δ efg1Δ mutants, switched under any of the four permutations of temperature and CO\(_2\). When the concentration of glucose was lowered to 0.1%, growth slowed and the two efg1Δ mutants (efg1Δ and sfl2Δ efg1Δ) underwent mass conversion (>95%) to opaque, suggesting that high levels of glucose repressed white-to-opaque switching in both mutants. We also found that when cells of the efg1Δ mutants were grown on mixtures of glucose and GlcNAc, tiny, elongate cells did not form. Moreover, high GlcNAc (2%) appeared to induce white-to-opaque switching in the presence of high glucose (2%). These results suggest that high concentrations of glucose repress switching from white to opaque in \( a/\alpha \) sfl2Δ and efg1Δ mutants and repress formation of tiny, elongate cells in efg1Δ mutants. Furthermore, the repression by glucose of white-to-opaque switching appears to be reversed by the addition of GlcNAc. It should be noted that Doedt and coworkers (38) have demonstrated that Efg1 affects expression of genes involved in sugar metabolism, acting as an inducer of genes involved in glycolysis, the citric acid cycle, and cell wall biosynthesis. On the other hand, Znaidi and coworkers (30) using ChIP binding assays did not identify a similar general increase in the expression of genes directly involved in sugar metabolism or the citric acid cycle in an sfl2Δ mutant but did find that many of the binding sites of Sfl2 were also binding sites of Efg1. These results do not distinguish between receptor-mediated mechanisms versus catabolic mechanisms for either glucose repression or GlcNAc induction.

The stability of opaque cells formed by the sfl2Δ and efg1Δ mutants (efg1Δ and sfl2Δ efg1Δ mutants) differed on glucose agar at 25°C. At 37°C, both the sfl2Δ and efg1Δ mutants underwent mass conversion from opaque to white in air or 5% CO\(_2\), but at 25°C, sfl2Δ mutants underwent mass conversion, but efg1Δ mutants were highly stable. This was true for the mutants derived from strain SC5314 and from strain P37039. These results demonstrate a fundamental difference in the stability of the opaque phenotype between sfl2Δ and efg1Δ mutants at physiological temperature (37°C), suggesting that efg1Δ mutants, but not sfl2Δ mutants, might stably express the opaque phenotype in the host.

The concentration of free glucose in the intestine varies dramatically in mammals, averaging 0.2% under normal feeding conditions (37). This concentration is well below that which has been used in the medium to grow *C. albicans* in vitro. GlcNAc is also found in the intestine, a product of mucin digestion by bacterial enzymes (39–41). Accurate measures of free GlcNAc could not be found. On GlcNAc agar, mass conversion from white to opaque occurred in both sfl2Δ and efg1Δ mutants at 37°C in 5% CO\(_2\). However, under the other three sets of conditions (25°C in air or CO\(_2\); 37°C in air), there were major differences in cellular phenotype. At 25°C in air, no switching to opaque occurred in any of the three mutants, but while white sfl2Δ cells continued to form white yeast phase cells, the efg1Δ and sfl2Δ efg1Δ mutants formed tiny, elongate cells. On GlcNAc agar at 25°C in 5% CO\(_2\), the efg1Δ mutants formed mixtures of tiny elongate and opaque phase cells. At 37°C in 5% CO\(_2\), however, the majority of white cells of all three mutants (sfl2Δ, efg1Δ, and sfl2Δ efg1Δ mutants) uniformly formed opaque cells. We show here by single cell analysis that if tiny, elongate cells formed at 25°C are placed under opaque-inducing conditions at 37°C, they differentiate into opaque cells.
Similar small, elongate cells have been reported in two previous studies. Langford et al. (42) found that when EFG1 and CZF1 were both deleted in an α/α strain originally derived from SC5314, they formed a tiny, elongate phenotype at 30°C, but not 37°C. Tao et al. (43) reported that the α/α strain BJ1097 switched between three colony phenotypes on YPD agar, white, opaque, and gray, and that a majority of cells in the gray colonies formed small, elongate cells that lacked cell wall pimples. It seems likely that these small, elongate cells represent the same phenotype we have found expressed by α/α efg1Δ mutants.

