The role of transcription factor, Ofi1 in the regulation of white-opaque switching and filamentation in *Candida albicans*

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Abstract

The role of transcription factor, Ofi1 in the regulation of white-opaque switching and filamentation in *Candida albicans*

Seyed Hananeh Ghafelehbashi

Candida albicans is a major fungal pathogen capable of undergoing phenotypic transitions in response to environmental cues. These morphological changes are regulated by transcription factors that coordinate key developmental pathways. In this study, we investigate the role of Opaque and Filamentation Inducer 1 (Ofi1) in modulating white-opaque switching and filamentous growth. Overexpression of *OFI1* under the *ACT1* promoter induced white-to-opaque switching up to 28% in normally non-switching *MTL* ***a/α*** cells, while in *MTL* homozygous strains, it significantly enhanced white-to-opaque switching, reaching 100%. Our study demonstrates that Ofi1 is a dosage-dependent transcription factor regulating the white-to-opaque and yeast-to-filament transition in *C. albicans*. Conversely, the deletion of *ofi1* greatly reduced the switching frequency, highlighting its essential role in both initiating and maintaining the opaque state. We propose that Ofi1 interacts with the transcription factors Wor2, Wor4, and Czf1, serving as a central regulator of white-opaque switching. Our results suggest that while Ofi1 and Wor2 independently regulate white-to-opaque switching, Wor2 does not contribute to filamentation. Also, the functional arrangement of Czf1 and Ofi1 is completely different in white-to-opaque switching and yeast-to-hyphae transition processes. Moreover, deleting *wor4* completely blocks white-to-opaque switching, but *OFI1* overexpression can partially bypass this defect. In filamentation, the ability of Ofi1 to compensate for *wor4* deletion depends on the medium, fully restoring filamentation in BSA-supplemented YPD but not in GlcNAc-induced conditions, suggesting Wor4 and Ofi1 regulate filamentation through distinct pathways. Furthermore, our findings highlight a potential role for *WOR4* in the Lee's GlcNAc solid media to induction of hyphal growth.

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List of abbreviations

Arginine	Arg
Base Pairs	Bp
Chromatin immunoprecipitation	ChIP
Clustered regularly interspaced short palindromic repeats	CRISPR

CRISPR-associated protein 9	Cas9
Cyclic AMP- protein kinase A	cAMP-PKA
Differential Interference Contrast	DIC
Luria-Bertani- Ampicillin	LB-AMP
Mating type-like	*MTL*
N-Acetylglucosamine	GlcNAc
Nourseothricin	NAT
Optical Density	OD
Phosphate-buffered saline	PBS
Polymerase chain reaction	PCR
Single guide RNA	sgRNA
Transcription Factors	TFs
Wild type	WT
Yeast extract, peptone, dextrose	YPD
YNB	Yeast Nitrogen Base

1. Introduction

Candida albicans is a commensal member of humans and is commonly found as part of the natural microbiota in areas such as the mouth (Ghannoum et al., 2010), gastrointestinal tract (Neville et al., 2015), and vagina (Peters et al., 2014), generally without causing harm in healthy individuals. However, *C. albican*s is a part of the family Candidaceae, which comprises a large number of yeast species known for their ability to cause opportunistic infections in humans, and under certain conditions, it can become pathogenic, leading to behaviours ranging from superficial mucosal infections to dangerous or life-threatening systemic infections (Mayer et al., 2013). *C. albicans* typically lives harmlessly in different parts of the body, but these infections, ranging from mild to serious, occur especially in immunocompromised individuals. *C. albicans* is one of the top causes of hospital-acquired infections in the U.S. and can lead to systemic infections with mortality rates up to 50% despite antifungal treatments (Doi et al., 2016). Fungal infections are a big health problem worldwide, causing death rates similar to those of tuberculosis or malaria. The global increase in Candidiasis is driven mainly by more people who have weakened immune systems, such as patients with HIV or cancer and organ transplant recipients (Kim & Sudbery, 2011).

C. albicans is a diploid yeast with eight pairs of chromosomes and a genome of 14.3 Mb, encoding 6,107 proteins (Jones et al., 2004). While both *C. albicans* and *S. cerevisiae* are yeasts, they diverged ~800 million years ago, which has resulted in extensive differences in their genome and physiology. *S. cerevisiae* is extensively used as a laboratory model organism due to its genetic flexibility. Yet, findings may not always be translatable to *C. albicans*, as it has different genes and regulatory pathways (Moran et al., 2011).

One major difference is in its codon usage: *C. albicans* interprets the CUG codon as serine instead of leucine, resulting in protein variants that might increase phenotypic diversity and stress adaptation (Gomes et al., 2007). The genetic diversity of *C. albicans* is also demonstrated by the fact that ~40% of *C. albicans* genes have no unequivocal homologs in *S. cerevisiae* as shown by BLAST searching with stringent criteria ($P < 1 \times 10^{-20}$) (Hartman IV, 2001), indicative of their evolutionary distance. This encompasses differences in drug susceptibility and also genetic

regulation- for example, gene deletions that are lethal in *S. cerevisiae* can be tolerated in *C. albicans*, which highlights its unique regulatory machinery (Roemer et al., 2003; De Backer et al., 2000). Overall, *C. albicans* and *S. cerevisiae* have many differences in their biological pathways, especially those related to drug resistance, virulence, and interactions with the host. These differences are important for *C. albicans*' ability to cause disease. For instance, *C. albicans* has specific pathways for forming biofilms and switching from a yeast to a hyphal form, processes essential for its pathogenicity (Tsui et al., 2016) or for white-to-opaque switching which is needed for mating, but also impacts its colonization properties (Miller & Johnson, 2002). Moreover, *C. albicans* has complex mechanisms of antifungal resistance that are often missing in *S. cerevisiae*. These genetic and functional differences highlight the need to study *C. albicans* directly to better understand its biology and how it causes disease. While *S. cerevisiae* is a valuable model organism for studying eukaryotic cell biology, the unique features of *C. albicans* show why research must focus on each organism, particularly when developing treatments for fungal infections.

Pathogenic and Commensal Roles of *C. albicans*

C. albicans is an opportunistic fungal pathogen in humans, commonly existing as a commensal organism in areas such as the gastrointestinal tract, genital mucosa, and oral cavity, usually without causing significant health issues (Pérez et al., 2013). Despite its typically benign presence, the pathogenic capacity of *C. albicans* becomes apparent in certain localized infections, particularly oral candidiasis and vaginitis, the latter of which significantly impacts females worldwide (Sobel, 2007). The potential for *C. albicans* to transition from a harmless commensal to a dangerous pathogen is a major health concern, especially for individuals with compromised immune systems, underscoring the need for vigilant monitoring in populations susceptible to such infections (Noble & Johnson, 2007). Due to *C. albicans'* phenotypic plasticity, it can adapt itself to various niches within the host organism through different polymorphic transitions, such as the white-to-opaque and the yeast-to-filamentous growth switches. As well, the reversible filamentous phenotypes of hyphae and pseudohyphae appear needed for *C. albicans* to effectively function in the mammalian host as a pathogen (Sudbery, 2011).

C. albicans was initially considered asexual, but it is now known to possess a sexual cycle hidden behind layers of regulation. *C. albicans* is a diploid organism that typically is heterozygous at the central regulator locus controlling mating type, the *MTL* (Mating Type Like) locus. Standard cells have the two forms of the *MTL* locus termed *MTL* **a** and *MTL* α; such heterozygotes are non-mating (Miller & Johnson, 2002). One regulatory layer that makes mating infrequent in *C. albicans* is the necessity of homozygosity of *MTL* genotypes (**a**/**a** or α/α) to permit activating mating competence through the formation of opaque cells, as cells of mating type (**a**/α) cannot switch from white-to-opaque and are not mating competent (Lohse & Johnson, 2009).

White vs Opaque; Phenotypic States of *C. albicans*

Switching from the white form to the opaque form is controlled by transcriptional regulatory proteins encoded by the Mating Type Like (*MTL*) loci and this switching is necessary for mating. Fig. 1 provides the white cells form, which is unstained, shiny, and dome-shaped colonies on solid agar containing the dye phloxine B, which is a pink color dye that helps distinguish white and opaque cells based on their color; the white cells express genes critical to the stability of the white form of the cells such as *EFG1* (Lachke et al., 2003). Opaque colonies are pink-stained and dull; they become flatter when they grow on agar, and they express opaque specific genes like *WOR1* (Xie et al., 2013).

To adapt to different environments within host cells, *C. albicans* relies on specific and complex transcriptional regulatory programs. Studying the transcription factors (TFs) that directly control its growth pathways is key to understanding how *C. albicans* colonizes and causes disease in its host. TFs regulate gene expression by binding to specific DNA sites or by coordinating feedback loops that can stabilize or change the cell's phenotype (Zordan et al., 2007). Six main TFs are known to regulate the white-opaque switch in *C. albicans*: Wor1, Wor2, Wor3, Wor4, Czf1, and Efg1 (Pérez et al., 2013). Among these, Wor1 is known as the master regulator. It binds to DNA sites upstream of over 200 genes and plays a critical role in maintaining the stability of opaque cells, where its expression is markedly higher (Zordan et al., 2006). This regulatory system operates as a feedback loop. Wor1 promotes the expression of itself, as well as *WOR2* and *CZF1* while repressing *EFG1* (Zordan et al., 2007). Other TFs, including Wor2, Wor3, Wor4, and Czf1, are more active in opaque cells than in white cells. They help stabilize and maintain the opaque

cell state but depend on Wor1 to function properly (Vinces & Kumamoto, 2007). In the next sections, we will examine the roles of these factors in detail, starting with Wor2, then Czf1, and ending with Wor4.

Zinc cluster TFs are an essential family of proteins in fungi, including *Candida* species. They possess a conserved Zn(2)-Cys(6) zinc cluster motif, a known DNA-binding domain in fungal transcriptional regulators. This unique structure enables them to bind DNA and regulate gene expression, playing pivotal roles in various cellular processes. In *Candida* species, these TFs are essential for regulating genes associated with virulence, including those involved in drug resistance and pathogenicity. Wor2, as a regulator of the white-opaque switching in *C. albicans*, is a member of this family. Formerly referred to as Zcf33, Wor2 is characterized by zinc cluster motifs, which are structural domains necessary for binding DNA. These motifs coordinate zinc ions to enhance DNA interaction, a defining feature of this group of TFs (MacPherson et al., 2006).

In the strains with homozygosity of *MTL* genotypes (**a/a** or α/α) which have *wor2*Δ/Δ mutation the switching frequency was decreased significantly (Zordan et al., 2007). However, after ectopic overexpression of Wor1, switching to the opaque state was observed in a *wor2*Δ/Δ mutant strain, but the cells did not remain stable in the opaque form. Wor2 has been shown to bind to 42% of genes that are associated with the opaque phenotype, and it is necessary for heritable maintenance of the opaque cell type (Hernday et al., 2013) and acts in concert with Wor1 in a positive feedback loop to enrich opaque cell identity (Sriram et al., 2009).

The *CZF1* gene also encodes a TF involved in the regulation of white-opaque switching in *C. albicans*. Czf1 also contains a zinc cluster motif, a common feature of fungal TFs. Similar to Wor2, Czf1 plays an important role in the white-opaque switch, highlighting the importance of zinc cluster TFs in governing such complex phenotypic transitions (Zordan et al., 2007). In *C. albicans*, Czf1 is an opaque-enriched transcriptional regulator that modulates the frequency of white-to-opaque switching. While not required for maintaining the heritable opaque state, Czf1 plays a key role in initiation to the opaque phase by repressing *EFG1*, the master regulator of the white state, and activating *WOR2*, a required factor in the maintenance of the cells in the opaque phase. Moreover, Czf1 binds to its own upstream regulatory region and those of *WOR2* and *EFG1*, highlighting its role in the switching process (Hernday et al., 2013). The *czf1* mutant highly reduces

the formation of opaque colonies, while ectopic expression of *CZF1* induces mass conversion to the opaque phase, showing its importance in phenotypic switching (Zordan et al., 2007).

Wor4 is a novel and critical regulator of white-opaque switching in *C. albicans*, playing an essential role in both the establishment and maintenance of the opaque cell type. Wor4 is part of the C2H2 zinc finger family and has a single zinc finger motif. This motif allows it to bind short DNA sequences, which is important for its regulatory role. Studies show that overexpressing *WOR4* can cause cells to switch from the white to the opaque state. In contrast, deleting *wor4* stops this transition entirely. This makes Wor4 function as an upstream regulator of key factors like Wor1 and an activator of the opaque state. Unlike other regulators, Wor4 has a unique role because its expression triggers switching, while its absence prevents it (Lohse & Johnson, 2016). ChIP-seq studies have found that Wor4 binds specific DNA regions. Its binding pattern is very similar to those of Wor1 and Wor2, suggesting that Wor4 is closely linked to the same regulatory network. Wor4 likely works with these TFs to stabilize DNA binding and keep the genes for the opaque state active. It also helps strengthen feedback loops that are necessary for maintaining opaque cells. Further studies on *WOR4* are expected to uncover more about how it works, offering a deeper understanding of the complex regulatory system behind *C. albicans* phenotypic switching (Lohse & Johnson, 2016). For example, the role of *WOR4* in the yeast-to-filament transition is still unclear.

Yeast to hyphae; Phenotypic States of *C. albicans*

C. albicans also has a reversible transition between cellular morphologies called the yeast-to-hyphae switch. This dimorphic trait, which involves the elongation of yeast cells to develop into filamentous hyphal structures, has great significance for paths of infection (Brown et al., 1999). So, this is not just a morphologic change but is really tied to the activation of virulence-related genes that can really maximize the invasiveness potential of the fungus. This transition is triggered by environmental conditions like enhanced temperatures, increased levels of CO_2, shifts in pH, serum, or the presence of N-acetylglucosamine (GlcNAc) (Sudbery, 2011). All these aspects persist and illustrate the complex strategies that *C. albicans* use to adapt to their environment and flourish as a pathogen under optimal conditions (Chow et al., 2021).

C. albicans adapts its morphology from yeast-to-hyphal forms through complex signaling pathways, including the cyclic adenosine monophosphate (cAMP) pathway (Leberer et al., 2001) and the mitogen-activated protein (MAP) kinase cascade (Vojtek et al., 1993), both influenced by environmental factors. The CaRas1 protein plays an important role in this switching by regulating these signalling pathways, thus facilitating the morphological shift necessary for infection (Leberer et al., 2001). Many TFs can influence yeast-to-hyphae switching, such as Cph1 (Liu et al., 1994) Efg1 (Lo et al., 1997) Czf1 (Brown et al., 1999) Ume6 (Banerjee et al., 2008) and Rha1 (Parvizi Omran et al., 2022). The activity of these TFs often depends on specific environmental signals. However, certain TFs, such as Efg1, can trigger the yeast-to-hyphae transition under a wide range of conditions (Lo et al., 1997). Interestingly, some TFs in *C. albicans*, including Efg1 and Czf1, have multiple functions in different morphology pathways. They are involved in regulating both the white-to-opaque switching and the yeast-to-hyphal transition (Huang, 2012). For example, Efg1 has a positive role in yeast-to-hyphae switching but has a negative role in white-to-opaque switching. Additionally, Czf1, as a key regulator of white-to-opaque switching in *C. albicans*, also plays a key role in the filamentation pathway, particularly under hypoxic conditions, with its deletion reducing filament formation in an embedded matrix (Brown et al., 1999). Research suggests that Czf1 may counteract the suppression of filamentation caused by Efg1. This idea is supported by epistasis-based genetic models and evidence from a yeast two-hybrid screen showing an interaction between Czf1 and Efg1 (Giusani et al., 2002). Additionally, changes in *CZF1* expression do not alter the hyper-filamentation phenotype observed in *efg1*Δ/Δ mutants under embedded conditions. This further indicates that Czf1's role in filamentation relies on its ability to modulate Efg1 activity.

Opaque to hyphae; Phenotypic States of *C. albicans*

In *C. albicans*, filamentation in white and opaque cells is regulated by distinct environmental and genetic factors (Sudbery, 2011). White cells primarily transition to filamentous forms at a higher temperature (37°C), responding to a variety of inducers such as serum, neutral pH, nutrient limitation, and high CO_2.

