

RESEARCH PAPER

Discovery of the gray phenotype and white-gray-opaque tristable phenotypic transitions in *Candida dubliniensis*

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ABSTRACT

Candida dubliniensis is closely related to *Candida albicans*, a major causative agent of candidiasis, and is primarily associated with oral colonization and infection in human immunodeficiency virus (HIV)-positive patients. Despite the high similarity of genomic and phenotypic features between the 2 species, *C. dubliniensis* is much less virulent and less prevalent than *C. albicans*. The ability to change morphological phenotypes is a striking feature of *Candida* species and is linked to virulence. In this study, we report a novel phenotype, the gray phenotype, in *C. dubliniensis*. Together with the previously reported white and opaque cell types, the gray phenotype forms a tristable phenotypic switching system in *C. dubliniensis* that is similar to the white-gray-opaque tristable switching system in *C. albicans*. Gray cells of *C. dubliniensis* are similar to their counterparts in *C. albicans* in terms of several biological aspects including cellular morphology, mating competence, and genetic regulatory mechanisms. However, the gray phenotypes of the 2 species have some distinguishing features. For example, the secreted aspartyl protease (Sap) activity is induced by bovine serum albumin (BSA) in gray cells of *C. albicans*, but not in gray cells of *C. dubliniensis*. Taken together, our results demonstrate that the biological features and regulatory mechanisms of white-gray-opaque tristable transitions are largely conserved in the 2 pathogenic *Candida* species.

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Introduction

Candida species are the most common agents of fungal infections in humans. Among them, *Candida albicans* is the best studied and most prevalent pathogen.^{1–5} *Candida dubliniensis*, which was identified as a new species in 1995, is closely related to *C. albicans*.^{6–8} The two species share many phenotypic characteristics, including the development of true hyphae and chlamydo spores.^{6,9} The genome sequences of these 2 species are highly similar and the gene order is largely collinear.¹⁰ However, *C. dubliniensis* is much less pathogenic and prevalent than *C. albicans*.^{3,11,12} Epidemiological studies indicate that *C. dubliniensis* is primarily associated with human immunodeficiency virus (HIV)-infected patients.^{6–9} A number of comparative genomic and phenotypic studies of *C. albicans* and *C. dubliniensis* have been performed in the previous 2 decades,^{12–15} but what underlying mechanisms or biological characteristics underlying the differences between the 2 species in


epidemiology, virulence, and the ability to develop antifungal resistance remain to be investigated.

A striking feature of the biology of *C. albicans* is its phenotypic plasticity: the ability to grow in several morphological forms.^{4,16,17} This ability is important for the fungus to rapidly adapt to the changing host environment and cause infections. White-opaque switching in *C. albicans* has been intensively investigated in the previous decade.^{18–21} White and opaque cells are 2 distinct and heritable cell types that differ in cellular morphologies, global gene expression profiles, susceptibility to host immune cells, and mating competency. White cells are round and relatively small, while opaque cells are elongated and large. The cell wall surface of white cells is smooth, whereas that of opaque cells is pimples.²² White cells are more virulent in systemic infections, while opaque cells have an enhanced ability to cause cutaneous damage due to high activity levels of secreted aspartyl proteases (Saps).^{23–25} In addition, opaque cells mate much more efficiently than white cells. Therefore, to

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mate, white cells must first switch to the opaque cell type.²⁶ Recently, we reported a novel cell type, the gray phenotype, in *C. albicans*.²⁵ Gray cells are elongated but smaller than white and opaque cells. Similar to the white and opaque cell types, gray cells are also heritable. The three cell types (white, gray, and opaque) form a tristable phenotypic switching system. The appearances of colonies formed by the 3 cell types are distinct on nutrient agar plates. On solid medium plates containing phloxine B, a red dye, white cells form white and shiny colonies and gray cells form light pink colonies, while opaque cells form “opaque” and red colonies. Gray cells of *C. albicans* are more virulent than white and opaque cells in models of ex vivo tongue infections and exhibit an intermediate level of mating competency. Many studies have demonstrated that different cell types evolved to adapt to different host niches.^{21,25,27}

In the present study, we report the discovery of the gray phenotype and the white-gray-opaque tristable switching system in *C. dubliniensis*. Despite differences in Sap activity and global gene expression profiles, the gray cells of *C. dubliniensis* are similar to the gray cells of *C. albicans* with regard to many biological features including cellular morphology, mating competency, and virulence in a mouse model of systemic infection. We further demonstrate that the Wor1 and Efg1 transcription factors play a critical role in the regulation of white-gray-opaque tristable transitions in *C. dubliniensis*.

Results

Discovery of a novel morphological phenotype, the gray phenotype, and white-gray-opaque tristable switching in *C. dubliniensis*

The gray phenotype of *C. dubliniensis* was initially observed in the clinical isolate PC35 when grown on Lee’s GlcNAc plates in 20% CO₂ for 7 d. Given that the 2 species are closely related phylogenetically, it is reasonable that *C. dubliniensis* undergoes white-gray-opaque transitions as *C. albicans* does.²⁵ To test whether other clinical strains could develop the gray phenotype, we performed white-gray-opaque switching assays in 10 clinical isolates of *C. dubliniensis* under several culture conditions (Fig. 1 and Fig. S1). Solid YPD and Lee’s media were used for cell growth. Four strains (PC35, P86, d163, and d172; 40% of the total tested strains) could undergo white-gray-opaque transitions. d163, P86, and d172 were isolated from HIV-infected patients, and PC35 was from a healthy individual.¹³ To verify that these strains were in fact *C. dubliniensis*, we sequenced the internal transcribed spacer (ITS) region of the rRNA genes. The colony and cellular morphology of the gray

cells of all 3 *C. dubliniensis* strains was similar to that of *C. albicans* gray cells (Fig. 1 and Fig. S1). The gray cells were bean-shaped and smaller than the white and opaque cells. The colonies of gray cells were smaller than those of white and opaque cells. The morphologies of the white and opaque cells of PC35 and P86 were similar to their counterparts in *C. albicans*. However, the opaque cells of d163 underwent clear filamentation and formed fluffy colonies on Lee’s GlcNAc medium (Fig. S1). White-opaque switching was not observed in d163 in a previous study.¹³ The discrepancy between our findings and the previous study could be due to the use of different media and culture conditions. Of note, d163 (a/a), P86 (α/α), and PC35 (a/a) are homozygous at the mating type locus (*MTL*), which controls white-opaque switching in *C. albicans*.²⁶ The opaque- and gray-competent strain, d172 (a/ α), is a *MTL* heterozygous strain, suggesting that this tristable switching in *C. dubliniensis* is independent of the mating type locus under our culture conditions.