Our results indicate that employing one set of environmental conditions may be inadequate for assessing the roles of many TFs in the process of white-opaque switching. To this point, a recent review by Noble et al. (44) on “switching and functional plasticity in the human host” emphasizes the importance of environmental conditions. Although we have focused on two transcription factors, it seems likely that mutational analyses of a number of additional transcription factors, including those regulated by Sfl2 and Efg1, will reveal similar as well as dissimilar patterns of switching, under different patterns of environmental conditions. In light of the results of Xie et al. (7) demonstrating that more than a third of tested natural α/α strains switch to opaque under inducing conditions, it seems likely that mutations in genes involved in the modulation of switching may have accumulated among natural strains of C. albicans, which has a highly clonal population structure. These different mutants may indeed provide specialized adaptive advantages in commensalism and pathogenesis. Indeed, switching of MTL-homozygous strains to the opaque phenotype has been shown to affect the capacity to colonize skin (45). It is likely no coincidence that the in vitro conditions necessary for inducing mass conversion from white to opaque in the two α/α mutants examined, the sfl2Δ mutant and efg1Δ mutant, correspond to the most common physiological conditions C. albicans experienced in the human host.

MATERIALS AND METHODS

Strains and media. The C. albicans strains used in this study are described in Table S1 in the supplemental material. The strains were maintained at room temperature on nutrient agar containing YPD (1% yeast extract, 2% peptone, 2% glucose) medium or agar containing supplemented Lee’s (sLee’s) medium, which contains 1.25% glucose (36). The latter (sLee’s agar) also contained 5 µg/liter of phloxine B, which differentially stains opaque colonies light red (21). Opaque cells of the sfl2Δ, efg1Δ, and sfl2Δ efg1Δ mutant strains were maintained on nutrient agar containing sLee’s-GlcNac (2%) medium (GlcNac agar) with phloxine B due to their instability in the presence of glucose. Prior to use, white cells were spread on fresh YPD or sLee’s agar and grown for 2 days at 30°C or 5 days at 25°C in air. Escherichia coli strain XL1-Blue (Agilent Technologies, TX, USA), used for the generation and maintenance of plasmids, was grown in LB medium (1% tryptone, 0.5% yeast extract, 1% NaCl) plus 100 µg/ml ampicillin.