In contrast, opaque cells, are more sensitive to lower temperatures (25°C) and demonstrate filamentation uniquely triggered by environmental cues such as GlcNAc and sorbitol (Fig. 2). This differential response highlights the organism's complex regulatory mechanisms that allow adaptation to various environments, with specific signalling pathways and TFs modulating the morphological changes in each cell state. In terms of filamentation signaling pathways in white cells, this is typically regulated through the MAPK and cAMP pathways with some key TFs that respond to various environmental conditions. For opaque cells, although some pathways overlap, the response to environmental stimuli and the role of certain TFs diverge, leading to filamentation under different conditions. For example, Cph1, recognized as the primary regulator within the MAPK signaling route, showed no significant effect on the filamentous transformation of opaque cells. This highlights the complexity of *C. albicans*' adaptive mechanisms, allowing it to thrive in diverse environments by utilizing alternate genetic regulation for morphological changes. Czf1, a transcription factor central to white-to-opaque switching and yeast-to-filament transitions, exhibits distinct regulatory roles in filamentation from opaque cells. While Czf1 drives filamentation in white cells under embedded conditions (Brown et al., 1999), it also regulates filamentation in opaque cells. Embedded conditions promote filamentation in both cell types, with *czf1* mutants showing a unique hyper-branching phenotype when grown on specific media, even without embedded cues. This suggests that Czf1 plays a key role in coordinating filamentation in opaque cells, operating through mechanisms distinct from its function in white cells (Si et al., 2013). So, TFs involved in the various morphogenetic transitions of *C. albicans* can exhibit overlapping roles, influencing each transition either positively or negatively.

During our research to create a library of activated TFs in *C. albicans*, we identified a particular TF whose activation led to an enhanced rate of white-to-opaque cell transition, suggesting an effect on this morphological switch (Shrivastava et al., 2021). It was named Ofi1 (Opaque and Filamentation Indicator 1) because of its role in switching cells from white-to-opaque and also regulating filamentation (Du et al., 2015). In one of the recent studies, it was found that 16 out of 90 clinical isolates of *MTL* **a**/α type possess the ability to form opaque colonies, and all of them have only Ofi1 overexpressed (Cui 2024). Ofi1 stands out as a key regulator in the white-to-opaque transition of *C. albicans*, essential for its adaptation and pathogenesis. Previous studies reported that *OFI1* overexpression resulted in a higher frequency of white-to-opaque switching, as well as higher filamentation and invasion, while *ofi1*Δ/Δ decreased the frequency of white-to-opaque

switching on GlcNAc medium but had no effect on filamentation (Du et al., 2015). Interestingly, *OFI1* does not have an ortholog with *S. cerevisiae* or other closely related fungi.

These studies have raised many questions about how the Ofi1 TF functions in collaboration with other key regulators of white-to-opaque switching and/or filamentation. Wor1, Wor2, Czf1, Wor4, and Wor3, all central regulators of white-to-opaque switching, demonstrate binding activity on the *OFI1* promoter (Hernday et al., 2013). The binding of Wor1 as a master regulator of white-to-opaque switching to the intergenic DNA upstream of the *OFI1* coding sequences and an observed value indicating up-regulation in the opaque state suggests a significant regulatory interaction. This indicates that Ofi1's activity is notably increased in the opaque phase of *C. albicans,* likely acting downstream of Wor1 (Zordan et al., 2007). Wor3 is another key regulator of white-to-opaque switching, which is activated by Ofi1 (Cui et al., 2024). Ofi1 influences a broader gene network, implying its position at the top of the regulatory hierarchy. Experimental evidence shows Ofi1 binding to *WOR1* and *WOR3* promoters, boosting their expression, even in the absence of Wor1, suggesting a direct regulatory capability. However, the function of Ofi1 cannot be replaced by overexpression *WOR3* (Cui et al., 2024).

To explore the genetic interactions between *OFI1* and the key transcriptional regulators of white-to-opaque switching, we focused on *WOR2*, *WOR4*, and *CZF1*. While *WOR1* and *WOR3* are also master regulators of this process, their interactions with *OFI1* have already been characterized in previous studies and are not part of our current investigation. We generated an extensive set of single and double mutants for *OFI1*, *WOR2*, *WOR4*, and *CZF1* and analyzed their roles in white-opaque switching and filamentation. Additionally, we overexpressed *OFI1* and these regulators in various mutant combinations to assess their effects on the transitions between white and opaque states, as well as on filamentation.

2. Materials and methods

Growth conditions

For the regular growth of *C. albicans*, the YPD medium contains 20 g/l glucose, 20 g/l yeast extract, and 10 g/l peptone, with 2% agar for the solid medium. Additionally, synthetic complete (SC) medium contains 0.17% Yeast Nitrogen Base (YNB) without amino acids and ammonium sulfate, 2% dextrose (glucose), 0.087% amino acid dropout mix, and 0.01% uridine. Semi-solid media contained 2% agar. This medium was tailored for auxotrophic strains by omitting specific amino acids as needed. In the preparation, N-acetylglucosamine (Lee's GlcNAc) was used at a concentration of 1.25%, ammonium sulfate ((NH4)2SO4) at 0.5%, magnesium sulfate heptahydrate (MgSO4·7H2O) at 0.02%, potassium phosphate dibasic (K2HPO4) at 0.35%, and sodium chloride (NaCl) at 0.5%. Agar, accounting for 2% of the mixture, was added to solidify the media. The components were initially dissolved in less than 200 ml of distilled water, and after complete dissolution, the volume was adjusted to a total of 200 ml. The medium was then sterilized by autoclaving at 121°C for 15 minutes. For solid media, the solution was allowed to cool to 50-55°C before being poured into petri dishes. Furthermore, Sorbitol medium (SOR), a variant of the synthetic complete dextrose medium, was enhanced with 1 M sorbitol. All the strains were stored as 20% (final concentration) of glycerol stocks in −80°C freezer.

Plasmid and strain construction

For all PCR amplifications involving *C. albicans* DNA in this study, genomic DNA from the SN152 strain which contains homozygous null mutations in *his1*, *leu2*, and *arg4* served as the template.

In this study, to generate mutant strains of *C. albicans*, we utilized the "solo system" CRISPR-Cas9 protocol as outlined in (Vyas et al., 2015). This method allows the creation of homozygous mutations in a single step. Three sets of primers were designed for each CRISPR mutant construction: sgRNA, repair DNA, and screening primers, as listed in Table S1.

The sgRNA (20 nt) featured extended sequences for cloning into the BsmBI site of plasmid pV1093, structured as Forward – 5'-atttgX20g-3' and Reverse – 5'-aaaacX20c-3' (All the plasmids used or constructed in this study are listed in Table S2). The sgRNA of *OFI1*, *WOR2*, *WOR4*, and *CZF1* were formed by annealing primers specific to each gene (*OFI1*-sg-F/*OFI1*-sg-R, *WOR2*-sg-F/*WOR2*-sg-R, *WOR4*-sg-F/*WOR4*-sg-R, and *CZF1*-sg-F/*CZF1*-sg-R) and cloning the fragments into the BsmBI site of pV1093 with the Golden Gate assembly technique to make plasmids pV1093-*OFI1*-sgRNA, pV1093-*WOR2*-sgRNA, pV1093-*WOR4*-sgRNA, and pV1093-*CZF1*-sgRNA, respectively.

In the Golden Gate assembly approach for *E. coli* DH5α transformation using BsmBI restriction sites, sgRNA, and pV1093 with BsmBI recognition sites are assembled in a single reaction. This process involves the simultaneous digestion and ligation of DNA parts, facilitated by BsmBI, a Type IIS restriction enzyme that cuts outside its recognition sequence, and T4 DNA ligase, which seals the DNA fragments. The reaction setup includes the *OFI1*-sg-F/*OFI1*-sg-R, *WOR2*-sg-F/*WOR2*-sg-R, *WOR4*-sg-F/*WOR4*-sg-R, and *CZF1*-sg-F/*CZF1*-sg-R, pV1093, BsmBI enzyme, T4 DNA ligase, and buffer, incubated initially at 37°C to allow cutting and joining, followed by a higher temperature step to inactivate the enzymes. Following assembly, the mixture is introduced into competent *E. coli* DH5α cells through a heat-shock procedure (Froger & Hall, 2007), allowing the bacteria to take up the recombinant DNA. The transformed cells are then cultured on LB + ampicillin agar plates to select for successful assemblies. Colonies are then examined for the precise insertion of sgRNA by leveraging the EcoRI restriction site strategically positioned between the BsmBI sites within the original pV1093 vector; a successful ligation results in the absence of this EcoRI site, indicating correct assembly (Fig. 3).

The selection of guide sequences was based on proximity to the stop codon (for gene replacement), high on-target, and low off-target scores. A supplemental file from Vyas et al., 2015, listing sequences targeting both alleles for any given gene, was referenced for optimal guide selection. The pV1093 plasmid housing CaCas9 DNA was synthesized by BioBasic, with codons optimized for expression in *C. albicans,* and containing an Amp marker for *E. coli* transformation was employed. The repair DNA, designed using Benchling, involved amplifying our gene of interest. The *ARG4* marker was PCR-amplified from plasmid pFA-Ca*ARG4*, with primers providing

homology to the flanking regions of the targeted gene (Fig.4. A & B). Additionally, the *ARG4* marker was transformed into the parental strain SN152, and transformants were selected on SD-Arg agar plates. Successful transformants were confirmed through colony PCR (Fig. 4 C & D).

To investigate the role of *OFI1* in white–opaque switching, we first aimed to construct the p*ACT1-OFI1* plasmid and subsequently test its overexpression function. First, the *OFI1* gene was amplified via PCR, and homology regions with restriction sites for HindIII and MluI were incorporated using primer extension. The PCR product was purified using the Thermo Scientific GeneJET Gel Extraction Kit, followed by digestion with HindIII and MluI. Simultaneously, the plasmid pCIP-*ACT1*, obtained from the Sellam laboratory (Tebbji et al., 2020), was linearized using the same restriction enzymes. The digested fragments of pCIP-*ACT1* and *OFI1* were gel-extracted and subsequently ligated to create the pCIP-*ACT1-OFI1* construct (Fig. 5 A). This construct was initially transformed into *E. coli* DH5α competent cells using the calcium chloride method (Mandel & Higa, 1970). Colonies were selected on ampicillin-containing media to propagate and amplify the plasmid and it was confirmed by colony PCR (Fig. 5 B). Because the yeast selection marker in this plasmid was *URA3* and our strains had no auxotroph to this marker we transferred the *ACT1- OFI1* into the plasmid pV1093, which contains the nourseothricin (NatR) selection marker for transformation in *C. albicans*. PCR amplification using primers designed with XmaI and BamHI restriction sites ensured proper orientation of the *ACT1-OFI1* insert. Colony PCR was performed with an external forward primer targeting the *ACT1* promoter and a reverse primer targeting the *OFI1* terminus to confirm successful plasmid assembly. The finalized plasmid was linearized with BamHI and XmaI and transformed into the *ENO1* locus of the *C. albicans* SN152 strain using the lithium acetate method of transformation protocol (Fig. 5 C & D) (Gietz et al., 1995). This approach allowed us to stably integrate the *OFI1* overexpression construct into the *C. albicans* genome. The resulting strains were used to evaluate the effect of *OFI1* overexpression on white–opaque switching and filamentation, providing critical insights into its regulatory function (Fig. 5 E).

MTL switching

To convert *MTL* heterozygosity to homozygosity in *C. albicans*, we employed a liquid sorbose medium (SOR) consisting of 1% yeast extract, 2% peptone, and 10% sorbose. The cultures were

incubated at 37 °C for a total duration of 48 hours, with shaking at 220 rpm in a 7 ml volume. Following incubation, the cultures were washed twice with 1x phosphate-buffered saline (PBS) to remove the residual medium. Subsequently, the cells were plated on a solid medium containing 2-4% sorbose, with an inoculum density of approximately 10^4 cells per plate. This step was pivotal in facilitating additional growth and diversification of the cell population. After allowing the colonies to grow, genomic DNA was extracted from selected colonies. This extracted DNA was then utilized for polymerase chain reaction (PCR) amplification using primers specific for the *MTL* **a** and *MTL* α genes (Fig. 8).

White–opaque switching assays

In the white-opaque switching assay for *C. albicans* using Lee's GlcNAc, the process starts with selecting a single colony, which is then cultured in YPD medium overnight. Following this, the cells are washed with 1X PBS to remove any residual media, and their concentration is adjusted to an optical density (OD600) of 1.0. These prepared cells are then plated onto agar media containing Lee's GlcNAc, which works as the primary carbon source and is instrumental in making the white-to-opaque phenotypic switch in *C. albicans*. To assist in distinguishing between the white and opaque cell forms, 5 μg/ml of phloxine-B is added to the Lee's GlcNAc medium. This dye selectively stains opaque cell colonies, allowing for a clear visual difference between the two phenotypes. The use of phloxine-B thus facilitates the subsequent incubation, observation, and detailed analysis of the colonies for morphological changes indicative of the transition from white-to-opaque. To determine the rate of white-to-opaque switching, the number of opaque colonies was counted and divided by the total colony count. This ratio provided the frequency of colonies undergoing the transition from white-to-opaque.

Filamentation assay

The environmental filament induction assays were conducted by culturing the selected strains overnight in liquid YPD at 30°C with shaking at 220 rpm overnight. The following day, the cells were washed twice with 1× PBS, resuspended to an OD600 of 0.1 in fresh liquid YPD supplemented with 20% fetal bovine serum (Sigma), and incubated at 37°C for 4 and 8 hours. For

solid medium, 5 μL of a culture adjusted to an OD600 of 0.1 was spotted onto Spider plates (1% nutrient broth, 0.4% potassium phosphate, 2% mannitol, 1.35% agar, pH 7.2) and incubated at 37°C for 3 days.

Invasion assay

Single colonies of the selected strains were grown overnight in 5 mL of YPD at 30 °C with shaking at 220 rpm. The cells were then washed twice with 1× PBS and diluted to an OD600 of 0.1. Five μL of the adjusted culture were spotted onto YPD plates and incubated at 30 °C for 1 day or spotted onto Spider agar plates and incubated at 37 °C for 3 days. The spots on the Spider plates were subsequently washed with sterile water for 15 seconds. The plates were scanned before and after washing using an Epson Perfection V500 Photo color scanner.

Microscopy

For microscopy analysis, we carefully selected colonies representing normal opaque, wrinkled opaque, and white from each strain. These colonies were each inoculated into 5 ml of liquid YPD medium and incubated overnight at 30°C with agitation at 220 rpm. Following incubation, the cells were gently washed twice with 1× PBS to remove any residual medium and debris. To ensure accurate characterization, optical microscopic images were captured using a Nikon Eclipse TS100 microscope. To visualize the cells, we employed differential interference contrast (DIC) microscopy, which provides a higher contrast image of unstained live cells. This technique is particularly useful for distinguishing the subtle morphological differences between normal opaque, wrinkled opaque, and white cells of *C. albicans*.

3. Results

A: White to opaque switching

A.1.1 The effects of *OFI1* manipulation on White-to-Opaque Switching in *MTL* Heterozygous strains

In *C. albicans*, the transition from white-to-opaque is typically regulated by *MTL* homozygosity. It thus involves key transcription factors (TFs) encoded by the *MTL* **a** and *MTL α* alleles. However, previous studies suggest that the protein encoded by *OFI1* (*ORF19.4972*) can influence this switching process independent of an alteration in *MTL* zygosity (Cui et al., 2024, Shrivastava et al., 2021).

To construct an *ofi1* deletion we employed the CRISPR-Cas9 system (Vyas et al., 2015), using a strategy where the *ARG4* replacement of *ORF1* begins just one nucleotide before the *ORF1* start codon and extends beyond the stop codon. This approach effectively removes the entire *OFI1* gene, ensuring that no residual functional elements remain, thereby allowing for a comprehensive assessment of Ofi1's role in white-to-opaque switching in *C. albicans* (Fig. 4 A). The transformed colonies were selected on SD-Arg plates, and colony PCR was conducted using both external (Ex) and internal (In) primers for *ARG4* and *OFI1*. This process allowed confirmation of homozygous null mutations, ensuring the complete disruption of *OFI1* function in the selected strains (Fig.4 B & C &D).

To further explore the role of *OFI1* in this context, we also first constructed a p*ACT1-OFI1* plasmid by cloning *OFI1* into the pCIP-*ACT1* vector, followed by ligation into the pV1093 plasmid, which contains the NatR marker for nourseothricin-based selection. This construct was then integrated into the *ENO1* locus of the heterozygous *MTL* **a**/α strain SN152 via lithium acetate transformation, generating a stable, overexpressed *OFI1* (Fig. 5).

These strains allowed us to assess the impact of *OFI1* manipulation on white-to-opaque switching under *MTL* heterozygous conditions, providing insights into the regulatory role of *OFI1* in this process without the need for *MTL* homozygosity. A recent study established that *OFI1* overexpression under the *TDH3* promoter led to 100% white-to-opaque switching (Cui et al., 2024)

when cells were grown on Lee's glucose medium. Building on these findings, we are exploring the impact of *OFI1* overexpression under the *ACT1* promoter (which is a weaker promoter than *TDH3*) to investigate how varying expression levels affect switching frequency.