Consistent with the previous report, SEM assays demonstrated that the cell wall surface of the white cells of *C. dubliniensis* was smooth and that of some opaque cells was pimpled (Fig. S2). However, there were some obvious “pits” on the cell wall surface of the gray cells. The functions of the “pimples” of opaque cells and “pits” of gray cells remain to be investigated.

Because the gray cells of *C. dubliniensis* are much smaller than white and opaque cells, we performed fluorescence-activated cell sorting (FACS) analysis to assess ploidy. As shown in Figure S3, all 3 cell types contained a comparable amount of DNA, which suggested that they are all diploid.

Effect of environmental conditions on white-gray-opaque tristable transitions in *C. dubliniensis*

To characterize the switching feature of *C. dubliniensis*, we performed quantitative switching assays using 9 culture conditions (Fig. 2). The switching frequencies between white-gray, white-opaque, and gray-opaque varied dramatically under different culture conditions. In air, the white cell type was more stable than the gray and opaque cell types. In fact, both gray and opaque cells underwent a mass conversion to the white cell type in all 3 culture media (YPD, Lee’s glucose, and Lee’s GlcNAc). In the presence of high levels of CO₂ (5% or 20%), the gray-to-white switching frequencies were remarkably decreased in all media, while the opaque-to-white switching frequencies were also reduced in Lee’s GlcNAc medium. In the presence of GlcNAc, gray cells became more stable in air. Furthermore, GlcNAc and CO₂ had a synergistic effect on preventing the switch from opaque-

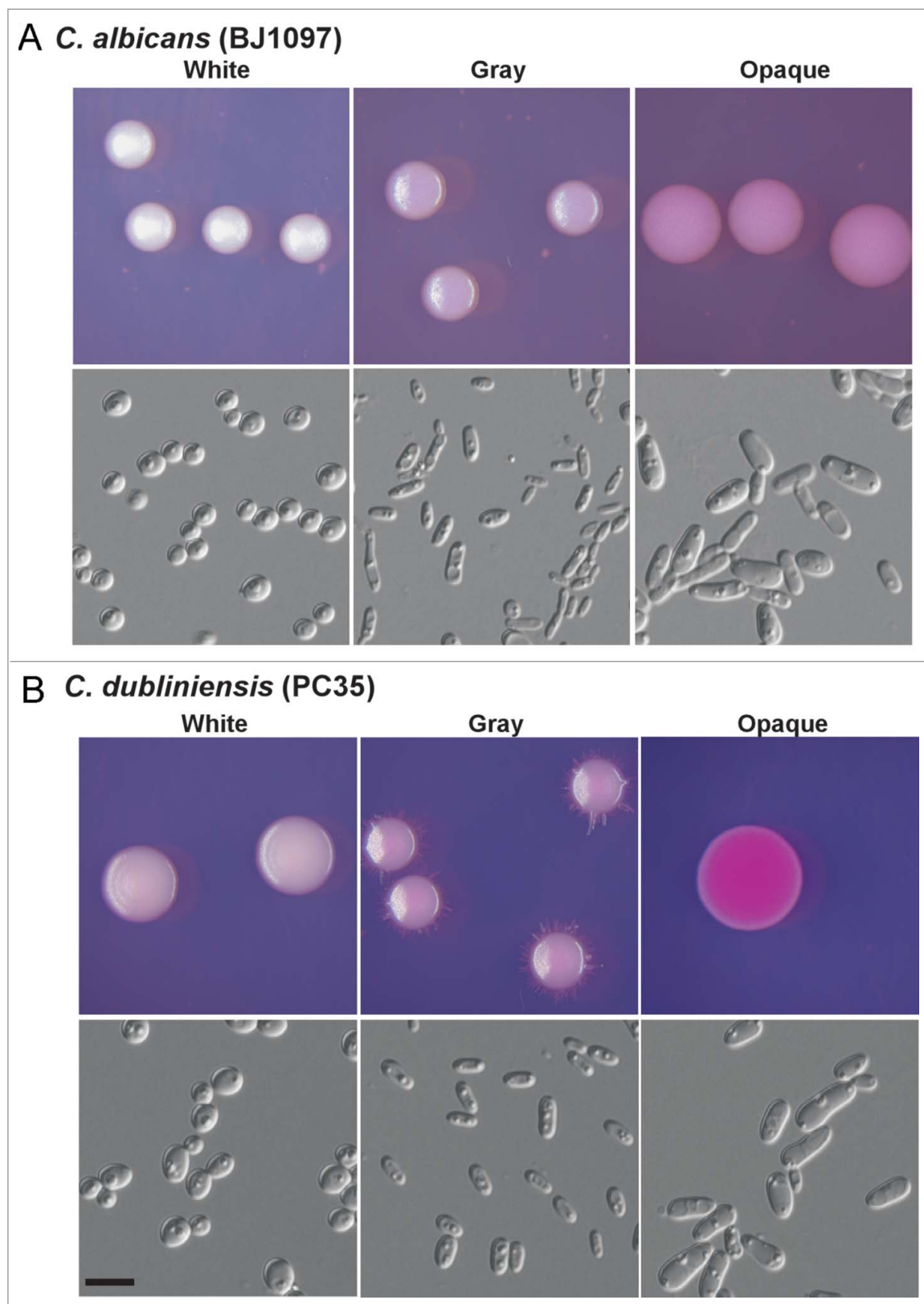


Figure 1. White-gray-opaque transitions in *C. albicans* (BJ1097, A) and *C. dubliniensis* (PC35, B). Homogeneous white, gray, or opaque cells were plated on agar plates. Colony and cellular morphologies of the 3 different phenotypes (white, gray, and opaque) are shown. The colonies and cells were imaged after 5 d of growth at 25°C in 5% CO₂. Scale bar, 10 μm. Phloxine B (a red dye that stains gray colonies light pink and opaque colonies red) was added at a concentration of 5 μg/mL to Lee's GlcNAc medium.