Construction of plasmids and generation of C. albicans strains. To delete the EFG1, SFL2, and WOR1 genes, in the wild-type C. albicans α/α strains SC5314 and P37039 (Table S1), we first generated the plasmids pEFG1SM, pSFL2SM, and pWOR1SM, respectively. To generate the plasmids, the 5′ and 3′ regions of the genes were amplified by PCR with the primer pairs listed in Table S2 using genomic DNA from strain SC5314 as the template. The 5′ and 3′ PCR-amplified DNA fragments of the genes and plasmid pSF52, which contains a C. albicans-adapted gene deletion cassette (46), were digested with the appropriate restriction enzymes (the target sequences of the enzymes are underlined in the sequences in Table S2). The DNA fragments into the vector plasmid were then ligated to clone the plasmids that have the gene deletion cassette flanked by the 5′ and 3′ regions of the genes. To reintroduce a wild-type EFG1 into its native locus in the efg1Δ mutants for complementation, we generated the pEFG1C plasmid. A coding region of the EFG1 gene was first amplified by PCR with the primers EFG1-F/-R (Table S2) and placed under the Tet-On promoter in the pNIM1 plasmid (47), resulting in the pNIM-EFG1 plasmid. A PstI/Sall-digested DNA fragment of the EFG1 and CaSAT1 selection marker genes from the pNIM-EFG1 plasmid was fused by ligation with a 5′ promoter region from the pEFG1SM plasmid and a 3′ region of the EFG1 gene, which were amplified by PCR with the primer pair EFG1-3′F1/-3′R (Table S2), into the pBluescript vector plasmid of pSF52 (46). This resulted in pEFG1C. To reintroduce a wild-type copy of SFL2 into the native locus in the sfl2Δ mutants for complementation, we similarly generated the pSFL2SC plasmid. A coding region of the SFL2 gene was first amplified by PCR with the primers SFL2-3′F1/-3′R (Table S2) and placed under the Tet-On promoter in the pNIM1 plasmid (47), resulting in the pNIM-SFL2 plasmid. A PstI/Sall-digested DNA fragment of the SFL2 and CaSAT1 genes from the pNIM-SFL2 plasmid was fused by ligation with a 5′ promoter region and a 3′ region of the SFL2 gene, which were amplified by PCR with the primer pairs, SFL2-3′F1/-3′R and SFL2-3′F1/-3′R (Table S2), respectively, into the vector plasmid of pSF52 (46). To complement both copies of SFL2, plasmid pSF2L2HC was generated by swapping the CaSAT1 gene in plasmid pSF2L2SC with the CaHygB gene. To create plasmid pMCherry-HygB, the mCherry gene, which was amplified by PCR with primers mCherry-F/-R from plasmid pMCherry-
SAT (48), was digested with SalI and BamHI, and inserted into the SalI/BglIII-digested vector pGFP-HygB (49). All sequences of the amplified DNA fragments were verified by sequencing after cloning into the plasmid pRS416. Finally, pGFP-SAT (48), and pGFP-HygB (49) were used to generate strains for mating experiments. Deletion of genes was performed as described by Reuss et al. (46). The gene deletion, complementation, and insertion cassettes from the generated plasmids were used to transform the two C. albicans a/a strains SC5314 and P37039 (Table S1). For selection of cells transformed with the gene deletion or insertion cassettes, YPD agar containing 200 μg/ml of nourseothricin or 1 mg/ml hygromycin B was employed, depending on the selection marker. CaSAT1 and CaHygB conferred resistance to nourseothricin and hygromycin B, respectively. Correct integration of the gene deletion and insertion cassettes, and deletion of the genes, were verified by PCR.

**Switching assays.** White-opaque switching was assessed on glucose agar or GlcNAc agar under four different sets of environmental conditions: 25°C, air; 25°C, 3% CO₂; 37°C, air; and 37°C, 5% CO₂. To assess switching from white to opaque, yeast (white) cells from YPD suspension cultures grown overnight at 25°C were plated at approximately 200 cells per 10-cm petri dish on glucose or GlcNAc agar. At 25°C in air or 5% CO₂, agar cultures were incubated 5 days. At 37°C in air or 5% CO₂, agar cultures were incubated for 3 days. For white-to-opaque switching, opaque-sectored as well as homogeneous opaque colonies were both counted as opaque. At the colony level, colonies formed by efg1Δ and sfl2Δ efg1Δ mutants at 25°C in 5% CO₂, and at 37°C in air were scored as “white” but contained mixtures of tiny, elongate cells and opaque colonies or mixtures of white and opaque cells, respectively. These observations are noted in Results. Therefore, random colonies, both white and opaque, and random opaque sectors were examined microscopically for cellular phenotypes in every experiment. To assess switching from opaque to white under the eight sets of environmental conditions, cells from homogeneous opaque colonies that formed on GlcNAc agar at 25°C in air for 5 days were plated on glucose or GlcNAc agar plates and analyzed for colony phenotype under the four sets of environmental conditions as described above for white-to-opaque switching. For opaque to white switching, white-sectored as well as homogeneous white colonies were scored as “white.”