The results of *OFI1* manipulation in *C. albicans MTL* **a**/α were assessed by comparing SN152 (wild type), the *ofi1* deletion, and the *OFI1* overexpression colonies in the presence of Phloxine B. The wild-type SN152 strain, which is *MTL* **a**/α heterozygous, did not undergo a transition to the opaque state, as expected, and all colonies remained white. Similarly, *ofi1* deletion resulted in colonies that were also entirely white, with no visible opaque or pink colonies, and microscopic examination confirmed that all cells remained white. In contrast, *OFI1* overexpression led to the appearance of pink colonies, suggestive of opaque switching (Fig. 6). When cells were examined using Differential Interference Contrast (DIC) microscopy at 63X and fluorescent microscopy at 100X magnification using a Leica DM 6000 microscope mounted with a TIR camera, it was observed that the colonies, despite having the pink (opaque) phenotype, were composed of a mixture of both white and opaque cells (Fig. 6). This suggests that overexpression of *OFI1* under the *ACT1* promoter in *MTL* **a**/α heterozygotes does induce white-to-opaque switching in *C. albicans* when grown on Lee's GlcNAc medium at 25°C for 4 days. However, not all cells switch, and the colonies exhibit a mixture of white and opaque cells. Overall, Ofi1 has an important role in white-to-opaque switching because switching to opaque in *MTL* heterozygotes strain is normally not possible.

A.1.2 Impact of Single Mutations of *wor2*, *wor4*, and *czf1* on *OFI1* Overexpression-driven White-to-Opaque Switching in *MTL* Heterozygotes

To determine how the observed white-to-opaque switching in *MTL* **a**/α heterozygotes is driven by *OFI1* overexpression, we assessed the effect of deleting three key regulators of white-to-opaque switching (*WOR2, WOR4,* and *CZF1*) in the presence of *OFI1* overexpression. This allows us to evaluate whether *OFI1* alone is responsible for the transition, providing deeper insights into its role in regulating white-to-opaque switching.

C. albicans white-to-opaque switching involves separate initiation of a phase switch and maintenance of the opaque state through multiple generations. This is crucial for allowing rare

stochastic switching (~1/10,000 cell divisions) to escape the epigenetic stability of the default white phase and attain environmental adaptation (Miller & Johnson, 2002). Maintenance of the opaque phenotype is stabilized by self-reinforcing transcriptional circuits, enabling cells to exploit niche-specific advantages including immune evasion and metabolic specialization. Essential transcriptional regulators known to control these processes are Wor1 and Czf1 which activate opaque-specific pathways and promote initiation, and Wor2 which is a key TF that acts to maintain the cells in the opaque phase through feedback loops. Other modulators of the switching process such as Wor3 and Wor4 appear to play roles in both initiation and stabilization (Lohse & Johnson, 2009; Zordan et al., 2007).

Previous studies have shown that in *OFI1* overexpression under the *TDH3* promoter, the deletion of *wor1* in *MTL* **a**/α heterozygous strains prevents the white-to-opaque switching, confirming that *WOR1* is essential for this process (Cui et al., 2024). To extend these observations, we investigated whether the other key regulators of white-to-opaque switching influence the process in *MTL* **a**/α heterozygous strains in the presence of *OFI1* overexpression. We constructed knockouts of *wor2*, *wor4*, and *czf1* in the presence of *OFI1*-overexpression. To make these deletions, we employed the CRISPR-Cas9 system (Vyas et al., 2015) with an *ARG4* replacement strategy beginning one nucleotide before the start codon and extending beyond the stop codon to completely remove the whole gene. Transformed colonies were selected on SD-Arg plates, and colony PCR with external and internal primers confirmed the presence of homozygous null mutations, ensuring a thorough disruption of each gene for assessing its role in white-to-opaque switching. All strains were cultured on Lee's GlcNAc medium plates and incubated at 25°C for four days. As expected, the wild-type SN152 strain heterozygous at *MTL* (*MTL* **a**/α) formed no opaque colonies; all colonies were completely white and displayed a typical yeast (round) morphology when examined microscopically. This aligns with the established understanding that *C. albicans* strains heterozygous at *MTL* are unable to undergo white-to-opaque switching. Similarly, in the *ofi1* deletion strain, the results matched the control, with no opaque cells observed under the microscope. However, *OFI1* overexpression **a**/α led to the appearance of pink colonies and checking the colonies under the microscope showed there was a mixture of white and opaque cells (Fig. 7 A). Then, we investigated the deletion of key regulators essential for white-to-opaque switching in the *OFI1* overexpression *MTL* **a**/α strain to determine whether the colonies remained pink and if opaque cells were still present. The intensity of the pink coloration varied depending

on the specific mutation: colonies overexpressing *OFI1* alone exhibited the most pronounced pink hue, whereas strains with *wor4* deletion displayed a reduced pinkish color, and the strains with *czf1* or *wor2* deletions generated even paler pink colonies. Microscopic examination confirmed that while the colonies appeared phenotypically distinct, the observed white-to-opaque switching frequency ranged between 20–35% (Fig. 7 B), suggesting that *OFI1* overexpression partially circumvents the regulatory barriers imposed by the *MTL* heterozygous state and key regulator deletions and it appears that the formation of opaque cells driven by *OFI1* overexpression occurs independently of *wor2*Δ/Δ, *wor4*Δ/Δ or *czf1*Δ/Δ deletions. However, *OFI1* under the *ACT1* promoter appears not as potent as under the *TDH3* promoter in inducing opaque cell formation.

A.2.1 Investigating the Role of *OFI1* in White-to-Opaque Switching in *MTL* Homozygous *C. albicans* Strains

After exploring the role of *OFI1* overexpression under the *ACT1* promoter and assessing its interplay with key transcriptional regulators such as *CZF1*, *WOR2*, and *WOR4*, it appeared that *OFI1* plays a critical role in facilitating white-to-opaque switching in *C. albicans MTL* **a**/α heterozygous strains. We were also interested in whether the same mechanism operated in *MTL* homozygous strains, where white-to-opaque switching is naturally permitted. In heterozygous strains, the **a**1/α2 repression complex typically locks the cells in the white state by silencing *wor1*, the master regulator of the opaque state. This repression is absent in homozygous strains, allowing switching to occur more readily (Fig. 8 A). By comparing the switching frequency and phenotypic outcomes of *OFI1* overexpression in *MTL* homozygous strains to the previously studied heterozygous strains, we can determine whether the regulation of this transition is consistent across mating-type configurations or if the absence of **a**1/α2 repression introduces new dynamics. This investigation provides insight into whether the dosage-dependent effects of *OFI1* overexpression differ significantly between *MTL* heterozygous and homozygous strains, further investigating the molecular framework governing white-to-opaque switching.

We identified *MTL* **a/a** homozygous strains after growth in 10% sorbose liquid followed by plating on 2% sorbose plates; the genomic DNA was extracted from candidates and homozygous strains were confirmed with PCR using *MTL* **a** and *MTL* α gene primers with two different sets of PCR

primers A1, (External region of *MTL* **a**1 forward primer) and A2, (External region of *MTL* **a**1 reverse primer). Using A1 A2 should generate a band of 1420 nucleotides. Similarly, PCR using A3, (External region of *MTL* α2 forward primer) and A4, (External region of *MTL* α2 reverse primer) would create a band of 868 nucleotides. However, if the strains became *MTL* **a/a** or *MTL* α/α they would not show one of these 2 sets of PCR products (Fig. 8 B).

While an *ofi1* knockout in a *MTL* heterozygous strain showed no switching to the opaque state, it remained unclear whether this was due to the *MTL* configuration itself or the absence of *OFI1*. By examining *ofi1* knockout strains in the *MTL* homozygous context, where switching naturally occurs, we can determine if *OFI1* is essential for the process or if its deletion eliminates switching regardless of the mating type configuration. This approach will help clarify whether *OFI1* is a critical regulator of white-to-opaque switching in *MTL* homozygous *C. albicans*.

The deletion of *ofi1*Δ/Δ in *MTL* **a/a** significantly impacted the white-to-opaque switching frequency in *C. albicans*, as observed on Lee's GlcNAc + phloxine B medium at 25°C after four days (Fig. 9 A). While the control strain SN152 **a/a** displayed a switching frequency of 47.7%, the *ofi1*Δ/Δ **a/a** mutant exhibited a substantially reduced switching frequency of 18%, underscoring the important role of *OFI1* in promoting this phenotypic transition. In contrast, *OFI1* overexpression in the **a/a** background resulted in a 100% switching frequency, indicating that elevated levels of *OFI1* can strongly drive the transition to the opaque phenotype (Fig. 9 B).

These findings align with previous reports (Du et al., 2015) that *OFI1* plays a role in promoting the opaque phenotype, likely as part of the regulatory network influenced by *WOR1*. The results suggest that *OFI1* overexpression enhances the regulatory feedback loop essential for white-to-opaque switching but still depends on *WOR1* function. Additionally, the phenotypic differences in colony morphology between these strains further emphasize the dosage-dependent role of *OFI1* in this process.

Following our observations of the impact of the *ofi1*Δ/Δ **a/a** construct on white-to-opaque switching, we investigated the role of Ofi1 within the complex network of interlocking transcriptional feedback loops governing this phenotypic switch in *C. albicans*. Currently, the interactions of Ofi1 with key regulators such as Wor1 and Wor3 have been established (Cui et al., 2024). The relationship between *WOR1* and *OFI1* shows that in cells with the *wor1*Δ/Δ mutation,

overexpression of *OFI1* does not facilitate opaque cell formation, while overexpression of *OFI1* alone does increase opaque cell formation, underscoring the role of Wor1 as the primary regulator essential for triggering the opaque phenotype, rather than Ofi1 (Du et al., 2015). It has been noted that a potential Ofi1 binding site is located 5.6 kb upstream of the *WOR3* coding region, and there is evidence that Ofi1 may play a direct role in the activation of *WOR3* expression (Cui et al., 2024). Despite known interactions of Ofi1 with *WOR1* and *WOR3* in the white-to-opaque switching pathway, both the precise role of Ofi1 and its interactions with other regulators remain unclear. This has motivated us to investigate deeper into the interactions between Ofi1 and other critical regulators, specifically *WOR2*, *WOR4*, and *CZF1*, to better understand how *OFI1* collaborates with or impacts these factors within the intricate regulatory network governing white-to-opaque switching. Accordingly, we sequentially explored the interactions of *OFI1* with *WOR2*, *WOR4*, and *CZF1*.

A.2.2 Regulatory Dynamics of *OFI1* and *WOR2* in White-Opaque Switching

We explored the regulatory dynamics of the interaction between *OFI1* and *WOR2* on white-opaque switching in *C. albicans*, focusing on *WOR2* as a critical regulator of the process. Previous research (Zordan et al., 2007) has demonstrated that *wor2*Δ/Δ impairs this process, with knockout strains forming opaque colonies less frequently than wild-type strains. Ectopic expression of *WOR2* from a p*MET3*-*WOR2* construct also failed to enhance white-opaque switching, as cells remained white, suggesting that *WOR2* alone, driven by the *MET3* promoter, is insufficient for opaque cell formation. This limitation of *WOR2* is noteworthy considering research that highlights the significant binding of Wor1 and Wor2 to the *OFI1* promoter, positioning *OFI1* function potentially to be regulated by Wor1, the master regulator of white-opaque switching, as well as by Wor2. Furthermore, Wor2 plays a specific role in maintaining the heritability of the opaque phenotype. Given these findings, investigating the interaction between *OFI1* and *WOR2* could provide new insights into the regulatory mechanisms driving white-opaque switching (Zordan et al., 2007). For this study, we constructed single and double mutants of *wor2* following the Vyas protocol (Vyas et al., 2015) to explore how *OFI1* and *WOR2* cooperate in regulating this phenotypic transition. This assay was performed as previously described, and white and opaque colonies were counted as shown in Fig. 10 A. The occurrence of opaque colonies in the *wor2* **a/a** strain was reduced

(15%) compared to the control strain (SN152 **a/a**), very similar to the level (18.5%) generated by the *ofi1* mutation. Intriguingly, the double mutant combining *ofi1* and *wor2* in the **a/a** background exhibited an even lower count of opaque colonies (3%), considerably less than the individual mutants of *ofi1* **a/a** and *wor2* **a/a** (Fig. 10 B & C). This frequency suggests that the two proteins are playing essentially independent roles in the process.

We further sought to investigate the frequency of opaque cells in the *OFI1* overexpression and *wor2*Δ/Δ **a/a** strain to clarify the interplay between these two genes. This examination evaluated white-opaque switching, showing 100% switching when overexpressing *OFI1* in the *wor2*Δ/Δ **a/a** background (Fig. 9). Thus, activating Ofi1 can bypass the need for Wor2. Overexpressing *WOR2* itself did not generate any opaque colonies or cells (Zordan et al., 2007). Therefore strong, independent expression of *OFI1* could make the Wor2 function in the white-opaque switch unnecessary. Interestingly, filamentous growth was observed only in opaque colonies of strains with *ofi1*Δ/Δ, in both the *ofi1* single mutant and *ofi1*Δ/Δ *wor2*Δ/Δ double mutant cells under conditions such as Lee's GlcNAc medium at 25°C. This phenomenon, the formation of filaments from opaque cells, which diverges from typical opaque cell behavior, will be investigated further in this study.

A.2.3 Regulatory Interplay Between *WOR4* and *OFI1* in White-to-Opaque Switching of *C. albicans*

Wor4 is a key transcriptional regulator genetically positioned upstream of *WOR1* and formally recognized as an activator of the opaque cell type in *C. albicans* (Lohse & Johnson, 2016). Similar to *OFI1,* ectopic expression of *WOR4* is sufficient to induce white-to-opaque switching, while in contrast to *ofi1*, its deletion totally blocks this transition, highlighting its critical role in regulating phenotypic switching (Zordan et al., 2006). ChIP-seq data shows that Wor4 binding is highly associated with Wor1 and Wor2 binding sites, meaning these TFs often bind to the same promoter regions. In opaque cells, Wor4 binding is significantly expanded compared to white cells, with 90% of Wor4-bound intergenic regions also bound by Wor1 or Wor2. Additionally, Wor4 binds to the promoter regions of all known white-opaque regulators, including *WOR1*, *WOR2*, *WOR3*, *CZF1*, *EFG1,* and *OFI1* (Lohse & Johnson, 2016). Since Czf1 and Wor4 are both involved in

promoting opaque formation, their interaction helps drive the initiation of the white-to-opaque transition. In contrast, Wor2 and Wor4 work together to maintain the opaque state, ensuring its stability across cell divisions. In white cells, Wor4 binding is more restricted, but it still interacts with key regulators such as Czf1 and Efg1. Notably, Wor4 binds upstream of *WOR1*, suggesting it may contribute to the activation of the opaque program, although its effect on *WOR1* expression appears to be modest in white cells (Lohse & Johnson, 2010, 2016). Given the unique role of *WOR4* in maintaining the opaque phenotype and its strong integration into the white-opaque circuit, *OFI1* may act downstream of *WOR4*, further influencing the dynamics of this phenotypic transition. To investigate this, we constructed a *wor4* knockout in the SN152 **a/a** background, as well as an *OFI1* overexpression **a/a** strain, using the CRISPR-Cas9 system (Vyas et al., 2015). These models allow us to examine the interaction between *WOR4* and *OFI1*. The same experimental setup as described previously was applied, with strains cultured in YPD, adjusted to OD600 0.1, plated on Lee's GlcNAc medium, and incubated at 25°C for four days to assess switching frequencies (Fig. 11 A).

Deletion of the *wor4* gene (*wor4*Δ/Δ **a/a**) had been shown to inhibit the formation of opaque colonies (Lohse & Johnson, 2016), which aligns with our experimental results. Specifically, when we cultured the *wor4*Δ/Δ **a/a** strain on Lee's GlcNAc plates, we observed a total absence of opaque colonies, reinforcing the assertion that *WOR4* is crucial for this phenotypic transition. This is consistent with previous studies that have identified *WOR4* as a key transcriptional regulator in the white-to-opaque switching process and suggested its role as an activator upstream of the Wor1 function in this regulatory pathway (Lohse & Johnson, 2016).

We also examined the switching rate of the double deletion strain *ofi1*Δ/Δ *wor4*Δ/Δ **a/a**. This combination, like the single *wor4*Δ/Δ **a/a** mutant, resulted in the absence of opaque colonies; the already reduced switching frequency of 18.5% in the *ofi1*Δ/Δ **a/a** strain was totally blocked in the double knockout strain. This observation suggests that while both Ofi1 and Wor4 are integral components of the switching mechanism, Wor4's role is more critical (Fig. 11 B & C) (Lohse & Johnson, 2016). The dynamics of switching frequency changed significantly when we overexpressed *OFI1* in the *wor4* knock-out *MTL* **a/a** strain. In this scenario, the white-to-opaque switching frequency was found to be increased to 58%; the block generated by the *wor4* deletion in *MTL* **a/a** was significantly bypassed. This implies that *OFI1* overexpression **a/a** can partially

compensate for the absence of *wor4*, indicating a potential relationship where *OFI1* may act downstream of *WOR4*.