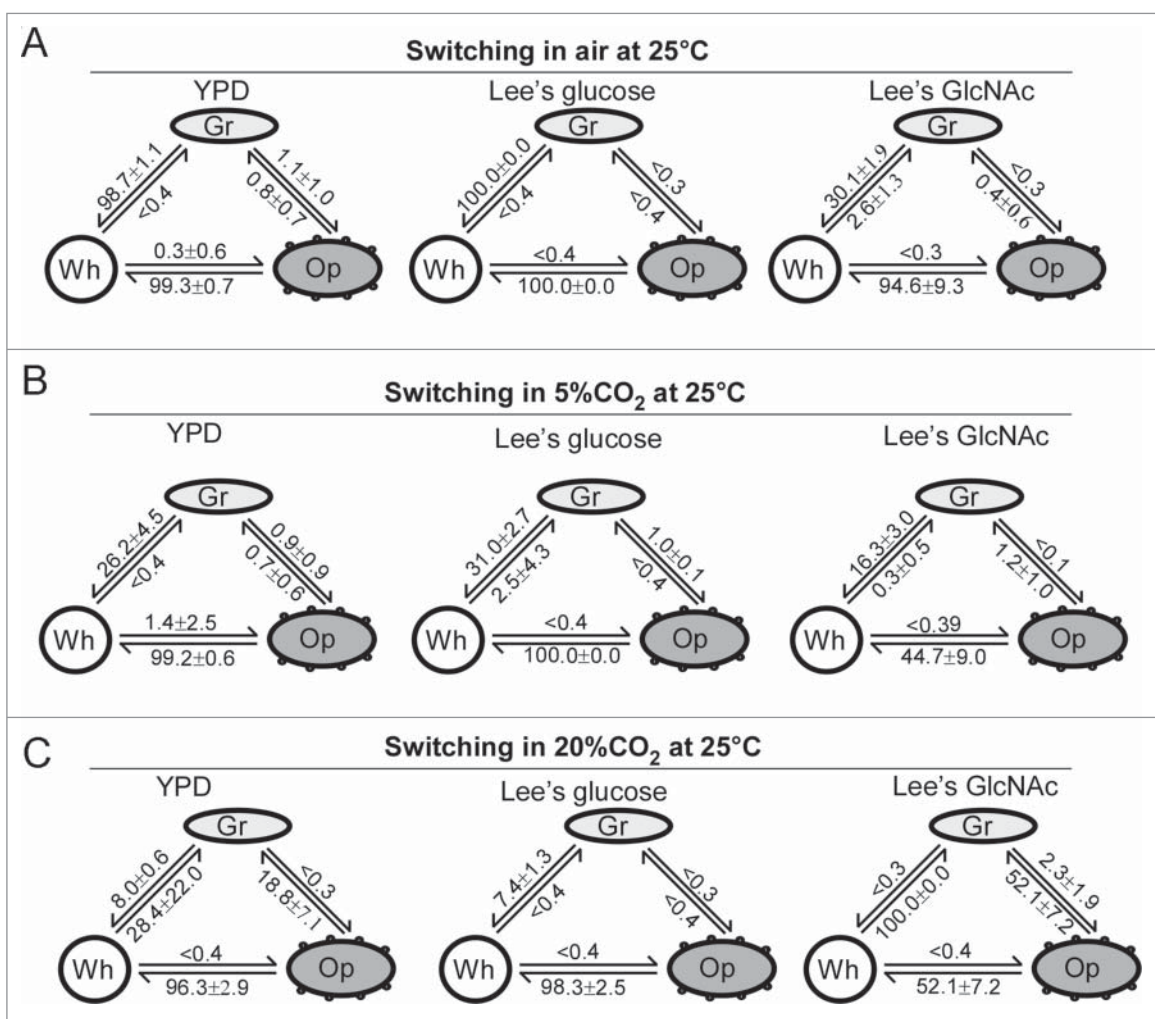


Figure 2. Switching frequencies of white-gray-opaque transitions in *C. dubliniensis* (PC35) under different culture conditions. Wh, white; Gr, gray; Op, opaque. Colonies (strain PC35) were grown under the indicated conditions at 25°C for 6 d. YPD, Lee's glucose, and Lee's GlcNAc media were used. Switching frequency = average ± standard deviation (SD). (A) Switching frequencies in air. (B) Switching frequencies in 5% CO₂. (C) Switching frequencies in 20% CO₂.

to-white. Although both GlcNAc and CO₂ promote white-to-opaque transitions in *C. albicans*,^{28,29} the inducing effect of GlcNAc and CO₂ on the white-to-opaque transition was not observed in *C. dubliniensis*.

Distinct global gene expression profiles in white, gray, and opaque cells

To characterize the global transcriptional profiles of white, gray, and opaque cells of *C. dubliniensis*, we performed RNA-Seq analysis. A comparative analysis indicated that despite some species-specific cell type-enriched genes, a large number of differentially expressed genes between white-gray, white-opaque, and gray-opaque cell types were similar to those found in *C. albicans* (Fig. 3 and Dataset S1). Several key findings are described below. In white cells, a number of genes involved in fermentative metabolism and the stress response (e.g. *HGT6*, *NAG3*, *HGT19*,

HSP21, *HSP70*, and *HSP78*) were exclusively enriched. Similar to the expression profile in white cells of *C. albicans*, the white-opaque regulator gene *EFG1* and white-specific gene *WH11* were upregulated in white cells of *C. dubliniensis*. The transcription factor gene *SFL2*, which is upregulated in gray cells of *C. albicans*,²⁵ was also enriched in gray cells of *C. dubliniensis*, which indicated that *Sfl2* plays a conserved role in this regulation. Most interestingly, at least 9 genes involved in ergosterol biosynthesis (*ERG1*, *ERG3*, *ERG4*, *ERG5*, *ERG6*, *ERG11*, *ERG13*, *ERG25*, and *ERG251*) and 3 mannanbiosynthesis-related genes (*MNN1*, *MNN4* and *MNN22*) were upregulated in gray cells of *C. dubliniensis*. These genes play a critical role in the regulation of antifungal resistance and other stresses. The global gene expression profile of opaque cells of *C. dubliniensis* was highly similar to that of opaque cells of *C. albicans*. Of the 55 opaque cells-enriched genes in *C. dubliniensis* (a 4-fold cut-off), 32 (58.2%) were also enriched in