**Quantitative RT-PCR.** To obtain RNA for performing quantitative reverse transcription-PCR (qRT-PCR), cells were harvested from colonies grown for 3 days on sLee’s-GlcNAc at 37°C in 5% CO₂ and then washed twice with water. Cells were then resuspended in RNAlater solution (Ambion, Life Technologies, Carlsbad, CA, USA) and incubated for 1 h at 4°C and stored at −80°C until use. RNA was extracted using the RNaseasy minikit (Qiagen) according to the manufacturer’s instructions. The TURBO DNA-free kit (Ambion, Life Technologies, Carlsbad, CA, USA) was used to remove DNA contamination. Finally, RNA quality was confirmed to be higher than 9.5 RQI (RNA quality indicator) with the Experion RNA StdSens and HighSens Analysis kit (Bio-Rad). For qRT-PCR assays, cDNA was first generated from the RNA samples using the iScript cDNA synthesis kit (Bio-Rad) as recommended by the manufacturer. qRT-PCR assays were performed with a LightCycler 480 SYBR Green I Master mix (Roche), according to the manufacturer’s instructions. The transcripts were quantified using a Roche LightCycler 480 real-time PCR detection system with SYBR green. The relative expression level of each gene was normalized to that of C. albicans TDH3 (50, 51). Primer pairs used for qRT-PCR are listed in Table S2.

**Mating assay.** To assess the capacity of opaque cells of the a/a sfl2Δ mutants to mate, the hygromycin B-resistant sfl2Δ derivatives, SC5314 sfl2Δ-Hr and P37039 sfl2Δ-Hr, and the a/a and a/a nourseothricin-resistant derivatives, P37005 Sr (a/a) and WO-1 Sr (a/a) derived from the a/a strain P37005 and a/a strain WO-1 (Table S1), respectively, were employed. To measure control levels of mating between a/a and a/a opaque cells, opaque cells of the hygromycin B-resistant strain a/a P37005-Hr and the nourseothricin-resistant strain a/a WO-1 Sr (Table S1) were crossed. Opaque cell suspensions of the strains were obtained from 5 to 10 homogeneous opaque colonies that were grown on a/a strain WO-1-Sr (Table S1) derived from the a/a strain P37005 and a/a strain WO-1 (Table S1), respectively, were employed. To measure control levels of mating between a/a and a/a opaque cells, opaque cells of the hygromycin B-resistant strain a/a P37005-Hr and the nourseothricin-resistant strain a/a WO-1 Sr (Table S1) were crossed. Opaque cell suspensions of the strains were obtained from 5 to 10 homogeneous opaque colonies that were grown on sLee’s-GlcNAc agar medium for 5 days at 25°C in air. The numbers of cells in the opaque cell suspensions were counted, and 1 × 10⁶ opaque cells of each of the mating partners was mixed in 10 μl of water. To facilitate mating, 10-μl portions of the mating mixtures were spotted on a nitrocellulose membrane placed on GlcNAc agar and incubated for 2 and 4 days at 25°C in air. The mating patches on the membrane were then resuspended in 1 ml of water, and 100-μl aliquots of the undiluted (10⁰) and diluted (10⁻¹ and 10⁻²) cell suspensions plated on YPD selection agar containing 200 μg/ml of nourseothricin and 1 mg/ml of hygromycin B. To measure total CFU in the recovered mating suspensions, 100-μl aliquots of 10⁻⁴ and 10⁻⁵ dilutions were plated on YPD agar in the absence of selection molecules. Mating events and total CFU were scored after 3 days of incubation at 30°C, and mating frequencies were computed.

**Immunolocalization of the opaque-specific pimple marker.** Rabbit-derived polyclonal antipimple antiserum (22) was used to visualize the formation of opaque-specific pimples. Opaque cells were heat killed in a 65°C water bath for 1 h. Pelleted cells were then resuspended in phosphate buffer solution (PBS) supplemented with 10% normal goat serum to block nonspecific binding. A 1:50 dilution of rabbit serum was preabsorbed five times with heat-killed MTL-homozygous white cells to remove antibodies to surface antigens common to white and opaque cells (22). Following staining with the primary antiserum, cells were washed with PBS and treated with Alexa Fluor 488-tagged goat anti-rabbit secondary antibody (Jackson ImmunoResearch, West Grove, PA). Fluorescent images were captured using a Bio-Rad 2100 multiphoton LSCM. Differential interference contrast (DIC) images were taken using a Canon Rebel T3i digital camera.