A.2.4 Investigating the Regulatory Interplay Between *OFI1* and *CZF1* in White-to-Opaque Switching of *C. albicans*

We also looked at the relationship between the TFs Ofi1 and Czf11 in *C. albicans* in the context of white-to-opaque switching. These two genes each have established involvement in the phenotypic switching process, but there is no direct DNA binding of Czf1 to the *OFI1* promoter as indicated by ChIP-chip data (Zordan et al., 2007). In our study, we examined the effects of *czf1* gene deletion and followed our standard protocol for the assessment of the interactions.

The data indicate that the *czf1*Δ/Δ **a/a** strain showed a significant reduction in the frequency of white-to-opaque switching, with a mean of 10.5%. This finding is consistent with the established role of *CZF1* as an opaque-enriched transcriptional regulator that stabilizes the opaque identity by repressing *EFG1* and activating *WOR2* (Lohse & Johnson, 2016). The diminished switching frequency observed in the *czf1*Δ/Δ **a/a** strain underscores the importance of *CZF1* in the regulatory network governing phenotypic transitions in *C. albicans*. Moreover, the double deletion of both *ofi1*Δ/Δ *czf1*Δ/Δ **a/a** resulted in an even more pronounced reduction in switching frequency, with a mean of only 1%. This dramatic decrease could suggest that both TFs are independently required for the white-to-opaque switching process, and their combined absence severely impairs the ability of *C. albicans* to transition between phenotypic states (Fig. 11).

Published data indicate that Czf1 does not exhibit DNA binding to the *OFI1* promoter, as indicated by ChIP-chip data (Zordan et al., 2007), but it is possible that Czf1 could exert influence on *OFI1* indirectly, given they both have roles in white opaque switching. This could involve the modulation of other TFs or regulatory pathways that ultimately impact on *OFI1* expression or activity. The observed high switching frequency in the *OFI1* overexpression strain in the absence of *czf1*Δ/Δ further supports this hypothesis, as the presence of *OFI1* appears to compensate for the loss of *CZF1*. Based on our data, deleting *ofi1* in both the *czf1*Δ/Δ and *wor2*Δ/Δ mutants had a similar effect, with the double deletion (*ofi1*Δ/Δ *czf1*Δ/Δ and *ofi1*Δ/Δ *wor2*Δ/Δ) reducing white-to-opaque switching more than either single mutant (*czf1*Δ/Δ and *wor2*Δ/Δ). Conversely,

overexpressing *OFI1* in *czf1*Δ/Δ or *wor2*Δ/Δ restored switching to 100%. Since Czf1 is required for initiating the opaque phase and Wor2 for maintaining it, these results suggest that Ofi1 could contribute to both processes, helping to establish and stabilize the opaque state.

B: Yeast to filament transition

B.1.1 Regulatory Effects of Ofi1 on Filamentation in *C. albicans*

Several TFs, such as Czf1 and Efg1, play key roles in both white-to-opaque switching and in the transition from yeast growth to filamentation in *C. albicans*. These TFs are essential for regulating the phenotypic transitions that contribute to the pathogenicity of this yeast. We aimed to examine the role of Ofi1 in filamentation, based on previous findings that indicated its involvement in this process (Du et al., 2015), through experiments across different media and temperature conditions. We examined the impact of *OFI1* overexpression under the control of the *ACT1* promoter and the interaction between the *ofi1* null mutation and mutations in the *WOR2*, *WOR4,* and *CZF1* genes. By investigating these conditions, we aimed to clarify the regulatory mechanisms underlying filamentation and its relationship with white-to-opaque switching, thereby contributing to a deeper understanding of the adaptive strategies applied by *C. albicans* in response to environmental cues. Unlike white opaque switching, where the *MTL* phenotype is important, filamentation initiation is *MTL* independent - *MTL* heterozygotes or homozygotes respond identically to filament-inducing conditions (Yi et al., 2011). For instance, the transcription factor Efg1 is known to be a central regulator of filamentation in *C. albicans*, and the level of Efg1 expression does not significantly change between different *MTL* configurations (i.e., **a**/α and **a**/**a** cells) (Yi et al., 2011). This indicates that the filamentation process should be more dependent on environmental cues and the presence of specific signaling pathways rather than on the mating type configuration. Moreover, the studies suggest that the regulatory networks governing filamentation are complex and involve multiple TFs and signaling pathways, including the Ras1/cAMP pathway, which is activated similarly in both *MTL* configurations (Lackey et al., 2013; Yi et al., 2011). This suggests that while the *MTL* locus is important for other aspects of *C. albicans* biology, such as mating (white-to-opaque switching) and biofilm formation, it may not be the primary determinant of

filamentation. We, therefore, carried out assays on *MTL* **a**/α strains, incorporating the *ofi1*Δ/Δ **a**/α mutant, the SN152 **a**/α control, and an *OFI1* overexpression **a**/α strain.

We performed the invasive growth test on YPD and Spider solid media at different temperatures to assess the ability of the strains to adhere and invade the agar surface, which is an important characteristic of their virulence. Additionally, we tested the constructs in YPD liquid supplemented with 20% Bovine Serum Albumin (BSA) to induce filamentation and captured microscopy images to analyze the morphological changes associated with this transition. We also checked the colony morphology on Lee's GlcNAc and Spider media at 25°C and 30°C to further evaluate the impact of different conditions on the strains' growth patterns.

B.1.2 *OFI1* specific analysis

For growth in liquid YPD plus serum, we monitored the *OFI1*-overexpression, *ofi1* deletion, and WT strains in liquid YPD medium at 4- and 8-hours post-inoculation, using microscopy to observe changes. Our results showed that overexpression of *OFI1* significantly enhanced filamentation as early as 4 hours, compared to the control in YPD liquid enriched with 20% serum (Fig. 13 A). With the deletion of *ofi1*, the frequency of yeast-to-filament transition decreased from 28% in the SN152 control strain to 21%, so the absence of *ofi1* appears to have a minor impact on filamentation under the tested conditions. In contrast, when *OFI1* was overexpressed, the frequency of the yeast-to-filament transition increased dramatically, reaching 100% (Fig. 13 B). This suggests that the overexpression of *OFI1* significantly promotes filamentation in YPD+ 20% serum at 37°C.

In an invasive growth assay, we tested the SN152 wild type (control), *OFI1* overexpression, and *ofi1*Δ/Δ strains on YPD and Spider solid media. Each strain was spotted with 10^4 cells and incubated at 25°C, 30°C, and 37°C for 3 days before washing to assess adhesion. On Spider medium at 25°C, both the control SN152 and *ofi1*Δ/Δ washed off completely and the *OFI1* overexpression strain did not wash off at all. At 30°C, the *ofi1*Δ/Δ strain washed off easily, while the control remained partially adhered and the *OFI1* overexpression strain remained strongly attached. At 37°C, both the control and *OFI1* overexpression strains stuck to the agar, whereas the

*ofi1*Δ/Δ strain washed off again. The results on YPD were similar, except that at 30°C, the control strain showed stronger adhesion compared to the Spider medium (Fig. 13 C). Growth on Spider and Lee's GlcNAc solid media generated no difference between *ofi1*Δ/Δ and the control colonies at both 25°C and 30°C. However, *OFI1* over-expression in both media resulted in wrinkled colonies (Fig. 13 D & 14). Our data showed Ofi1 plays a role in filamentation under different filament inducers: YPD liquid supplemented with 20% Bovine Serum Albumin (BSA) and on solid Spider, YPD, and GlcNAc media. Additionally, in our invasive growth assays, *OFI1* overexpression at 25°C, 30°C, and 37°C in Spider and YPD solid media resulted in colonies that remained adhered to the agar even after washing, indicating that Ofi1 enhances adhesion and invasive growth.

B.1.3 *WOR2*'s Role in Regulating Filamentation

WOR2 homologs are found in filamentous fungi such as *Aspergillus nidulans* and *Neurospora crassa*, as well as in the fission yeast *Schizosaccharomyces pombe*. However, *WOR2* has no identifiable ortholog in *S. cerevisiae* (CGD- http://www.candidagenome.org/). While some *Candida* species homologs of *WOR2* have been annotated, its functional role outside *C. albicans* remains unclear and requires further investigation. Based on the many TFs such as Efg1, and Ofi1 that are involved in more than one morphogenesis transition in *C. albicans* (Du et al., 2015; Lo et al., 1997), Wor2, as a key TF of the white-to-opaque switching, could potentially play a role in the filamentation transition. We tested if Wor2 was involved in the filamentation pathway and if there is any filamentation regulatory interaction between Ofi1 and Wor2 with the combination of single and double mutations of these two genes and *OFI1* overexpression in cells lacking *wor2* in filamentation-inducing media such as Spider and Lee's GlcNAc solid media and liquid YPD+ 20% serum. An invasive growth assay on Spider media was performed using a spot assay, with cell concentrations that ranged from 10^7 to 10^3. This test evaluates the ability of strains to adhere and invade the agar surface during growth at 25°C, 30°C and 37°C.

When *OFI1* was overexpressed, the colonies became completely wrinkled on Spider and Lee's GlcNAc media at 30°C, independent of the presence of Wor2. This suggests that Wor2 does not play a role in filamentation under these conditions (Fig. 14).

In liquid YPD + 20% serum at 37°C, the *ofi1* mutation reduced filamentation, and the double deletion mutant (*ofi1*Δ/Δ *wor2*Δ/Δ) exhibited the same phenotype as the *ofi1* single mutant. This indicates that the absence of Wor2 did not further alter the reduced filamentation observed in the *ofi1* mutant. Additionally, *OFI1* overexpression significantly increased filamentation in YPD + 20% serum, and the deletion of *wor2* did not influence this effect (Fig. 15), suggesting that Ofi1 enhances filamentation independently of *WOR2* under these conditions. We found the yeast-to-filament transition frequency in the *wor2*Δ/Δ mutant was similar to the control at 37°C after 8 hours in YPD+ 20% serum. Overexpression of *OFI1*, with or without *wor2*, resulted in a 100% transition frequency and the double deletion of *wor2* Δ/Δ *ofi1* Δ/Δ showed 21% switching to filaments, which was the same as the single mutant of *ofi1* (Fig. 16).

Similarly, the deletion of *wor2* did not cause a significant difference from the wild-type (SN152) response in the invasive growth test on Spider solid media at 37°C. In contrast, the deletion of *ofi1* affected invasiveness across all tested temperatures (25°C, 30°C, and 37°C) on Spider solid media (Fig. 17). Therefore, we find no evidence that Wor2 influences the yeast-to-hyphal transition in *C. albicans*.

B.1.4.1 Exploring the Role of *WOR4* in Filamentation and Its Interaction with *OFI1* in *C. albicans*

WOR4 was first identified in 2016 as a key TF in the white-to-opaque switching process of *C. albicans* (Lohse & Johnson, 2016). While its role in this transition is known, there is still a lot to learn about how it functions in other pathways and under different environmental conditions. *WOR4* homologs are present across various fungal species, including filamentous fungi and members of the *Basidiomycota* and *Mucoromycotina*, which points to its broader evolutionary importance. However, the absence of *WOR4* in fungi like *S. cerevisiae*, *Kluyveromyces lactis*, and *Schizosaccharomyces pombe* highlights its unique evolutionary path and possibly distinct functions.

We investigated how the transcription factor Wor4 impacts filamentation in *C. albicans* under different conditions, including Spider and Lee's GlcNAc solid medium and YPD with 20% serum

liquid, across a range of temperatures. To assess the invasive growth of the strains, an assay was performed on Spider media using a spot assay with cell concentrations ranging from 10^7 to 10^3 at 25°C, 30°C, and 37°C. On Spider medium at 30°C, the *OFI1* overexpression strain showed more wrinkled colonies compared to the wild-type strain, consistent with earlier tests. The other strains didn't show significant changes on Spider at 25 and 30°C (Fig. 18).

In YPD + 20% serum, the *ofi1*Δ/Δ mutant strain showed a somewhat reduced filamentation frequency, as did the *wor4*Δ/Δ strain. Also, the double deletion resulted in an even greater reduction than either single mutant (Fig. 19). When *OFI1* was overexpressed in a *wor4*Δ/Δ background, filamentation reached 100%, indicating that *wor4* deletion did not block the enhancement of filamentation by *OFI1* activation. This filamentation frequency was significantly higher compared to the 20% observed in the *wor4*Δ/Δ single mutant, suggesting that *WOR4* is not required for the *OFI1*-induced filamentation enhancement.

Interestingly, on Lee's GlcNAc medium, the *wor4*Δ/Δ strain formed fuzzy colonies, as did the *ofi1*Δ/Δ *wor4*Δ/Δ double-deletion strain and the *OFI1* overexpression strain with *wor4*Δ/Δ. These colonies looked distinct from the control and the *ofi1*Δ/Δ strain, which both exhibited a more typical and non-fuzzy colony morphology. These findings suggest Wor4 might have a role in Lee's GlcNAc signaling pathway for filamentation (Fig. 18).

In invasive growth assays on Spider solid media at 25°C, 30°C and 37°C, strains with *ofi1*Δ/Δ and the double-deletion strain *ofi1*Δ/Δ *wor4*Δ/Δ failed to show invasive growth as they were easily washed off the agar reflecting the *ofi1*Δ/Δ phenotype. However, the *wor4*Δ/Δ strain alone retained invasive growth, suggesting the *wor4*Δ/Δ mutation has no impact on invasive growth on Spider solid media at 25°C, 30°C and 37°C (Fig. 17).

In our study regarding the potential interaction between Wor4 and Ofi1, it appears as though the environment and the media that we were using are critical to the final result, but that in contrast to Wor2, under some conditions, Wor4 can impact filamentous growth.

B.1.4.2 *WOR4* Knockout Affects Filamentation in the Lee GlcNAc Pathway?

We followed up on the observation that the *wor4*Δ/Δ strain showed considerable filamentation at 30°C (Fig. 18). While Lee's GlcNAc medium is not traditionally recognized as a strong filamentation inducer, the wild-type strain displayed some filamentous growth, although it was limited. In contrast, the filamentation observed in the *wor4*Δ/Δ strain was significantly enhanced, suggesting that the absence of *wor4* may activate alternative regulatory pathways that trigger filamentation in response to Lee's GlcNAc. This highlights the complex interplay of transcriptional regulators in filamentation and raises interesting questions about the compensatory mechanisms that operate in the absence of *wor4*.

To further explore the role of Wor4 in filamentation, we systematically compared all strains related to *WOR4* and *OFI1* under various temperatures in Lee's GlcNAc medium, with a focus on colony morphology at 37°C to determine whether the results observed at 30°C remain consistent at a higher temperature. The strains included *wor4*Δ/Δ, *wor4*Δ/Δ *ofi1*Δ/Δ double deletion, *OFI1* overexpression, and *OFI1* overexpression in a *wor4*Δ/Δ background. Notably, all strains with *wor4*Δ/Δ, including the double deletion and *OFI1* overexpression in the *wor4*Δ/Δ background, displayed fuzzy and wrinkled colony phenotypes (Fig.21). Interestingly, *wor4* deletion resulted in fuzzy colonies, while *OFI1* overexpression led to wrinkled colonies. Finally, the combination of *wor4* deletion and *OFI1* overexpression produced colonies that were both wrinkled and fuzzy. This suggests that Wor4 and Ofi1 may both be involved in filamentation but through distinct regulatory pathways.

The findings provide strong evidence that the absence of *wor4* stimulates filamentation under Lee's GlcNAc-inducing conditions. These insights not only enhance our understanding of the regulatory mechanisms governing filamentation in *C. albicans* but also highlight the importance of Wor4 as a key player in this process. Further research into the specific interactions between *WOR4*, *OFI1*, and Lee's GlcNAc-induced signaling pathways will be essential to unravel the full extent of their roles.

B.1.5 The Essential Role of *CZF1* in Regulating Filamentation in *C. albicans*

Czf1 is a transcription factor that plays a role in both white-to-opaque switching and filamentation in *C. albicans*. Unlike many other regulatory genes, *CZF1* appears to be unique to the *Candida* clade, with homologs found in *C. tropicalis* and *C. dubliniensis* (Blandin et al., 2000) but not in model yeasts like *S. cerevisiae* (Seoighe et al., 2000). Interestingly, while the genes surrounding *CZF1* are arranged in the same order across different ascomycete fungi, *CZF1* itself is missing in most species outside *Candida*. A related gene has been found in *Debaryomyces hansenii*, but with notable differences in its regulatory region. The fact that *CZF1* is mostly limited to *Candida* species suggests it evolved for functions specific to their unique lifestyles, possibly helping them adapt to different environments or regulate morphological changes in ways that aren't needed in other fungi. Previously, we checked the role of Czf1 in white-to-opaque switching and its interaction with *OFI1*. Now, to further investigate the interaction between *CZF1* and *OFI1*, we examined their roles in filamentation growth under various filament-inducing conditions, including Lee's GlcNAc, Spider solid medium at 25°C and 30°C, and liquid YPD with 20% serum at 37°C. Also, we checked the invasive growth on Spider to evaluate the ability of strains to adhere and invade the agar surface at 25°C, 30°C and 37°C.