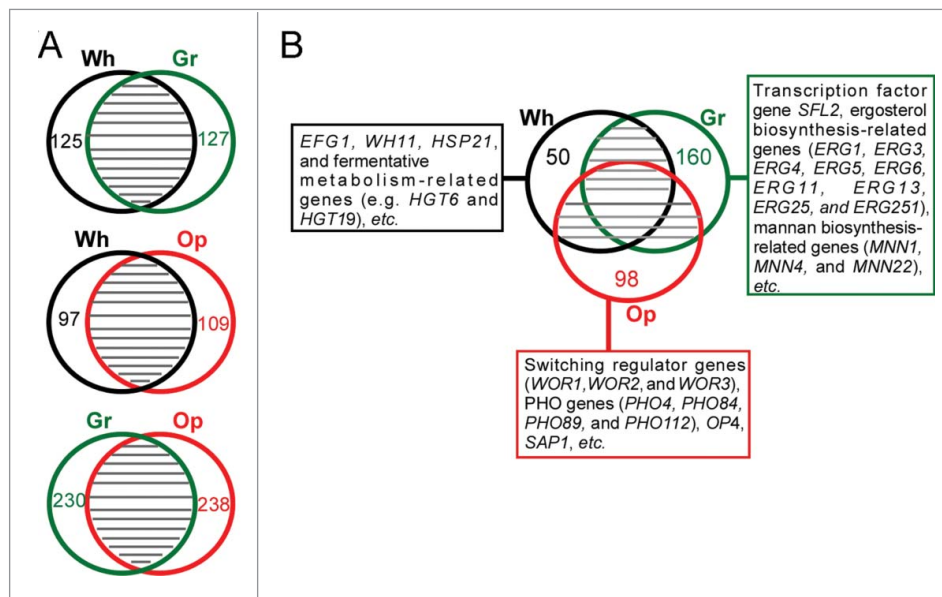


Figure 3. Global gene expression profiles in white, gray, and opaque cells of *C. dubliniensis*. Differentially expressed genes were identified by RNA-Seq analysis using a 2-fold cut-off. Wh, white; Gr, gray; Op, opaque. (A) Venn diagrams of differentially expressed genes between white-gray, white-opaque, and gray-opaque cell types. The numbers of cell type-specific genes are indicated. (B) White-, gray-, and opaque-specific genes.

opaque cells of *C. albicans*.^{24,25,30} These genes included *SAP1*, *OP4*, *WOR2*, *WOR3*, and some oxidative metabolism-related genes (such as *PXP2* and *ALD6*). Of note, 4 genes involved in phosphate metabolism (*PHO4*, *PHO84*, *PHO89*, and *PHO112*) were upregulated in opaque cells of *C. dubliniensis*. The relative expression levels of 7 cell type-enriched genes (*ERG11*, *ERG251*, *RPL10A*, *RPL12*, *WOR1*, *OP4*, and *WH11*) in the 3 different cell types were verified using quantitative RT-PCR assays (Fig. S4). A detailed analysis of differentially expressed genes in the 3 cell types is presented in Dataset S1.

White, gray, and opaque cells exhibit different secreted aspartyl protease (Sap) activities

Because several *SAP* genes were differentially expressed in different cell types (*SAP9* and *SAP10* in white, *SAP7* and *SAP10* in gray, and *SAP1* in opaque cells, Dataset S1), we tested the Sap activities of white, gray, and opaque cells in 3 clinical isolates of *C. dubliniensis* (PC35, P86, and d163) using YCB-BSA assays.³¹ Opaque cells exhibited the highest Sap activity, while gray cells had the lowest Sap activity (Fig. S5). The Sap activities of white, gray, and opaque cells in the 3 clinical strains were equivalent (opaque > white > gray), suggesting that this feature is not strain-dependent. The RNA-Seq analysis demonstrated that *SAP1* was exclusively expressed in opaque cells and its absolute expression level was very high (the RPKM value was 1,341 in opaque cells and was only approximately 4 in white and gray cells). Although

SAP9 and *SAP10* were upregulated in white cells and *SAP7* and *SAP10* were upregulated in gray cells, the absolute expression levels of these genes were very low in all cell types (RPKM values <220). These results for the expression levels of *SAP* genes in different cell types are consistent with the Sap activities observed on YCB-BSA plates (Fig. S5).

White, gray, and opaque cells differ in virulence in systemic and ex vivo tongue infection models in mice

As shown in Figure 4A, at the higher inoculation concentration (1×10^7 cells per mouse), mice infected with white cells died faster than those infected with opaque cells, while none of the mice infected with gray cells died. At the lower inoculation concentration (4×10^6 cells per mouse), the mice began to die at the fifth day after infection with white cells, but no mice infected with either gray or opaque cells died even at 15th day after inoculation. Therefore, the order of virulence from highest to lowest in the mouse systemic infection model was as follows: white cells > opaque cells > gray cells. Moreover, the growth rates of white, gray, and opaque cells in Lee's GlcNAc medium at 37°C were comparable (data not shown), suggesting that this difference of virulence is not due to the different growth rates of cell of the 3 cell types.

We further examined the fungal burdens in different organs in a mouse systemic infection model (Fig. 4B). Consistent with the findings observed in *C. albicans*, the