**Imaging colonies and cells.** Colonies of strains, which were grown on agar plates under the indicated conditions in Results, were imaged through a stereo microscope equipped with a Nikon E990 digital camera. Cells from the colonies were imaged with a Canon Rebel T3i digital camera attached to a Bio-Rad Radiance 2000MP upright microscope with a 60 × plan water immersion objective. Cells
Imaging the transition from the tiny, elongate phenotype to the opaque phenotype. To follow the transition from individual tiny, elongate cells to opaque cells, at the cellular level, tiny, elongate cells of \( \alpha \) efG1Δ were plated under a thin layer of agarose in GlcNAc medium in a glass-bottomed, gridded 30 μl-dish (ibidi GmbH, Germany) and then incubated for 30 h at 37°C in 5% CO₂. At time points, cells were imaged with a Canon Rebel T3i digital camera attached to a Bio-Rad Radiance 2000MP upright microscope with a 60× plan water immersion objective. At least 10 tiny, elongated cells were individually followed until the cells developed microcolonies.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/mSphere.00703-18.

FIG S1, TIF file, 2.8 MB.

TABLE S1, DOCX file, 0.02 MB.

TABLE S2, DOCX file, 0.02 MB.

TABLE S3, DOCX file, 0.02 MB.

TABLE S4, DOCX file, 0.01 MB.

TABLE S5, DOCX file, 0.02 MB.

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REFERENCES

1. Odds FC. 1988. Candida and candidosis: a review and bibliography, 2nd ed. Baillière Tindall, London, United Kingdom.
2. Mukherjee PK, Sendid B, Hoarau G, Colombel JF, Poulain D, Ghanounou MA. 2015. Mycobacteria in gastrointestinal diseases. Nat Rev Gastroenterol Hepatol 12:77–87. https://doi.org/10.1038/nrgastro.2014.188.
3. Merseguel KB, Nishikaku A5, Rodrigues AM, Padovan AC, e Ferreira RC, de Azevedo Melo A5, Briones MR, Colombo AL. 2015. Genetic diversity of medically important and emerging Candida species causing invasive infection. BMC Infect Dis 15:57. https://doi.org/10.1186/s12879-015-0793-3.
4. Lockhart SR, Pujol C, Daniels KJ, Miller MG, Johnson AD, Pfaffer MA, Soll DR. 2002. In Candida albicans, white-opaque switchers are homozygous for mating type. Genetics 162:737–745.
5. Ropers J, Maurais C, Diogo D, Marcret-Houben M, Perin A, Sertour N, Mosca K, Pernal E, Laval G, Boucher C, Ma L, Schwartz K, Voelz K, May RC, Poulain J, Battail C, Wincker G, Buzonou ME, d’Enfert C. 2018. Gene flow contributes to diversification of the major fungal pathogen Candida albicans. Nat Commun 9:2253. https://doi.org/10.1038/s41467-018-04787-4.
6. Odds FC, Bougnoux ME, Shaw DJ, Bain JM, Davidson AD, Diogo D, Jacobsen MD, Lecomte M, Li SY, Tavanti A, Maiden MC, Gow NA, d’Enfert C. 2007. Molecular phylogenetics of Candida albicans. Eukaryot Cell 6:1041–1052. https://doi.org/10.1128/EC.00041-07.
7. Xie J, Tao L, Nobile CJJ, Tong Y, Guan G, Sun Y, Cao C, Hernday AD, Johnson AD, Zhang L, Bai FY, Huang G. 2013. White-opaque switching in natural MTLα/α isolates of Candida albicans: evolutionary implications for roles in host adaptation, pathogenesis, and sex. PLoS Biol 11:1001525. https://doi.org/10.1371/journal.pbio.1001525.
8. Magee BB, Magee PT. 2000. Induction of mating in Candida albicans by construction of MTLα and MTLα strains. Science 289:310–313. https://doi.org/10.1126/science.289.5477.310.
9. Hull CM, Raisner RM, Johnson AD. 2000. Evidence for mating of the “asexual” yeast Candida albicans in a mammalian host. Science 289:307–310. https://doi.org/10.1126/science.289.5477.307.
10. Miller MG, Johnson AD. 2002. White-opaque switching in Candida albicans is controlled by mating-type locus homeodomain proteins and allows efficient mating. Cell 110:293–302. https://doi.org/10.1016/S0092-8674(02)00837-1.
11. Lockhart SR, Zhao R, Daniels KJ, Soll DR. 2003. Alpha-pheromone-induced “shmooing” and gene regulation require white-opaque switch-
24. Zordan RE, Galgocy DJ, Johnson AD. 2006. Epigenetic properties of white-opaque switching in Candida albicans are based on a self-sustaining transcriptional feedback loop. Proc Natl Acad Sci U S A 103:12807–12812. https://doi.org/10.1073/pnas.0605138103.