OFI1 overexpression led to wrinkled colonies on both Lee's GlcNAc and Spider solid media at 25°C. However, when *czf1* was deleted in the *OFI1* overexpression strain, the colonies no longer exhibited the wrinkled phenotype. This suggests that the absence of *czf1* reduces the wrinkling effect typically induced by *OFI1* overexpression (Fig. 22). In 20% serum-induced filamentation, the yeast-to-filament switching rate for the *czf1*Δ/Δ mutant was 10.8%, which was not significantly different from the *czf1*Δ/Δ *ofi1*Δ/Δ double mutant at 9.8%, indicating that the absence of *czf1* inhibits filamentation and that the additional deletion of *ofi1* does not worsen this defect (Fig. 23 & 24). We further explored whether overexpressing *OFI1* could enhance filamentation in the absence of *czf1*; however, the results indicated that this was not the case, as *OFI1* overexpression without *czf1* did not increase filamentation. Although our previous data showed that *OFI1* overexpression enhances filamentation to nearly 100%, this effect was not observed when *czf1* was deleted. In the invasive growth assays, we observed where the *czf1*Δ/Δ mutant and *czf1*Δ/Δ *ofi1*Δ/Δ double mutant were unable to invade the agar surface, and *OFI1* overexpression in the *czf1*Δ/Δ background could not restore invasive growth (Fig. 17).

C: Filamentation of Opaque Cells

C.1 Exploring the Role of *OFI1* in the Filamentation of Opaque Cells

Traditionally, opaque cells are not seen to undergo filamentation under conditions that typically induce such growth in white cells. Filamentous growth in opaque cells was observed only when cells were cultured in environments such as Lee's GlcNAc or Sorbitol medium at 25°C — conditions quite divergent from those commonly associated with white cell filamentation (Si et al., 2013). However, in our investigation into the morphological behavior of opaque *C. albicans*, we noted that in opaque colonies, the *ofi1* mutant strain consistently showed a very fuzzy appearance when grown on Lee's GlcNAc medium at 25°C. This phenotype persisted even in the double-deletion strain lacking both *ofi1* and *wor2*, as these colonies also exhibited a fuzzy morphology. However, we were unable to determine whether this phenotype extends to double-deletion strains lacking both *ofi1* and *wor4*, as this construct failed to produce any opaque cells for analysis. We selected confirmed opaque colonies from Lee's GlcNAc plates, washed them, and restreaked them onto fresh Lee's GlcNAc plates. After four days, the *ofi1*Δ/Δ strain exhibited a fuzzy colony morphology, which differed from the SN152 control. To further investigate the role of Ofi1, we tested SN152, *ofi1*Δ/Δ, and *OFI1* overexpression strains under another filament-inducing condition specific to opaque cells—sorbitol medium. Again, *ofi1*Δ/Δ colonies exhibited a fuzzy phenotype, whereas the *OFI1* overexpression and control strains did not. Microscopy images confirmed the presence of filamentous cells in the *ofi1*Δ/Δ strain (Fig. 25). Examining the role of Czf11 in *OFI1*-mediated filamentation in opaque cells could provide valuable insights into their regulatory relationship.

This work provides insight into the distinct morphological pathways between opaque and white cells. This suggests that although the absence of *ofi1* may reduce the overall number of opaque colonies, it also seems to promote filamentation in the opaque form.

4. Discussion

Regulation of White-Opaque Switching and Morphogenesis by Ofi1 in *C. albicans*

In the fungal pathogen *C. albicans*, transcription factors (TFs) regulate processes such as morphological transitions, virulence, and stress response. In this study, we aimed to better understand the role of the transcription factor Ofi1 in two processes involved in morphological transitions in *C. albicans*: the white-to-opaque switch and the yeast-to-filamentous growth transition.

In *C. albicans*, the two distinct cellular morphologies known as white and opaque have been characterized, with the opaque form being crucial for mating. These cells exhibit notable differences in shape and biological function. White cells are typically round and form smooth, dome-shaped colonies, whereas opaque cells are elongated and produce flatter, darker colonies with a rough surface texture. The transition between these two forms is significantly influenced by environmental factors, with GlcNAc media promoting this switching more effectively than glucose. This phenomenon is attributed to GlcNAc's role in activating signaling pathways that enhance the expression of key TFs involved in the switching process, such as Wor1. The use of phloxine B in GlcNAc solid media allows for the visual differentiation of these cell types, as it stains opaque colonies pink, facilitating the study of switching dynamics.

In *C. albicans*, the *MTL* locus determines the cell's ability to switch between the standard white and opaque forms. Cells with both **a** and α alleles (**a**/α) produce a repressor that, in general, keeps them in the white, non-mating state. If a cell loses one allele, becoming either **a** or α, or homozygosis the locus, becoming **a**/**a** or α/α, repression will be removed, giving the cell the potential to switch to the opaque form and become mating-competent. This system ensures that only cells capable of mating undergo the white-opaque transition. After mating, the resulting cells return to *MTL* heterozygosity, revert to the white state, and cannot switch back to opaque unless they once again become homozygous for one of the *MTL* alleles. Interestingly, some natural **a**/α

isolates can switch to the opaque form under specific conditions, such as the presence of GlcNAc, even though they remain mating incompetent.

The regulation of this switching mechanism is complex, involving a network of TFs that respond to environmental cues, enabling *C. albicans* to adapt to various host niches and evade immune responses. Control of the white-to-opaque switch in *C. albicans* cells is an example of a well-studied, complex transcriptional regulatory network, where several TFs, each with a unique role in the phenotypic switch, work together in a coordinated manner to not only regulate this transition but also maintain the cells in opaque phase. The switch to the opaque state is regulated by the master regulator, Wor1, and additional TFs, including Wor2, Wor3, Wor4, Ahr1, Czf1, and Efg1 have different roles in the control of this transition. Wor2, Wor4, and Wor3 are known as maintenance TFs, directing cells to remain in the opaque phase (Zordan et al., 2007; Lohse & Johnson, 2010). While Czf1, Wor4, Wor3, and Wor1 are the set of initiation of the opaque state.

Scientists suspected that *OFI1* (orf19.4972) might play a role in white-to-opaque switching because it was identified as an opaque-enriched gene in microarray analyses and contains multiple C2H2 zinc fingers, a known DNA-binding domain associated with transcriptional regulation (Zordan et al., 2007). Previous work showed that the gene *OFI1* under *TDH3* promoter (Cui et al., 2024) or the Ofi1 protein fused to an activation module (Shrivastava, 2021) can direct switching to opaque phase in up to 100% of cells grown in SD-Glucose solid media even when they are *MTL* **a**/α. *TDH3* is mainly active in glucose due to its role in glycolysis, and it directs high-level expression of *OFI1* under those conditions. In our study, we used the *ACT1* promoter for overexpressing *OFI1* which normally controls the *ACT1* gene encoding actin, a key component of the cytoskeleton. The *ACT1* promoter is subject to stringent regulatory mechanisms, which can potentially limit its effectiveness in driving high-level expression of transgenes (Uppuluri et al., 2007). However, *OFI1* overexpression under the *ACT1* promoter is capable of driving *MTL* **a**/α cells into the opaque form at a switching rate of 28%. This suggests that *OFI1* is a dosage-dependent TF for the white-opaque transition, because, in the generally non-switching *MTL* **a**/α cells, the stronger promoter *TDH3* generated more switching to the opaque phase (100%) than did the *ACT1* promoter (28%). In our study *OFI1* overexpression in a *MTL* homozygous strain increased white-to-opaque frequency to 100% while deletion of the gene reduced this frequency

significantly; down to 21% compared with the control frequency of 47% in Lee's GlcNAc after 4 days. Demonstrating that Ofi1 can both initiate and maintain the opaque phase.

We were interested in positioning Ofi1 in the network of TFs controlling the white-to-opaque transition in *MTL* homozygous strains. We first looked at its interaction with Wor2, a component required for stabilizing the opaque state once initiated. *OFI1* overexpression in a *wor2*Δ/Δ mutant resulted in 100% opaque cells in *MTL* **a/a** strains, suggesting that Ofi1 can compensate for Wor2's role in stabilizing the opaque state. Our analysis suggests that the reason Ofi1 can maintain opaque cells in the opaque state is its ability to bind to the WOR3 promoter, which is essential for opaque maintenance, as well as the *WOR1* promoter, the master regulator of the opaque state, increasing their expression (Cui et al., 2024), it may contribute to opaque cell stability either directly or indirectly. Also, high level of Ofi1 can bypass the *wor2* mutation, allowing the cells to remain in the opaque phase. However, the double deletion of *ofi1*Δ/Δ *wor2*Δ/Δ reduced switching blow the frequency of individual mutation and this suggests the genes have independent function. Because overproduction of Ofi1 bypasses the *wor2* mutation and thus may function in opaque cell stability, but the double mutant suggests Wor2 and Ofi1 play different function, it may be that Ofi1 functions in both initiating and stabilizing the opaque state.

Similarly, Wor4 appears involved in both the establishment and maintenance of the opaque phase. It regulates *EFG1*, and when Wor4 is deleted (*wor4*Δ/Δ), there is no suppression of *EFG1*, allowing Efg1 function to keep the cell in the white phase since it is the master regulator directing the white cells state (Lohse & Johnson, 2016). Because the *wor4* mutant totally blocks switching, the double deletion of *wor4*Δ/Δ *ofi1*Δ/Δ is uninformative. However, overexpressing *OFI1* in the strain lacking *wor4* bypassed the mutation and increased the number of opaque cells from 0% to 58%. We believe it did not reach 100% because the deletion of *wor4*Δ/Δ removed its suppression on *EFG1*. Efg1 directs the cells to remain in the white phase, preventing a full transition to the opaque state. It is possible that using a stronger promoter, such as *TDH3*, for *OFI1* overexpression could enhance switching more than *ACT1*, depending on the dosage-dependent effect of *OFI1*.

Czf1 is important in driving the white-to-opaque transition in *C. albicans*. Ectopic expression of *CZF1* can activate the white-to-opaque switching pathway and generate 100% of opaque cells

(Zordan et al., 2007) as does *OFI1* overexpression, while deletion of *czf1*Δ/Δ reduces the switching frequency to approximately 10%. This reduction is further decreased in the double deletion of *ofi1*Δ/Δ *czf1*Δ/Δ, resulting in a switching frequency of about 1.16%, consistent with both TFs playing distinct functions. In white-to-opaque switching, Ofi1 and Czf1 function independently. Knocking out *ofi1* reduces switching and knocking out *czf1* also decreases it. When both genes are deleted together, the switching frequency declines even further, suggesting their independent roles in this process.

As a result, creating single and double mutants of *ofi1*Δ/Δ with *wor2*Δ/Δ, *czf1*Δ/Δ, and *wor4*Δ/Δ suggests that Ofi1 can initiate white-to-opaque switching independently of these factors. Since Ofi1 can activate *WOR3* and *WOR1* (Cui et al., 2024), its maintenance of the opaque phase may occur indirectly through these two TFs. Additionally, *OFI1* binding to the *WOR1* promoter is essential for white-to-opaque switching, as increased binding leads to higher *WOR1* expression, further promoting the transition to the opaque state (Fig. 26).

Regulation of Filamentation by Ofi1 in *C. albicans*

Hyphal growth, also known as filamentation, is a key virulence factor in *C. albicans* because the pathogen can switch between yeast, pseudohyphae, and hyphal phases to promote invasion of tissue, immune evasion, and biofilm development (Whiteway & Bachewich, 2007) which are necessary for the success of the fungus. In contrast to *S. cerevisiae*, which can differentiate into pseudohyphae under nitrogen starvation, but does not form true hyphae, *C. albicans* instead utilizes hyphal morphogenesis as an important mechanism of pathogenesis (Cullen & Sprague, 2012). True hyphae differ from pseudohyphae in structure and biological function: true hyphae contain long parallel-sided cells containing septa without constrictions, directional growth, and infiltration potential, whereas pseudohyphae are elongated and constricted cells that form string-like structures (Ruggero et al., 2018). The differentiation into true hyphae is induced environmentally by serum, elevated temperature, neutral pH, etc., activating evolutionarily conserved signaling pathways. Filamentation is governed in part by the cAMP-protein kinase A (PKA) pathway. Upon receiving environmental signals, adenylate cyclase (Cyr1) generates cAMP, which activates PKA (Tpk1/Tpk2), leading to the activation of a set of TFs, including Efg1

and Flo8, that regulate hypha-specific genes (Rocha et al., 2001). Ras1 is a GTPase that acts upstream of Cyr1 and amplifies cAMP signaling and also interacts with MAP kinase pathways, (for example the MAPK, Cek1), which allows for the integration of several signals (Thompson et al., 2018). Ras1 also promotes polarized growth by regulating cytoskeletal reorganization through effectors such as Cdc24 (Sudbery, 2011). The dynamic interaction between these pathways provides precise control over filamentation and is critical for *C. albicans* pathogenicity compared to less virulent pathogenic fungi, which are not filamentous at infective sites. Many TFs coordinate these morphogenetic transitions, most prominently Efg1 (Stoldt, 1997). While Ofi1 is known to play a role (Du et al., 2015), our aim was to clarify its specific role, building on previously demonstrated effects of Ofi1 on filamentation. We aimed to elucidate the regulatory network determining the outcomes of filamentation and its coordination with white-to-opaque switching, through the analysis of the phenotypic effects of *OFI1* overexpression and the *ofi1* null mutation, on filamentation and on mutations in the *wor2*, *wor4*, and *czf1* genes.

We showed that Ofi1 strongly promotes filamentation in *C. albicans* under filament-inducing conditions such as 20% BSA-supplemented YPD liquid, Spider and Lee's GlcNAc at 37°C, and that yeast-to-filamentation was seen in almost all cases when *OFI1* was overexpressed, and this frequency was decreased in the case of the *ofi1* mutant.

Our analysis into an interaction of Ofi1 with the other TFs showed that, Wor2 functioned independently of Ofi1 in regulating white-to-opaque switching. Knocking out *wor2* reduces white-to-opaque switching to 15%, and knocking out *ofi1* decreases it to 16%, compared to the control frequency of 47%. When both *wor2* and *ofi1* are deleted together, the switching frequency drops further to 2.9%, suggesting that these factors contribute independently to the process. However, while Wor2 plays a role in white-to-opaque switching, it does not contribute to filamentation. Our investigation of white-to-opaque switching revealed that *OFI1* overexpression in a *wor4* deletion background could bypass the switching block caused by *wor4* deletion, increasing the switching frequency from 0% to 58%. This led us to ask whether Ofi1 could similarly bypass the filamentation defect of *wor4*Δ/Δ. The answer depended on the medium used to induce filamentation. In YPD liquid medium supplemented with 20% BSA at 37°C, high levels of Ofi1 fully compensated for the absence of *wor4*, increasing filamentation from 21% to 100%. However, on Lee's GlcNAc solid medium, the *wor4*Δ/Δ strain displayed a distinct fuzzy colony morphology,

while *OFI1* overexpression produced wrinkled colonies. Interestingly, in the *wor4*Δ/Δ strain with *OFI1* overexpression, colonies appeared both wrinkled and fuzzy, suggesting that both factors promote filamentation but likely through different pathways.

GlcNAc can induce filamentation through a distinct pathway independent of classical hyphal regulators. Brg1 plays a crucial role in this process as a suppressor of filamentation, acting as a key transcription factor required for GlcNAc-induced hyphal growth (Su et al., 2018). Our analysis suggests that Wor4 has a strong DNA-binding site in the *BRG1* promoter, indicating that Wor4 may activate *BRG1*. In *wor4*Δ/Δ strains, reduced *BRG1* activity may contribute to increased filamentation by relieving its suppressive effect.

The functional relationship between Czf1 and Ofi1 in white-to-opaque switching is distinct from its role in filamentation. While Czf1 contributes to white-to-opaque switching, its absence can be compensated by *OFI1* overexpression, allowing the process to continue. This suggests that Ofi1 can bypass the requirement for Czf1 in white-to-opaque switching. In contrast to its role in white-to-opaque switching, Czf1 is essential for filamentation. Unlike in switching, where Ofi1 can compensate for *czf1* loss, in filamentation, the absence of *czf1* completely blocks the Ofi1 function. This indicates that Czf1 plays a dominant role in the yeast-to-hypha transition, and its interaction with Ofi1 follows a fundamentally different regulatory arrangement compared to white-to-opaque switching. It seems the cell has chosen these two factors for very different roles in these two separate switching processes (white-to-opaque/ yeast-to-filament), likely because of how they are linked to the rest of the signaling network.