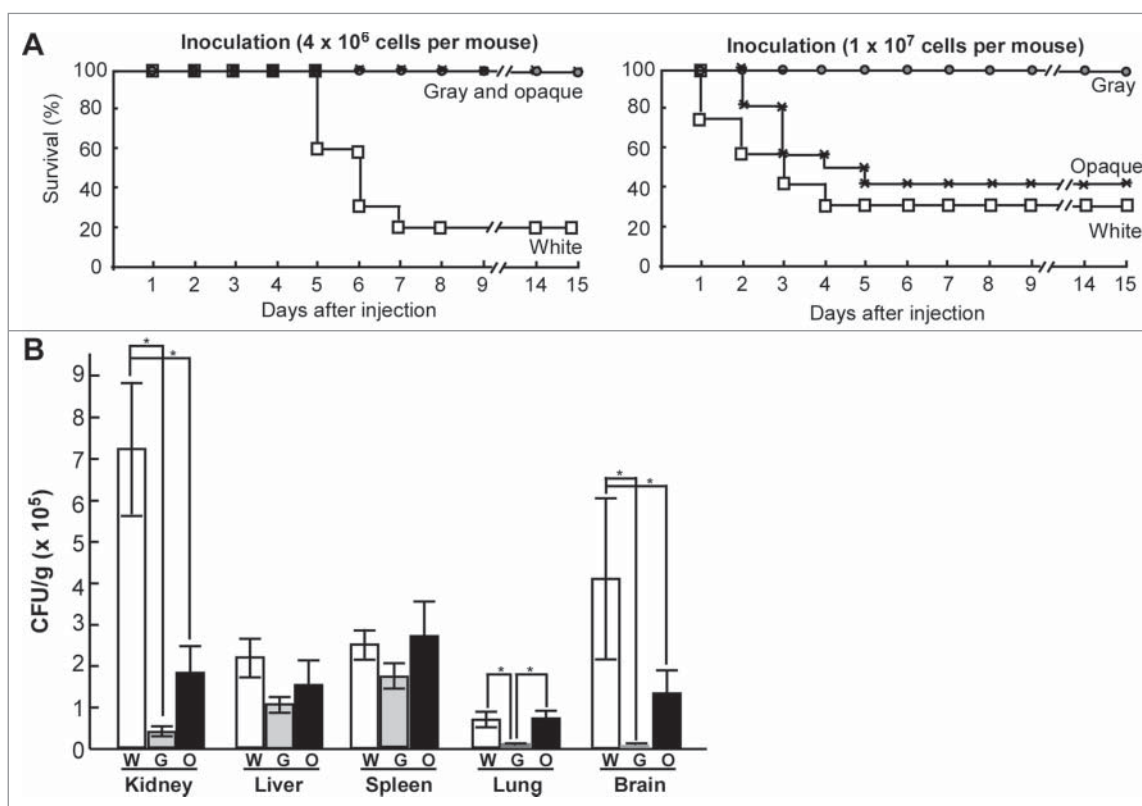


Figure 4. White, gray, and opaque cells differ in virulence in mouse systemic infections. (A) Survival curves for white, gray, and opaque cells of strain PC35. Each cell type (4×10^6 , left, 10 mice used for each cell type; or 1×10^7 , right, 12 mice used for each cell type) was injected into each mouse via the tail vein. (B) Fungal burdens of white, gray, and opaque cells in different organs in a mouse systemic infection system. Each cell type (2×10^6 cells in $200 \mu\text{L}$ of PBS) was injected into each mouse via the tail vein. Six mice were used for each cell type. The mice were killed at 24 hours after injection and 5 organs (liver, kidney, spleen, lung, and brain) were used for the fungal burden assays. The average numbers and standard deviations of the CFU per gram of different tissues are indicated. * indicates significant difference (P value < 0.05 , Student's *t*-test, 2-tailed).

fungal burdens of white cells of *C. dubliniensis* were higher than those of gray and opaque cells in the kidney, while the relative levels of fungal burdens of gray and opaque cells were higher in the spleen and liver compared with other organs. To monitor the stability of the 3 cell types in vivo, homogenized kidneys of infected mice were suspended and plated on Lee's GlcNAc plates. We found that the majority ($>90\%$) of white and gray cells maintained their original cell type, while about 40% of opaque cells maintain the original cell type and about 60% of opaque cells switched to the white cell type.

Saps are one of the major virulence factors of *Candida* species in mucosal infection. These secreted enzymes degrade animal tissues and facilitate fungal growth and penetration during infections.^{23,25,32,33} We performed ex vivo tongue infection assays and found that the order of growth rates for the 3 cell types was opaque cells $>$ gray cells $>$ white cells at both 30°C (Fig. S6A) and 37°C (Fig. S6B), which indicated that opaque cells have a greater ability to degrade animal tissues. White and gray cells were very stable at both temperatures, while opaque cells underwent mass conversion to the white phenotype

at 37°C (Fig. S6C). Together with the findings for the systemic infection assays, these results suggest that different cell types may be better at colonizing different animal tissues.

White, gray, and opaque cells differ in mating competence

White-opaque switching and sexual mating are linked processes in *C. albicans*, *C. dubliniensis*, and *C. tropicalis*.^{13,26,34,35} Opaque cells mate much more efficiently than white cells in these species. We tested the mating competence of white, gray, and opaque cells of *C. dubliniensis*. As shown in Figure 5A, the formation of mating projections (long filament-like tubes) was observed in the mixed culture of opaque "a" cells (PC35) \times opaque "a" cells (P86), but this formation was rarely observed in mixed cultures of white "a" cells \times white "a" cells or gray "a" cells \times gray "a" cells. We then performed quantitative mating assays to compare the competences among the 3 cell types. As shown in Figure 5B, similar to the gray cells of *C. albicans*, *C. dubliniensis* gray cells exhibited an