25. Huang G, Wang H, Chou S, Nie X, Chen J, Liu H. 2006. Bistable expression in Candida albicans. Eukaryot Cell 5:1674–1687. https://doi.org/10.1128/EC.00311-12.

26. Zordan RE, Miller MG, Galgocy DJ, Tuch BB, Johnson AD. 2007. Interlocking transcriptional feedback loops control white-opaque switching in Candida albicans. PLoS Biol 5:e256. https://doi.org/10.1038/plosbio.0050256.

27. Lohse MB, Hernday AD, Fordyce PM, Noiman L, Sorrells TR, Hanson-Smith V, Nobile CJ, DeRisi JL. 2013. Identiﬁcation and characterization of a previously undescribed family of sequence-speciﬁc DNA-binding domains. Proc Natl Acad Sci U S A 110:7660–7665. https://doi.org/10.1073/pnas.1217341110.

28. Balan I, Alarco AM, Raymond M. 1997. The Candida albicans CDR3 gene codes for an opaque-phase ABC transporter. J Bacteriol 179:7210–7218.

29. Tailford LE, Crost EH, Kavanaugh D, Juge N. 2015. Mucin glycan foraging in the human gut microbiome. Front Genet 6:81. https://doi.org/10.3389/fgene.2015.00081.

30. Znaidi S, Nesseir A, Chauvel M, Rossignol T, d’Enfert C. 2013. A comprehensive functional portrait of two heat shock factor-type transcriptional regulators of the Candida albicans white-opaque switch. Genetics 203:1679–1692. https://doi.org/10.1534/genetics.116.190657.

31. Hernday AD, Lohse MB, Fordyce PM, Nobile CJ, DeRisi JL, Johnson AD. 2013. Structure of the transcriptional network controlling white-opaque switching in Candida albicans. Mol Microbiol 90:22–35. https://doi.org/10.1042/MMI20130190.

32. Alkafeef SS, Yu C, Huang L, Liu H. 2018. Wor1 establishes opaque cell fate through inhibition of the general co-repressor Tup1 in Candida albicans. PLoS Genet 14:e1007176. https://doi.org/10.1371/journal.pgen.1007176.

33. Solis NV, Park YN, Swidergall M, Daniels KJ, Filler SG, Soll DR. 2013. Candida albicans white-opaque switching influences virulence but not mating during oopharyngeal candidiasis. Infect Immun 81:e00774-17. https://doi.org/10.1128/IAI.00774-17.

34. Rauceo JM. 2013. Sko1 regulates white-opaque switching in Candida albicans. Mol Microbiol 7:e01565-15. https://doi.org/10.1128/MMI.01565-15.