In summary, our data demonstrate the complex regulatory roles of Ofi1, Wor2, Wor4, and Czf1 in filamentation and white-to-opaque switching in *C. albicans*. We find that Ofi1's interaction with other TFs in white-to-opaque switching may differ from its interaction with the same factors in the yeast-to-filament transition. The response of such TFs to environmental signals is vital for the adaptability of this opportunistic pathogen. This further highlights the need for future studies to elucidate the specific molecular pathways in which these TFs interact and regulate filamentation, particularly during host interactions and responses to environmental stressors.

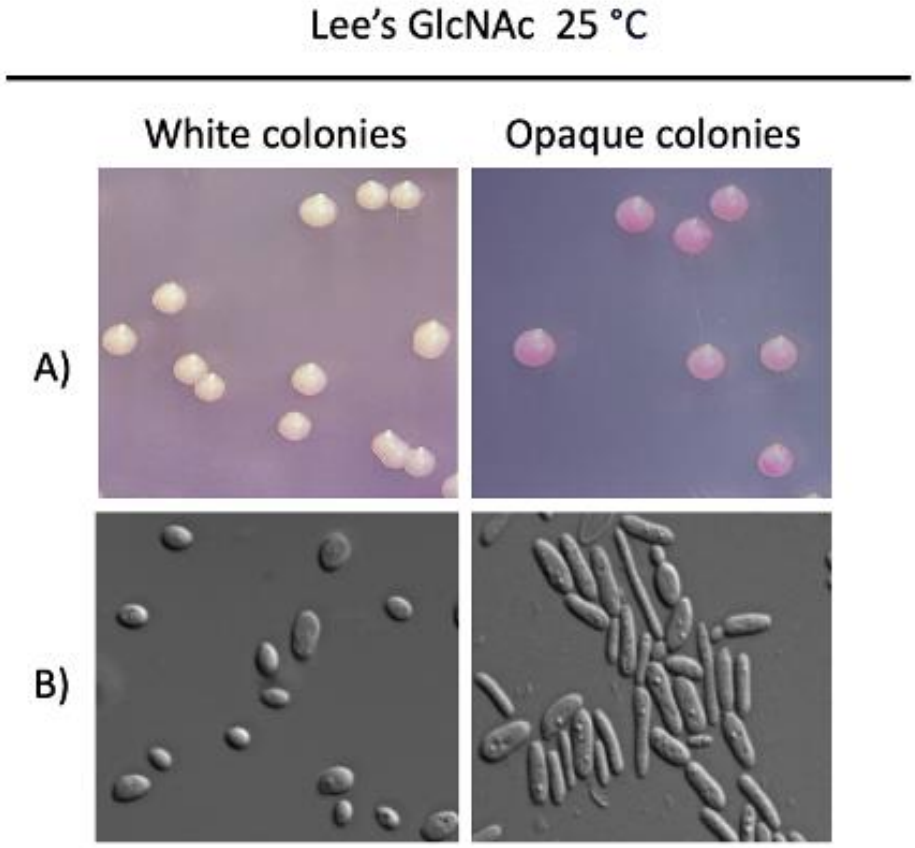


Figure 1: A) Differential appearance of *C. albicans* white and opaque colonies on Lee's GlcNAc with phloxine B plate at 25 °C for 4 days; White cell colonies remain uncolored (white), while opaque cell colonies are selectively stained pink. B) Microscopic images of *C. albicans* white and opaque cells, showing the distinct morphological characteristics of each cell type. The white colonies are formed from cells with the *wor1*Δ/Δ **a/a** genotype, where Wor1 is recognized as the master regulator of the opaque state. The opaque colonies are generated by cells with the *efg1*Δ/Δ **a/a** genotype, with Efg1 acting as the master regulator of the white state.

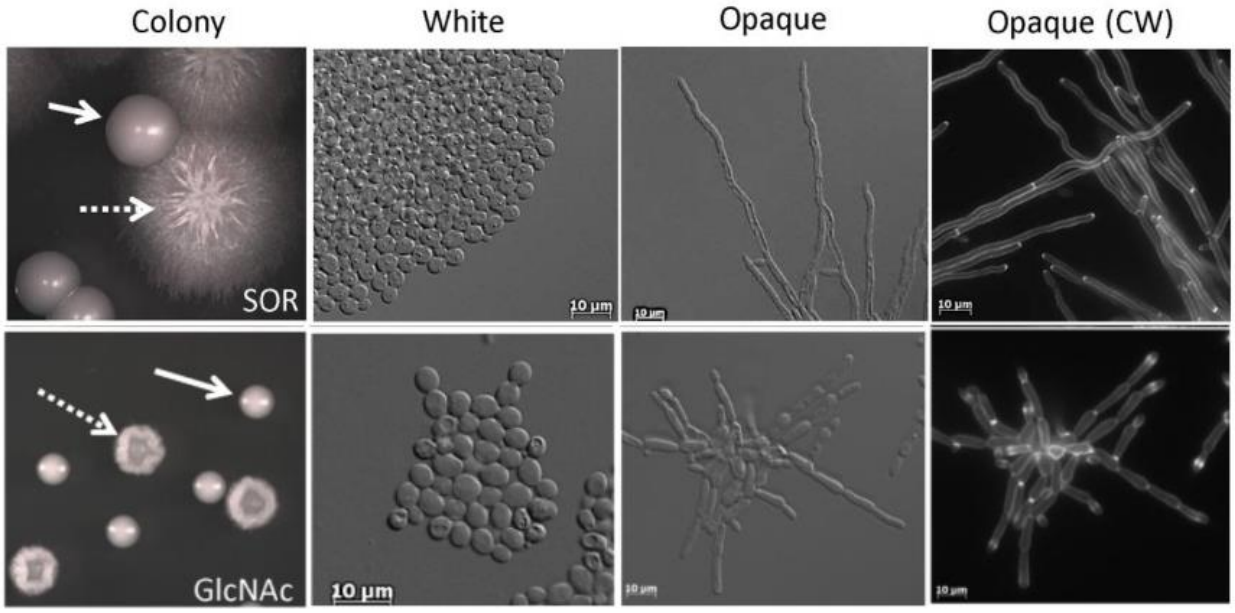


Figure 2: The impact of temperature and medium on filamentous growth in *C. albicans* SC5314 strains, comparing white and opaque cell responses to sorbitol and GlcNAc at temperatures of 25°C for four days (Si et al., 2013).

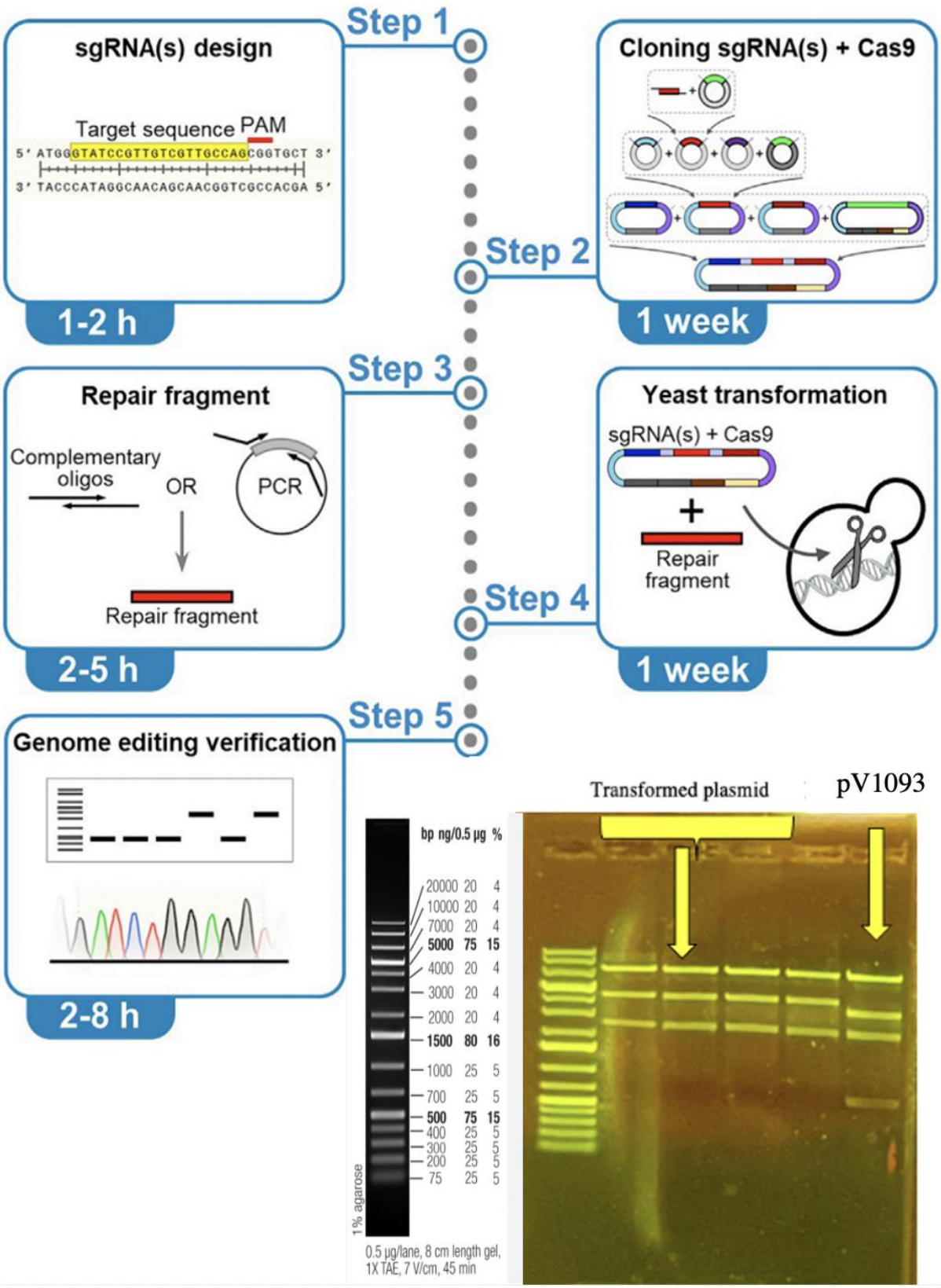

Step 1
sgRNA(s) design
Target sequence PAM
5' ATGGGTATCCGTTGTCGTTGCCAGCGGTGCT 3'
3' TACCCATAGGCAACAGCAACGGTCGCCACGA 5'
1-2 h
Step 2
Cloning sgRNA(s) + Cas9
1 week
Step 3
Repair fragment
Complementary oligos
OR
PCR
Repair fragment
2-5 h
Step 4
Yeast transformation
sgRNA(s) + Cas9
+
Repair fragment
1 week
Step 5
Genome editing verification
2-8 h
bp ng/0.5 µg %
20000 20 4
10000 20 4
7000 20 4
5000 75 15
4000 20 4
3000 20 4
2000 20 4
1500 80 16
1000 25 5
700 25 5
500 75 15
400 25 5
300 25 5
200 25 5
75 25 5
1% agarose
0.5 µg/lane, 8 cm length gel,
1X TAE, 7 V/cm, 45 min
Transformed plasmid
pV1093

Figure 3: Schematic representation of CRISPR-Cas9-mediated genome editing in *C. albicans*. (1) Design of sgRNA using Benchling. (2) Cloning of sgRNA into the *C. albicans* CRISPR plasmid pV1093 via Golden Gate assembly. (3) Amplification of the *ARG4* repair template using extension primers from the PFA-*ARG4* plasmid. (4) Transformation of *C. albicans* strains with digested pV1093 (containing Cas9 and sgRNA) along with the repair template. (5) Agarose gel confirmation of successful sgRNA ligation. The plasmid pV1093 has four EcoRI restriction sites, one of which is located between the BsmBI sites where the sgRNA is inserted. Successful ligation of sgRNA removes this EcoRI site, leading to a gel pattern with three bands instead of four, with the second band shifting up by 80 bp.

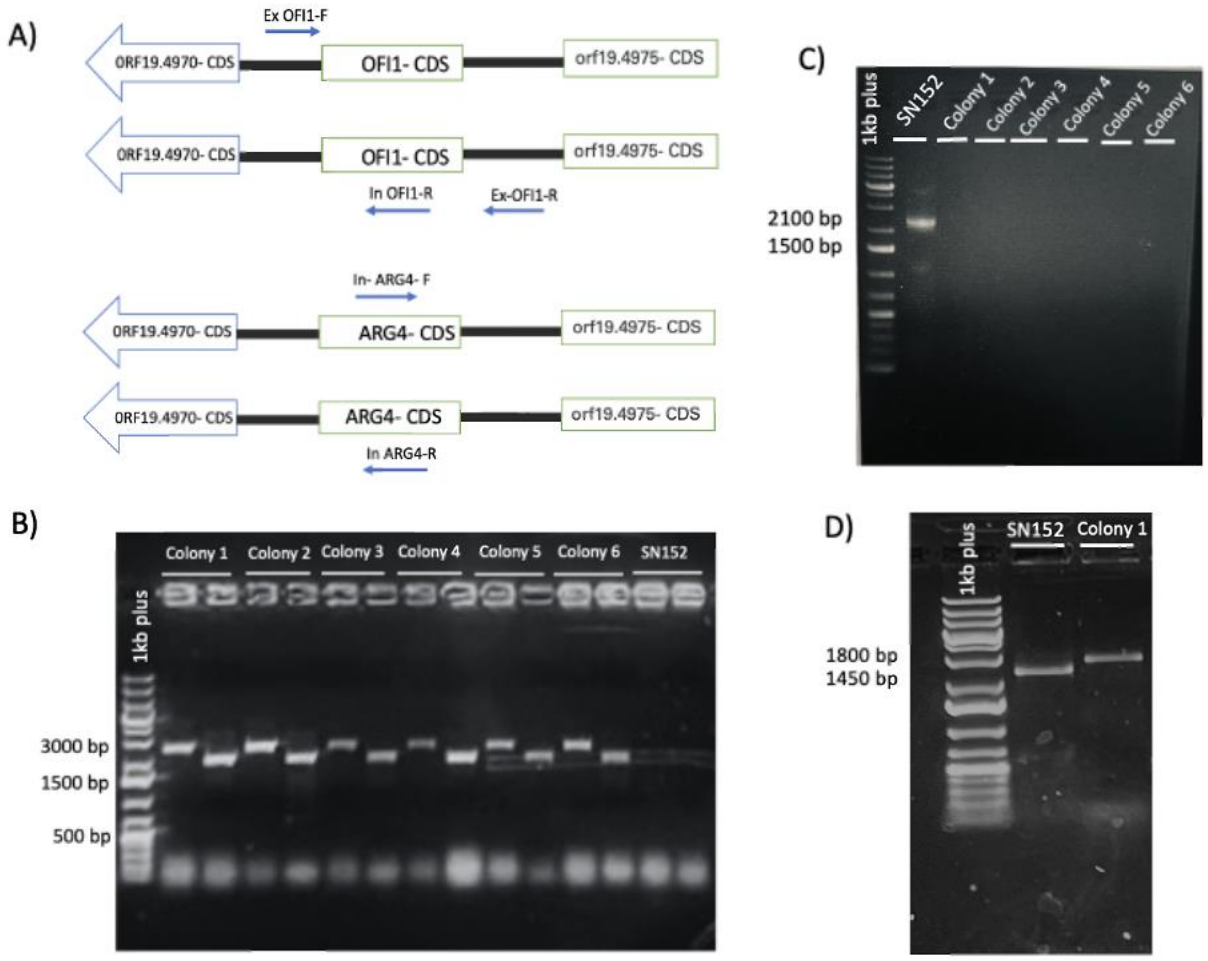


Figure 4: A) Structure of the genomic region of the *OFI1* gene replaced with *ARG4* as a repair template. B) Candidate-transformed colonies were tested for the insertion of *ARG4* at the *OFI1* locus. For each colony, the first lane shows amplification using primers Ex-*OFI1*-F (external region *OFI1* forward primer) and In-*ARG4*-R (internal region *ARG4* reverse primer), which generates a 2900 base pair band uniquely in strains with a proper insertion of *ARG4*. The second lane is an amplification using primers In-*ARG4*-F (internal region *ARG4* forward primer) and Ex-*OFI1*-R (external region *OFI1* reverse primer), producing a PCR product of 2100 base pairs unique to strains with the correct insertion of *ARG4*. Six colonies show positive results, and the last two lanes display the wild-type control: SN152. C) To confirm homozygous replacements in the colonies, candidates were tested using Ex-*OFI1*-F and In-*OFI1*-R. The control exhibited the expected 2100 bp band, while the other colonies lacked the wild-type alleles. D) The final confirmation is PCR amplification with Ex-*OFI1*-F and Ex-*OFI1*-R, which shows a 350 bp

difference between SN152 as a control and the selected construct. For this last assay, we tested the first colony and confirmed it was positive. 1 kb Plus GenRuler was employed as the ladder in these analyses.