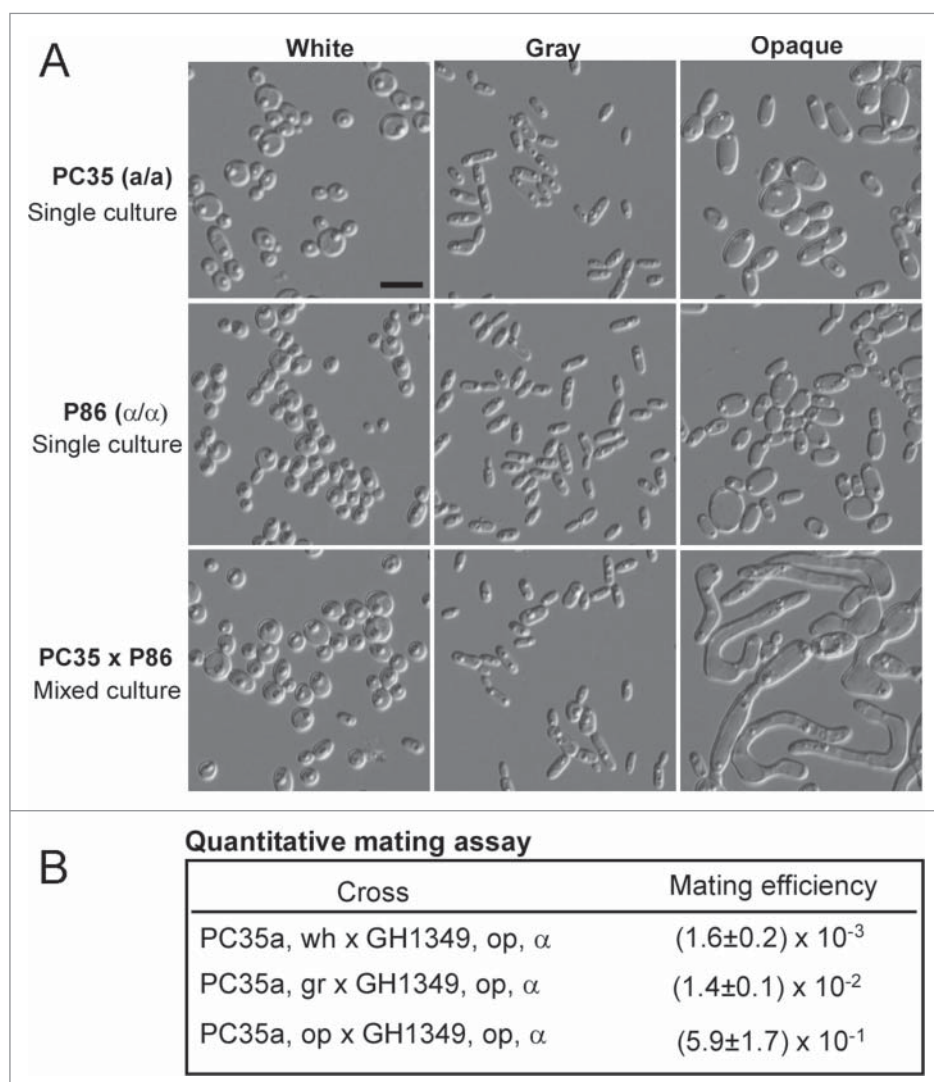


Figure 5. White, gray, and opaque cells exhibit different mating competence. (A) Mating assays performed in liquid Lee's GlcNAc medium between PC35 (*MTLa/a*, *C. dubliniensis*) and P86 (*MTLα/α*, *C. dubliniensis*). Cellular morphologies of single strain cultures and mixed cultures are shown. Single strain cultures: 5×10^6 white, gray, or opaque cells of PC35 or P86 grown in 1 mL of Lee's GlcNAc medium at 25°C for 24 hours. Mixed cultures: 5×10^6 white, gray, or opaque cells of PC35 mixed with 5×10^6 cells of the same cell type of P86 and grown in 1 mL of Lee's GlcNAc medium at 25°C for 24 hours. Scale bar, 10 μm. (B) Quantitative mating assays. Experimental strain: PC35u (*MTLa/a*, *ura3*⁻). Tester: *C. albicans* strain GH1349 (*MTLα/α*, *arg4*⁻). White, gray, or opaque a/a cells of PC35 (5×10^6 cells) were mixed with an equivalent number of opaque α cells (GH1349) in 10 μL of ddH₂O, spotted onto Lee's GlcNAc medium plates and cultured at 25°C for 48 hours. The mating mixtures were replated onto SC-uridine, SC-arginine, and SC-uridine-arginine medium plates for prototrophic selection. Mating efficiency = average ± standard deviation (SD).

intermediate level of mating competency. Opaque cells of PC35 mated hundreds of times and gray cells mated approximately 10 times more efficiently than white cells.

The *Wor1* and *Efg1* transcription factors regulate white-gray-opaque transitions

Wor1 and *Efg1* play a critical role in the regulation of white-opaque and white-gray-opaque transitions in *C. albicans*.^{25,36-40} We investigated the role of these 2 transcription factors in the regulation of white-gray-opaque transitions in *C. dubliniensis*. As mentioned

previously, *WOR1* is exclusively expressed in opaque cells, while *EFG1* is enriched in white cells of *C. dubliniensis* (Dataset S1). Deletion of *WOR1* blocked the formation of opaque cells and the mutant could only switch between the white and gray cell types. Gray cells of the *wor1/wor1* mutant became more stable than those of the WT control on Lee's GlcNAc medium (Figs. 2, 6, and Fig. S7). Deletion of *EFG1* blocked the formation of white cells and the mutant could only switch between the gray and opaque cell types. Compared with the WT control, the switching frequency of the *efg1/efg1* mutant increased in the

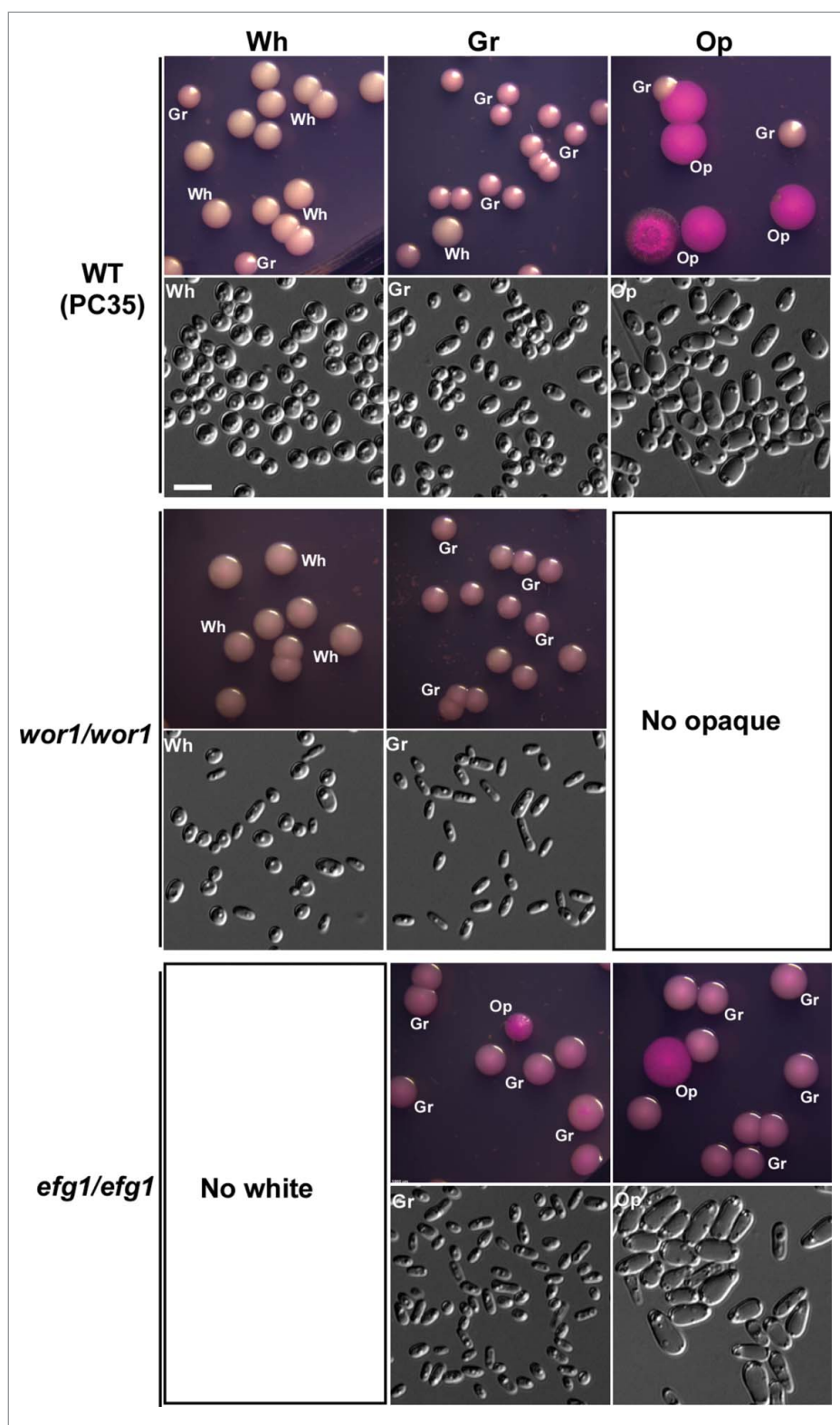


Figure 6. Wor1 and Efg1 regulate white-gray-opaque transitions in *C. dubliniensis*. Wh, white; Gr, gray; Op, opaque. Cells were grown on Lee's GlcNAc medium in air at 25°C for 5 d. The switching frequency data are shown in Figure S7. No opaque colonies were observed in the *wor1/wor1* mutant, and no white colonies were observed in the *efg1/efg1* mutant.

presence of GlcNAc (Figs. 2, 6, and Fig. S7). These results suggest that Wor1 is essential for the opaque phenotype, while Efg1 is required for the white

phenotype. The two transcription factors could coordinately regulate the white-gray-opaque tristable switching system in *C. dubliniensis*.