35. Alkafeef SS, Yu C, Huang L, Liu H. 2018. Wor1 establishes opaque cell fate through inhibition of the general co-repressor Tup1 in Candida albicans. PLoS Genet 14:e1007176. https://doi.org/10.1371/journal.pgen.1007176.

36. Bedell GW, Soll DR. 1979. Effects of low concentrations of zinc on the growth and dimorphism of Candida albicans: evidence for zinc-resistant and -sensitive pathways for mycelium formation. Infect Immun 26: 348–354.

37. Ferraris RP, Yasharpour S, Lloyd KC, Mirzayan R, Diamond JM. 1990. Luminal glucose concentrations in the gut under normal conditions. Am J Physiol 259:G822–G837. https://doi.org/10.1152/ajpgi.1990.259.5.G822.

38. Ferraris RP, Yasharpour S, Lloyd KC, Mirzayan R, Diamond JM. 1990. Luminal glucose concentrations in the gut under normal conditions. Am J Physiol 259:G822–G837. https://doi.org/10.1152/ajpgi.1990.259.5.G822.

39. Alkafeef SS, Yu C, Huang L, Liu H. 2018. Wor1 establishes opaque cell fate through inhibition of the general co-repressor Tup1 in Candida albicans. PLoS Genet 14:e1007176. https://doi.org/10.1371/journal.pgen.1007176.

40. Reuss O, Vik A, Kolter R, Morschhauser J. 2004. The SAT1 flipper, an optimal tool for gene disruption in Candida albicans. Gene 341: 119–127. https://doi.org/10.1016/j.gene.2004.06.021.

41. Tailford LE, Crost EH, Kavanaugh D, Juge N. 2015. Mucin glycan foraging in the human gut microbiome. Front Genet 6:81. https://doi.org/10.3389/fgene.2015.00081.

42. Kvaal C, Lachke SA, Srikantha T, Daniels K, McCoy J, Soll DR. 1999. Misexpression of the opaque-phase-specific gene PEP1 (SAPI) in the white phase of Candida albicans confers increased virulence in a mouse model of cutaneous infection. Infect Immun 67:6652–6662.

43. Ferraris RP, Yasharpour S, Lloyd KC, Mirzayan R, Diamond JM. 1990. Luminal glucose concentrations in the gut under normal conditions. Am J Physiol 259:G822–G837. https://doi.org/10.1152/ajpgi.1990.259.5.G822.

44. Noble SM, Gianetti BA, Witchley JN. 2017. Candida albicans cell-type switching and functional plasticity in the mammalian host. Nat Rev Microbiol 15:96–108. https://doi.org/10.1038/nrmicro.2016.157.

45. Ferraris RP, Yasharpour S, Lloyd KC, Mirzayan R, Diamond JM. 1990. Luminal glucose concentrations in the gut under normal conditions. Am J Physiol 259:G822–G837. https://doi.org/10.1152/ajpgi.1990.259.5.G822.

46. Tailford LE, Crost EH, Kavanaugh D, Juge N. 2015. Mucin glycan foraging in the human gut microbiome. Front Genet 6:81. https://doi.org/10.3389/fgene.2015.00081.

47. Tailford LE, Crost EH, Kavanaugh D, Juge N. 2015. Mucin glycan foraging in the human gut microbiome. Front Genet 6:81. https://doi.org/10.3389/fgene.2015.00081.

48. Tailford LE, Crost EH, Kavanaugh D, Juge N. 2015. Mucin glycan foraging in the human gut microbiome. Front Genet 6:81. https://doi.org/10.3389/fgene.2015.00081.

49. Tailford LE, Crost EH, Kavanaugh D, Juge N. 2015. Mucin glycan foraging in the human gut microbiome. Front Genet 6:81. https://doi.org/10.3389/fgene.2015.00081.

50. Tailford LE, Crost EH, Kavanaugh D, Juge N. 2015. Mucin glycan foraging in the human gut microbiome. Front Genet 6:81. https://doi.org/10.3389/fgene.2015.00081.