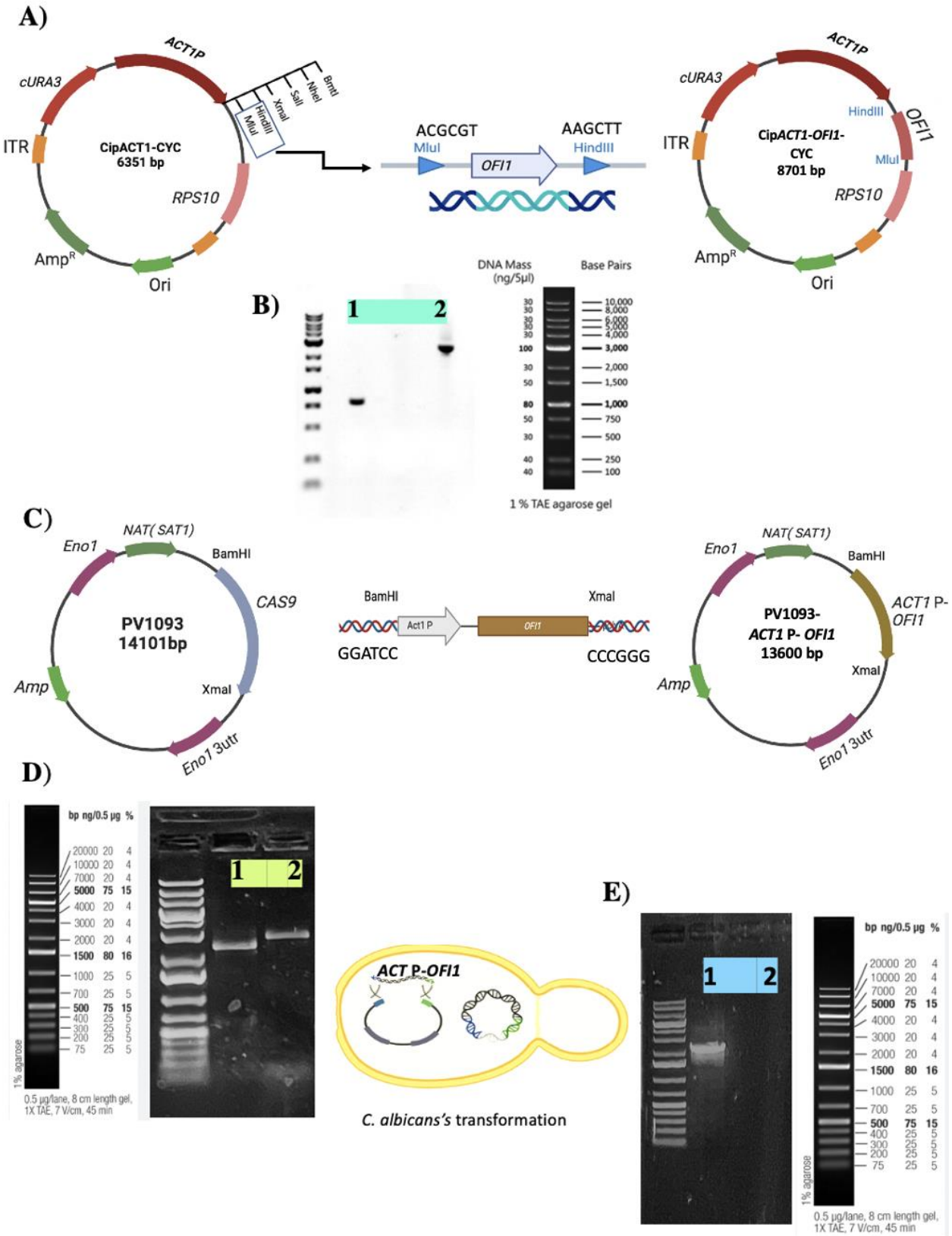

A)
ACT1P
cURA3
ITR
CipACT1-CYC
6351 bp
RPS10
AmpR
Ori
BmtI
NheI
SalI
XmaI
HindIII
MluI
ACGCGT
MluI
OFI1
AAGCTT
HindIII
CipACT1-OFI1-CYC
8701 bp
B)
1
2
DNA Mass (ng/5µl)
Base Pairs
1 % TAE agarose gel
C)
Eno1
NAT(SAT1)
BamHI
CAS9
PV1093
14101bp
Amp
XmaI
Eno1 3utr
Act1 P
GGATCC
CCCGGG
PV1093-
ACT1 P- OFI1
13600 bp
ACT1 P-OFI1
D)
bp ng/0.5 µg %
1% agarose
0.5 µg/lane, 8 cm length gel, 1X TAE, 7 V/cm, 45 min
C. albicans's transformation
E)

Figure 5: Construction and confirmation of the *OFI1* expression plasmid and its integration into *C. albicans*. A) Schematic representation of the cip*ACT1*-CYC plasmid carrying the *ACT1* promoter, an ampicillin resistance gene for selection on LB+Amp media, and a multiple cloning site with *MluI* and *HindIII* restriction sites. *OFI1* was amplified with these restriction sites, digested, and ligated into the plasmid. B) Agarose gel confirmation of *OFI1* integration into the plasmid. Band 1: PCR product using internal *ACT1* forward and internal *OFI1* reverse primers (800 bp). Band 2: PCR product using external *ACT1* forward and external *OFI1* reverse primers (2300 bp). C) Replacement of *ACT1* P-*OFI1* with Cas9 in the PV1093 plasmid, incorporating the Nat marker for *C. albicans* selection via digestion and ligation. D) Gel confirmation of successful plasmid modification. Band 1: PCR product using external *ACT1* forward and external *OFI1* reverse primers (2200 bp). Band 2: PCR product confirming Cas9 insertion (3000 bp). E) Agarose gel confirmation of pV1093 transformation into the *C. albicans* genome. Band 1: PCR product using external *ACT1* forward and external *OFI1* reverse primers. Band 2: Untransformed control strain. All PCR products were sequence-verified.

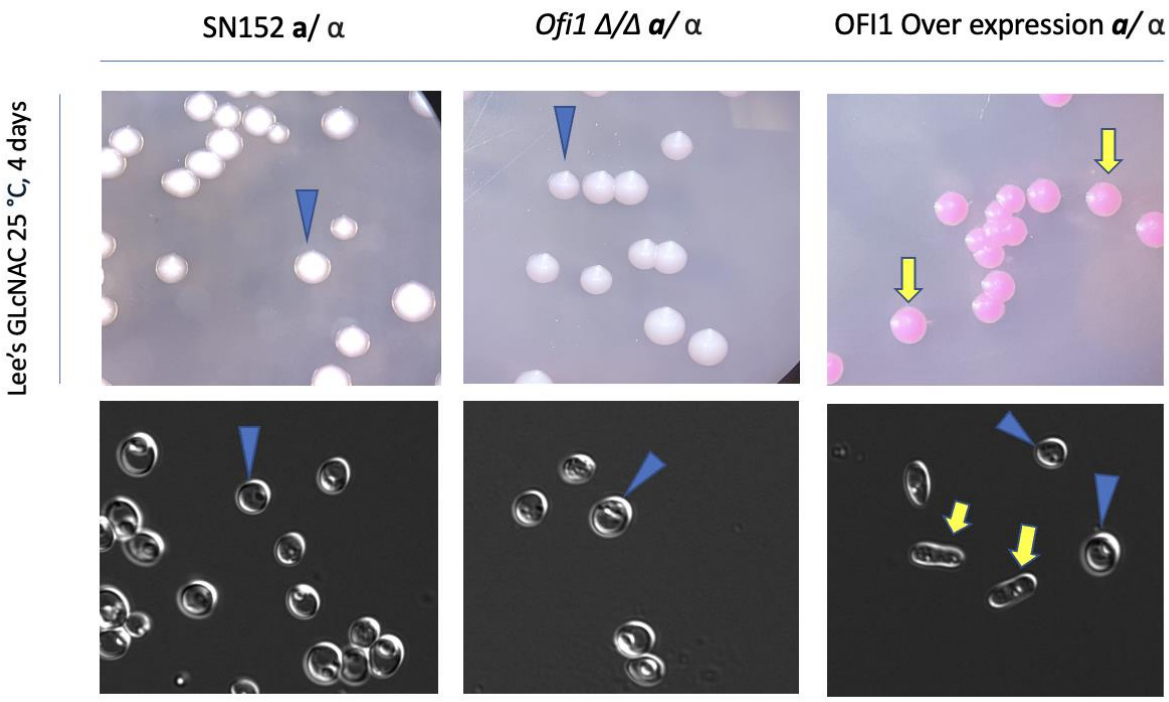


Figure 6: Colonies of *C. albicans* strains *MTL* **a**/α (wild-type SN152, *ofi1*Δ/Δ, and *OFI1* overexpression) grown on Lee's GlcNAC medium at 25°C for 4 days in the presence of Phloxine B. SN152 and *ofi1*Δ/Δ with *MTL* **a**/α colonies remained white, while *OFI1* overexpression resulted in pink colonies, suggesting opaque switching. Cellular morphology was assessed after washing with 1x PBS, and microscopic examination revealed a mixture of white and opaque cells in the *OFI1* overexpression colonies, despite the colony's overall opaque appearance. Blue arrows represent white colonies and cells; yellow arrows represent opaque colonies and cells.

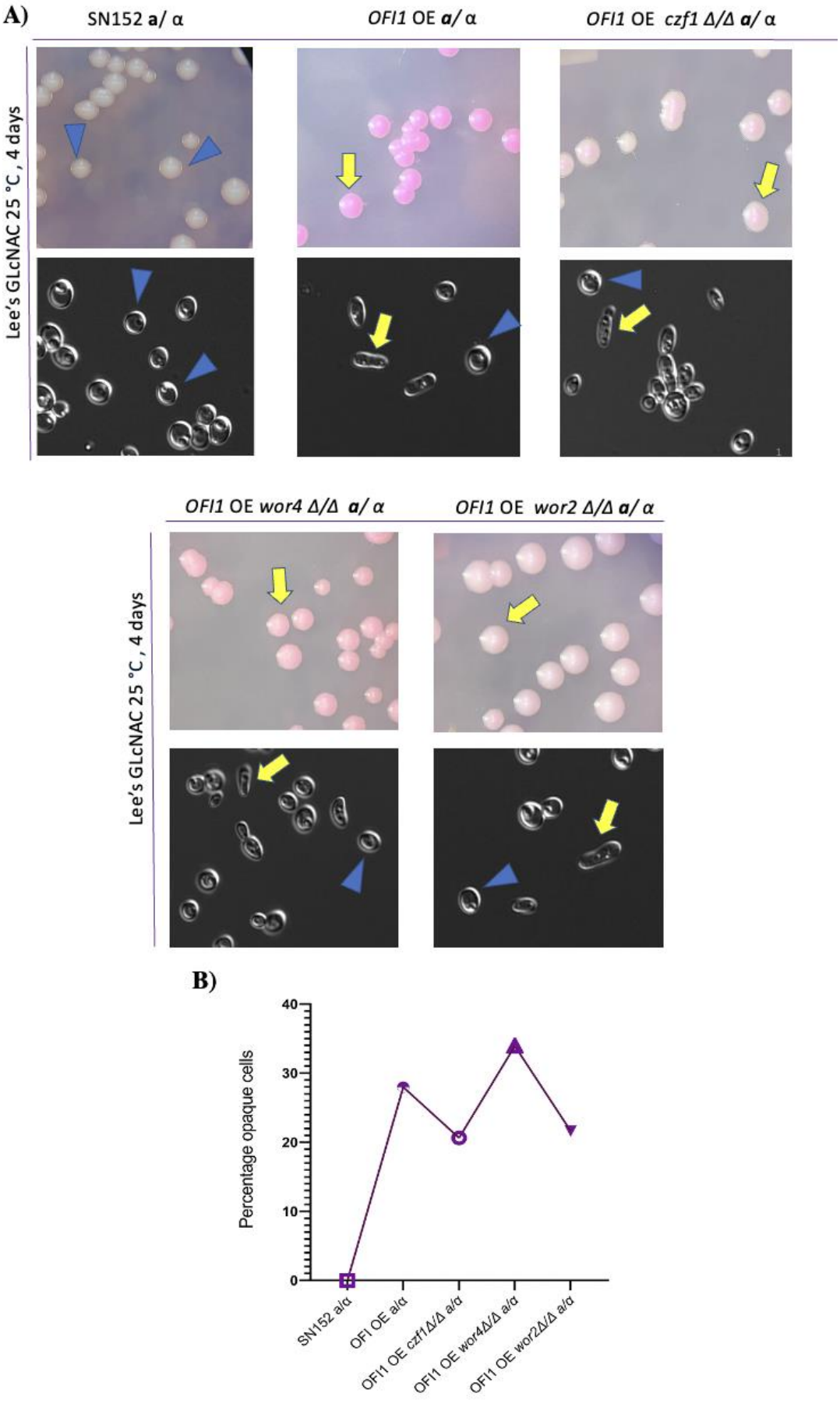

A)
SN152 a/ α
OFI1 OE a/ α
OFI1 OE czf1 Δ/Δ a/ α
Lee's GLcNAC 25 °C , 4 days
OFI1 OE wor4 Δ/Δ a/ α
OFI1 OE wor2 Δ/Δ a/ α
Lee's GLcNAC 25 °C , 4 days
B)
Percentage opaque cells
40
30
20
10
0
SN152 a/α
OFI OE a/α
OFI1 OE czf1Δ/Δ a/α
OFI1 OE wor4Δ/Δ a/α
OFI1 OE wor2Δ/Δ a/α

Figure 7: Effect of *OFI1* overexpression in *MTL* heterozygous **a**/α strains with knockouts of white-opaque switching regulators (*WOR2*, *WOR4*, and *CZF1*). A) SN152 wild type and strains with *OFI1* overexpression, and *OFI1* overexpression in *wor2*Δ/Δ, *wor4*Δ/Δ, and *czf1*Δ/Δ mutants were cultured at 25°C with GlcNAc for 4 days. SN152 **a**/α colonies remained white, while *OFI1* overexpression **a**/α and the single mutations resulted in frequent pink colonies. Microscopy images show a mixture of white and opaque cells in the pink colonies, with cells washed with 1x PBS before imaging. Blue arrows represent white colonies and cells; yellow arrows represent opaque colonies and cells. B) Graph showing the opaque frequency of all strains, indicating an increase in white-to-opaque switching compared to the wild type, though none exceeded 35%.