Discussion

Phenotypic switching allows pathogenic fungi to rapidly adapt to the changing environment of the host. It has been reported that 3 *Candida* species (*C. albicans*, *C. dubliniensis*, and *C. tropicalis*) can undergo white-opaque switching, which is linked to the ability to cause systemic and cutaneous infections and to undergo sexual mating.^{13,19-21,25,34,35} We have recently discovered that *C. albicans* can also exist as a novel cell type, the gray phenotype,²⁵ which is distinct from the well-studied white and opaque cell types.²¹ In the current study, we report the discovery of the gray phenotype and white-gray-opaque transitions in *C. dubliniensis*. These findings imply that the white-gray-opaque tristable switching system is conserved in the 2 closely related *Candida* species.

The gray cell type of *C. dubliniensis* is similar to that of *C. albicans* in terms of several biological features, such as cell morphology, reduced virulence in systemic infection models, and mating competence. Four of the 10 clinical isolates of *C. dubliniensis* (40%) were found to be able to form gray cells. This ratio of gray-competent strains is much higher than that of *C. albicans* (10%, unpublished data). The regulatory mechanisms of the white-gray-opaque transitions in the 2 species are also similar. Minimally, the 2 master regulators, *Wor1* and *Efg1*, play similar regulatory roles in the 2 species.²⁵ However, the gray cells of the 2 species also have their own species-specific characteristics. For example, the rich YPD medium has an inducing effect on the gray phenotype in *C. albicans*,²⁵ but not in *C. dubliniensis* (Fig. 2). Interestingly, it has been shown that, unlike *C. albicans*, *C. dubliniensis* could not undergo filamentation in YPDS medium (YPD medium supplemented with 10% v/v fetal calf serum), but could filament in water plus 10% (vol/vol) fetal calf serum.⁴¹ Given its primary niche is the oral cavity, which represents a poor nutritional environment, it is possible that rich nutrient conditions repress phenotypic transitions in *C. dubliniensis*. It has been shown that both GlcNAc and CO₂ induce the opaque phenotype in *C. albicans*. Combined use of GlcNAc and CO₂ has a synergistic effect on this induction.^{24,28,29} In *C. dubliniensis*, GlcNAc or CO₂ alone does not promote the opaque phenotype. However, combined use of GlcNAc and CO₂ (20%) promotes the gray phenotype and stabilizes the opaque phenotype (Fig. 2). Given that both CO₂ and GlcNAc are important host-related environmental cues (released by commensal bacteria), the different abilities of *C. albicans* and *C. dubliniensis* to undergo morphological changes in response to these inducers could contribute to their virulence and colonization ability. *C. dubliniensis* primarily colonizes the oral cavity. The switch to the gray phenotype could help this

fungus to compete with other bacterial members of the same niche.

There are 10 SAP genes in *C. albicans* but only 8 in *C. dubliniensis*.¹⁴ Interestingly, Sap activity is induced by the protein BSA in gray cells of *C. albicans* but not in the gray cells of *C. dubliniensis*. The expression level of *SAP2* is increased thousand times in the presence of BSA in gray cells of *C. albicans*.²⁵ This difference of Sap activity is consistent with the result of ex vivo tongue infection assays. Gray cells grow faster than white and opaque cells of *C. albicans*,⁴² while the growth rate of opaque cells is the fastest in *C. dubliniensis* (Fig. S6). Given that Saps are major virulence factors in pathogenic *Candida* species, the difference in Sap activity between the 2 species may contribute to their pathogenesis. The inducible feature of Sap activity in *C. albicans* may contribute to its higher virulence compared with *C. dubliniensis* in infection settings.

A global gene expression analysis demonstrated that white, gray, and opaque cells of *C. dubliniensis* have a set of cell type-specific genes (Dataset S1). A large number of white and opaque cell-enriched genes found in *C. dubliniensis* are also enriched in the corresponding cells of *C. albicans*, especially those genes with a highly differential expression pattern. However, only a small number of gray cell-enriched genes in *C. dubliniensis* were also found to be enriched in the gray cells of *C. albicans*. These genes include the transcription factor *SFL2*, *CAR1*, *ERG251*, and *ORF19.5933*. It has been suggested that *C. dubliniensis* is more capable of developing antifungal resistance (e.g., to azoles) than *C. albicans*.^{9,12,14} This feature of *C. dubliniensis* may be associated with its prevalence in AIDS patients, who are often subjected to antifungal treatments. Our results showing the upregulated expression of a number of ergosterol biosynthesis genes in gray cells of *C. dubliniensis* provide a potential explanation for this phenomenon.

As in *C. albicans*, white, gray, and opaque cells of *C. dubliniensis* differ in mating competence. Gray cells exhibit an intermediate level of mating competence in both species. The link between phenotypic switching and sexual mating could play a balancing role in the sexual and non-sexual reproduction lifestyles in the 2 species. Phenotypic switching facilitates a rapid adaptation to new environments, while sexual reproduction may play a role in the evolution of new traits and maintaining a diverse population to gain advantages over other species over time. Therefore, different cell types may play a distinct role in the processes of infecting the host and adapting to unique ecological niches and different lifestyle stages. Understanding the regulatory mechanisms of these morphological transitions would not only provide insights into the adaptive strategies of pathogenic pathogens but

would also be important for the development of new antifungal drugs.

In summary, the 2 closely related species, *C. dubliniensis* and *C. albicans*, share many common biological features. In the present study, we report the white-gray-opaque phenotypic switching system in *C. dubliniensis*, which is highly similar to that in *C. albicans*. The new gray cell type of *C. dubliniensis* is similar to the gray cell type of *C. albicans* in terms of cellular morphology, mating competence, and genetic regulatory mechanisms. These findings are reasonable given the high similarity of the genomic DNA sequences of the 2 species. There are indeed some unique features of gray cells of *C. dubliniensis*, such as its low Sap activity and unique global gene expression profile. Our results suggest that the heritable white-gray-opaque phenotypic switching system is conserved in the 2 closely related species, *C. dubliniensis* and *C. albicans*.

Materials and methods

Strains and plasmids

The strains used in this study are listed in Table S1. All clinical strains of *C. dubliniensis* were isolated from the oral cavity. YPD (20 g/L glucose, 20 g/L peptone, 10 g/L yeast extract, w/v) and Lee's medium^{24,29} were used for the routine growth of yeast cells. The solid medium contained 2% agar and 5 μ g/mL of the dye phloxine B, which stains opaque colonies red and gray colonies light pink.

To delete the first allele of the *URA3* gene, *C. dubliniensis* PC35 was transformed with the PCR product amplified from the *CaSAT1* cassette (in plasmid pNIM1⁴³) with primers URA3-SAT1Fwd and URA3-SAT1Rev (Table S2). The *URA3/ura3::SAT1* heterozygous strain was streaked onto 5-fluoroorotic acid (5-FOA)-containing plates to select for uridine-auxotrophic isolates. Fusion PCR strategies⁴⁴ were used to delete the first allele of *WOR1* or *EFG1* in PC35. The primers used for PCR are listed in Table S2. The *Cahph* marker was used for positive selection on plates containing medium supplemented with hygromycin (the *Candida hph* hygromycin-resistance gene was a gift from Dr. Xiaoqin Lai, Institute of Microbiology, CAS, Beijing, China). To delete the second copy of *WOR1* or *EFG1*, we constructed plasmids pSFS2a-WOR1KO and pSFS2a-EFG1KO. Two fragments that are homologous to the 5'- and 3'-sequences of *WOR1* were inserted into the *ApaI/XhoI* and *SacII/SacI* sites of pSFS2A⁴⁵ to generate plasmid pSFS2a-WOR1KO. Two fragments that are homologous to the 5'- and 3'-sequences of *EFG1* were inserted into the *KpnI/XhoI* and *SacII/SacI* sites to generate

plasmid pSFS2a-EFG1KO. The plasmids were linearized with *ApaI* (or *KpnI*) and *SacI* and used to delete the second allele of *WOR1* or *EFG1*.

Phenotype switching assays

White-gray-opaque switching assays were performed as described previously.²⁵ Lee's glucose, Lee's GlcNAc, and YPD media were used to quantitatively assess the switching frequency. White, gray, or opaque cells were first grown on solid Lee's GlcNAc medium at 25°C for 5 d. Homogeneous white, gray, or opaque colonies were resuspended, plated on nutrient agar plates and incubated in air, 5% CO₂, or 20% CO₂ at 25°C for 5 to 6 d. The number of colony of different cell types was counted, and the switching frequencies between different phenotypes were calculated.

SEM assays

SEM assays were performed as previously reported.^{24,25} White, gray, and opaque cells were grown in liquid Lee's glucose medium at 25°C for 24 hours. The cells were gently washed with 1 \times PBS and fixed with 2.5% glutaraldehyde. The samples were then washed 3 times with 0.1 M Na₃PO₄ buffer (pH 7.2), dehydrated with gradually increasing concentrations of ethanol (30% – 50% – 70% – 85% – 95% – 100%) and coated with gold. The cells were imaged with a scanning electron microscope (FEI QUANTA 200).

Mating assays

C. dubliniensis cells were grown in liquid Lee's GlcNAc medium to the stationary phase at 25°C. To compare the mating responses in different cell types, 5 \times 10⁶ white, gray, or opaque "a" cells (PC35) were mixed with 5 \times 10⁶ of the corresponding "α" cells (P86) in 1 mL of liquid Lee's GlcNAc medium and cultured at 25°C for 24 hours. The cellular morphologies were then examined using a microscope.

For the quantitative mating assays, *C. albicans* GH1349 (*MTLα/α*, *arg4/arg4*) was used as the tester strain because its opaque cells are very stable and *C. albicans* can mate efficiently with *C. dubliniensis*.¹³ Opaque cells (5 \times 10⁶) of GH1349 were mixed with 5 \times 10⁶ experimental white, gray, or opaque cells of *C. dubliniensis* PC35 in 10 μ L of ddH₂O and spotted onto solid Lee's GlcNAc medium plates. The mating mixtures were cultured in air at 25°C for 48 hours and then resuspended, diluted, and plated on 3 types of selectable plates (SC-uridine, SC-arginine, and SC-uridine-arginine) for

growth. The mating efficiencies were calculated according to previous reports.^{26,37}

Sap activity and FACS analysis assays

Sap activity and FACS analysis assays were performed according to our previous study.²⁵ Cells were grown in liquid Lee's glucose medium to the exponential phase and used for FACS analysis. YCB agar containing 0.2% BSA as the sole nitrogen source was used for the Sap activity assays. White, gray, and opaque cells were grown in liquid Lee's glucose medium at 25°C to the exponential phase and then collected and washed twice with ddH₂O. The cells of each type (5×10^6 cells in 5 μ L of ddH₂O) were spotted onto the YCB-BSA agar plates and incubated in air at 25°C for 5 d. The activity of the secreted enzyme was determined based on the size of the white halo rings.

Virulence assays

All of the animal experiments were performed according to the guidelines approved by the Animal Care and Use Committee of the Institute of Microbiology, Chinese Academy of Sciences. Systemic infection and ex vivo tongue (mucosal) infection assays were performed according to previous reports.²⁵ Female BALB/c mice aged 4 to 5 weeks were used in this study. Ex vivo tongue infection assays were performed in air at 30°C or 37°C. For the survival rate tests, 10 to 12 mice were used for each cell type (white, gray, or opaque). For the fungal burden assays, 2×10^6 cells of each cell type in 200 μ L of PBS were injected into each mouse via the tail vein. Six mice were used for each cell type (white, gray, or opaque). The mice were humanely killed at 24 hours after injection. Organ tissues (liver, kidney, spleen, lung, and brain) were removed and used for the colony-forming unit (CFU) analysis.

RNA extraction, RNA-Seq, and quantitative real-time PCR (Q-RT-PCR) assays

White, gray, and opaque cells were grown at 25°C in liquid Lee's glucose medium for 24 hours. Total RNA was extracted using GeneJET RNA Purification kits according to the manufacturer's instructions. The RNA-Seq analysis was performed by the company BGI-Shenzhen according to the company's protocol (<http://www.genomics.cn/>).¹⁰ Approximately 10 million (M) reads were obtained by sequencing each library. The gene expression level was calculated using the RPKM method.⁴⁶ Quantitative RT-PCR assays were performed to verify the relative gene expression levels in different cell types

according to our previous report.⁴² The signal from each experimental sample was normalized to expression of the *ACT1* gene. Three repeats were performed for quantitative RT-PCR experiments.

Statistical analysis

In the switching and mating assays (Figs. 2, 5 and Fig. S7), 3 biological repeats were performed. Switching frequency (or mating efficiency) = average \pm standard deviation (SD). In Figure 4B, 6 mice were used for each cell type. In Figure S6, 3 mice were used for each cell type. Average values \pm standard deviations are shown. Two-tailed Student's *t*-tests were performed.

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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