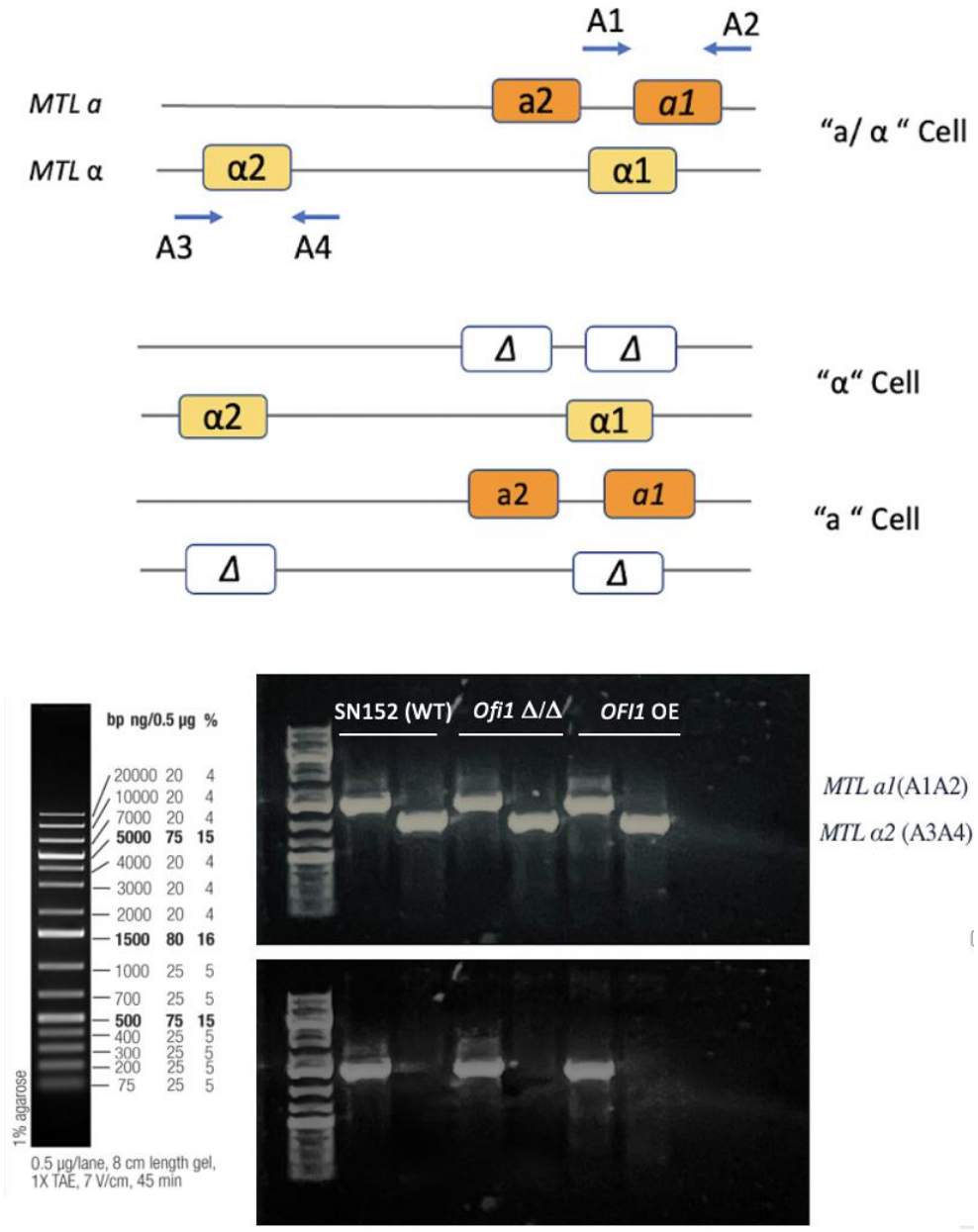
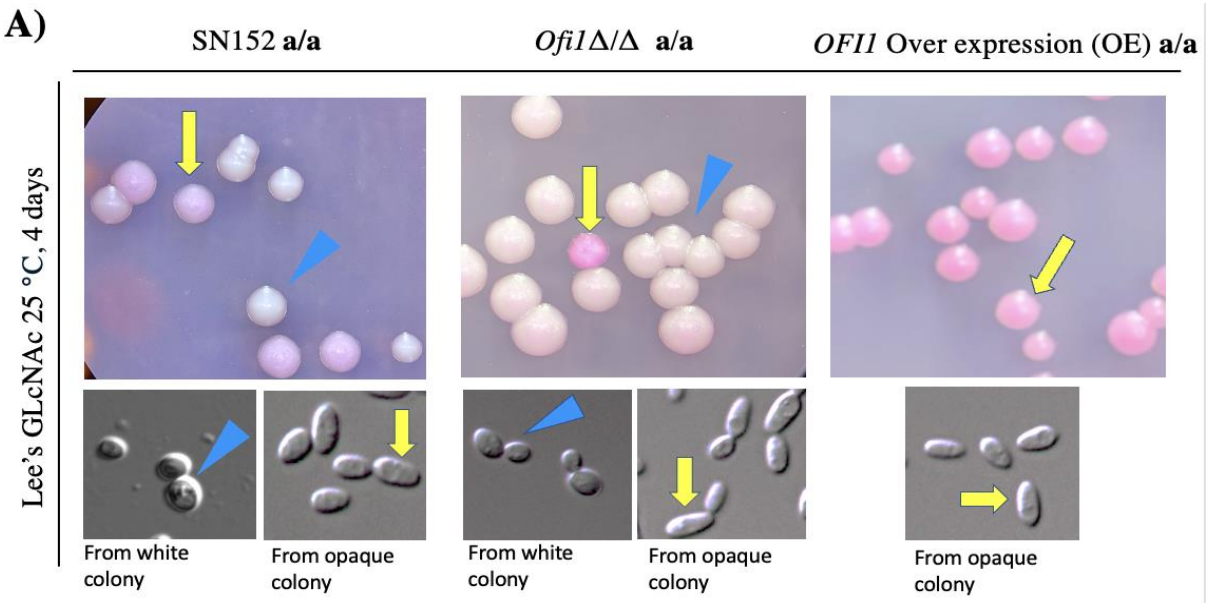


Figure 8: A) Structure of the *MTL* locus showing *MTL* **a** and *MTL* α and the primers designed to detect these two genes. B) Colony PCR to detect the *MTL* **a**1 and *MTL* α2 genes; Band 1) defines *MTL* **a** amplified with A1 and A2 primers and the size of PCR product is 1420 bp; Band 2) defines *MTL* α and amplified with A3 and A4 primers, the size of PCR product is 868 bp. The ladder used in this experiment was 1kb plus.



White to Opaque Switching frequency (%)

SN152 **a/a**	47.7±0.6 (n=652)
Ofi1 Δ/Δ **a/a**	18± 6 (n = 605)
OFI1 Overexpression (OE) **a/a**	100.0±0.0 (n=500)

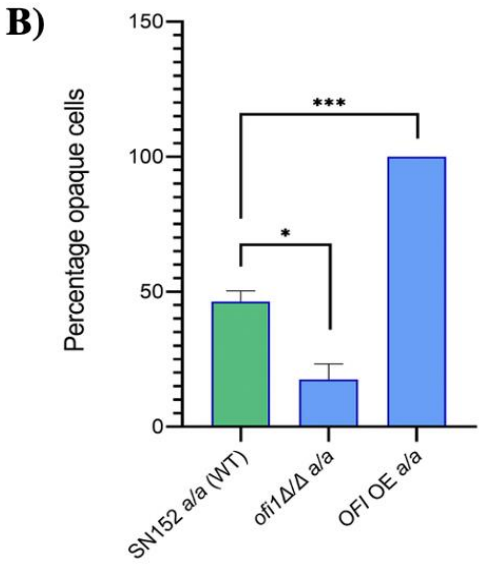
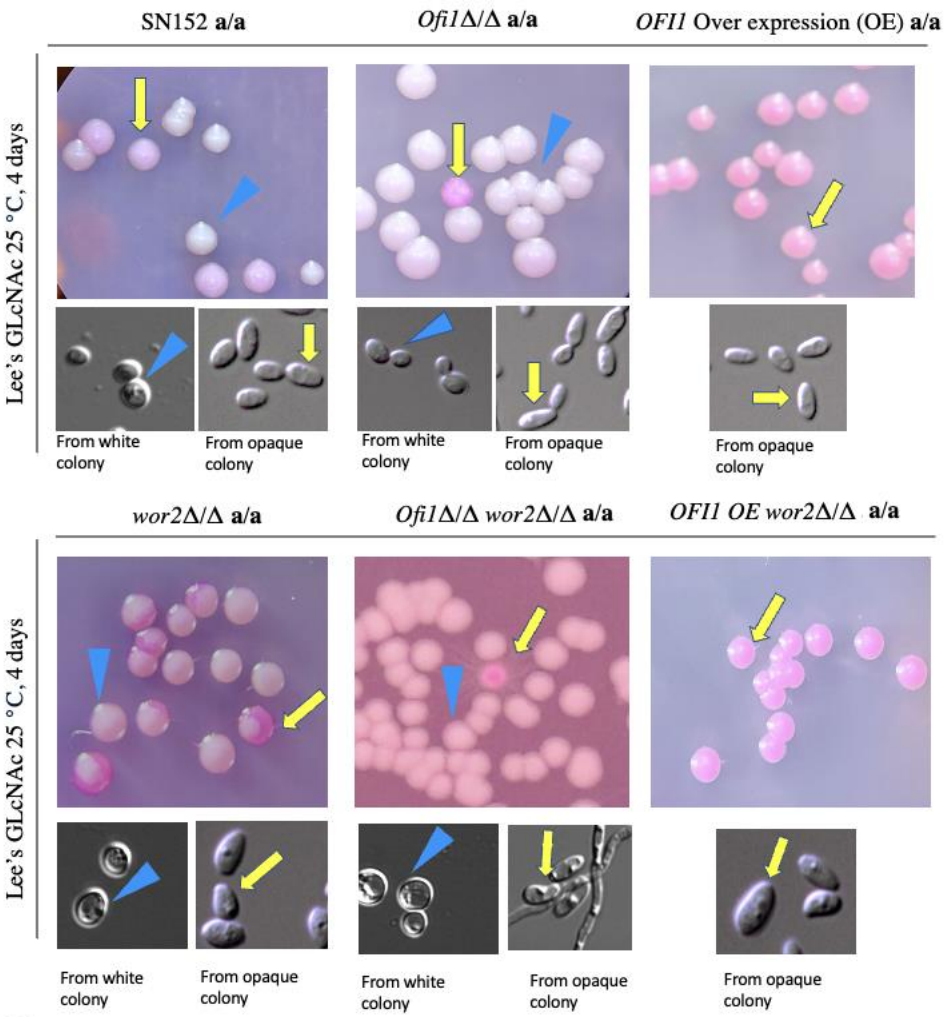


Figure 9: Impact of *ofi1*Δ/Δ **a/a** and *OFI1* overexpression **a/a** on white-to-opaque switching. A) Strains were plated on Lee's GlcNAc + phloxine B medium and incubated at 25°C for 4 days illustrating phenotypic differences in colony morphology. B) The bar graph shows the number of white versus opaque colonies for each strain. The *ofi1*Δ/Δ **a/a** mutant exhibited a significantly reduced white-to-opaque switching frequency compared to the control strain (SN152 **a/a**), which displayed a 47.7% switching frequency. In contrast, *OFI1* overexpression (*OFI1* overexpression **a/a**) resulted in a heightened switching frequency of 100%. n = total colonies counted; blue arrows represent white colonies and cells; yellow arrows represent opaque colonies and cells.

A)



B)

White to Opaque Switching frequency (%)

SN152 a/a	47.7± 0.6 (n=652)
*ofi1*Δ/Δ a/a	18± 6 (n = 605)
*wor2*Δ/Δ a/a	15.09 ± 4.0 (n= 583)
*ofi1 wor2*Δ/Δ a/a	2.90± 0.6 (n=649)
OFI1 OE a/a	100.0±0.0 (n=500)
OFI1 OE *wor2*Δ/Δ a/a	100.0±0.00 (n= 510)

C)

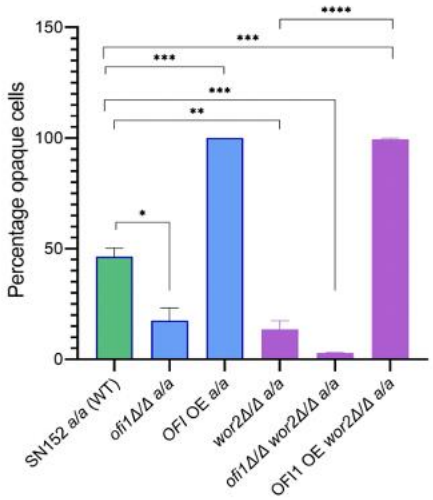
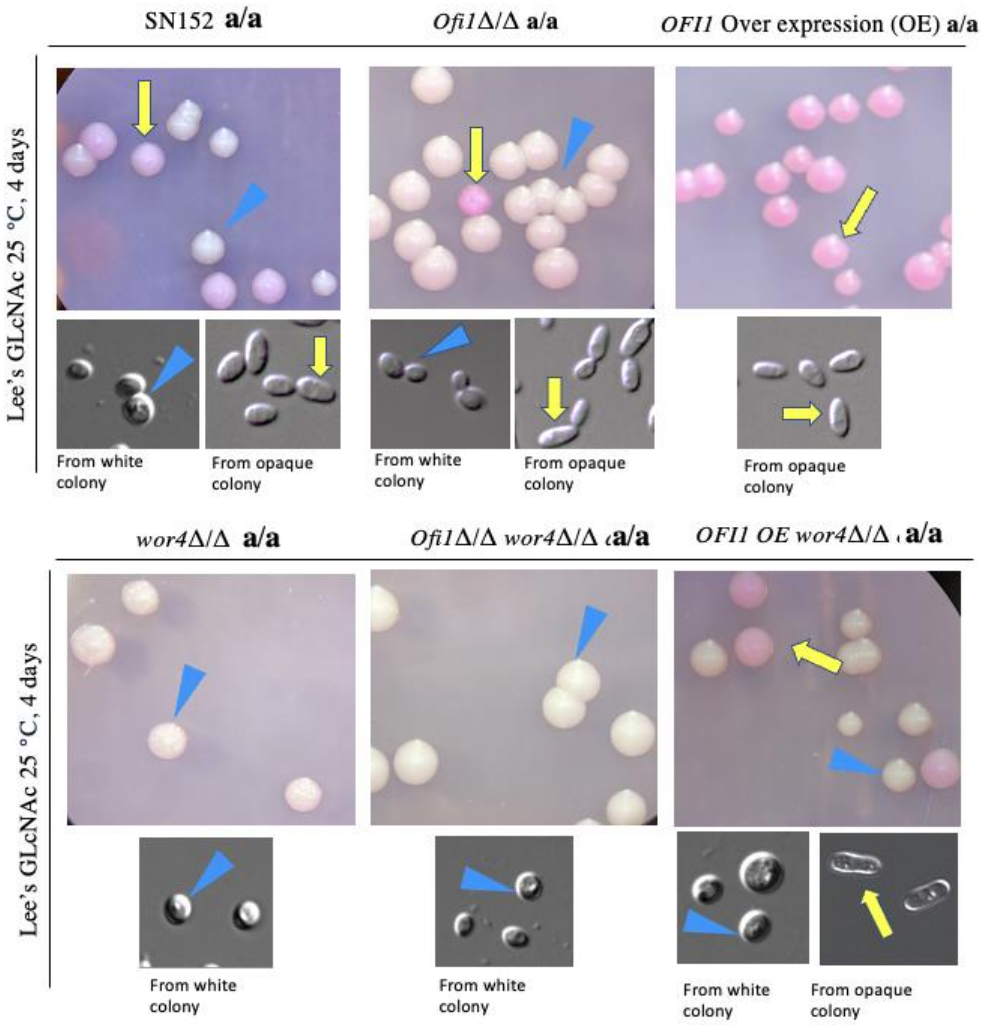


Figure 10: A) Colony morphologies of SN152 **a/a**: wild type, *ofi1*Δ/Δ **a/a**, *wor2*Δ/Δ **a/a**, *ofi1*Δ/Δ *wor2*Δ/Δ **a/a**, *OFI1* overexpression **a/a**, and *OFI1* overexpression *wor2*Δ/Δ **a/a** strains after 4 days on Lee's GlcNAc medium at 25°C, showing white and opaque colonies. B) The rate of white-to-opaque switching (W/OP) and the total number of colonies for each strain. C) Comparison of W/OP switching frequencies among *ofi1*Δ/Δ **a/a**, *wor2Δ/Δ* **a/a**, *ofi1*Δ/Δ *wor2*Δ/Δ **a/a**, *OFI1* overexpression **a/a**, and *OFI1* overexpression *wor2*Δ/Δ **a/a** strains relative to the SN152 **a/a** control strain. The data points represent mean values from at least three independent experiments. Significant reductions in W/OP switching frequencies in the mutants compared to SN152 **a/a** are denoted by asterisks (****P < 0.0005), highlighting the impact of gene deletions on this phenotypic transition. Statistical significance was determined using Fisher's exact test. Blue arrows represent white colonies and cells; yellow arrows represent opaque colonies and cells.

A)



B)

White to Opaque Switching frequency (%)

SN152 **a/a**	47.7±0.6 (n=652)
*ofi1*Δ/Δ **a/a**	18± 6 (n = 605)
*wor4*Δ/Δ **a/a**	0.00 ± 0.0 (n= 1750)
*ofi1 wor4*Δ/Δ **a/a**	0.00± 0.0 (n=1310)
OFI1 OE **a/a**	100.0±0.0 (n=500)
OFI1 OE *wor4* Δ/Δ **a/a**	58.47±3.03 (n= 785)

C)

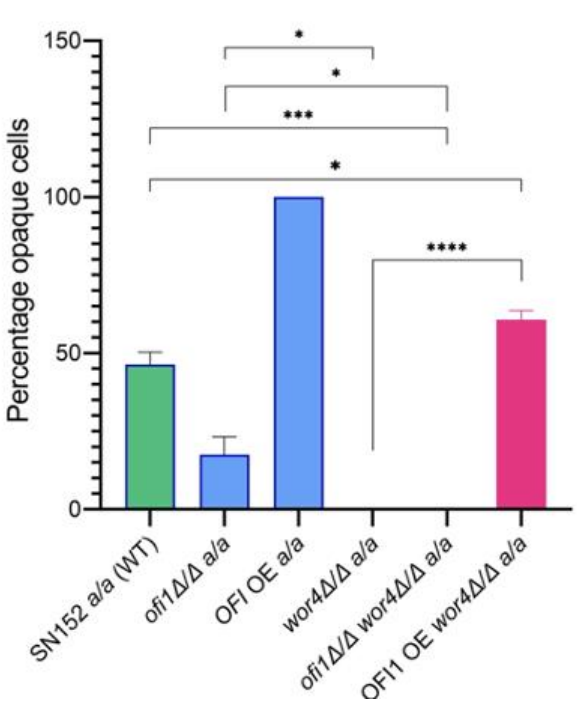
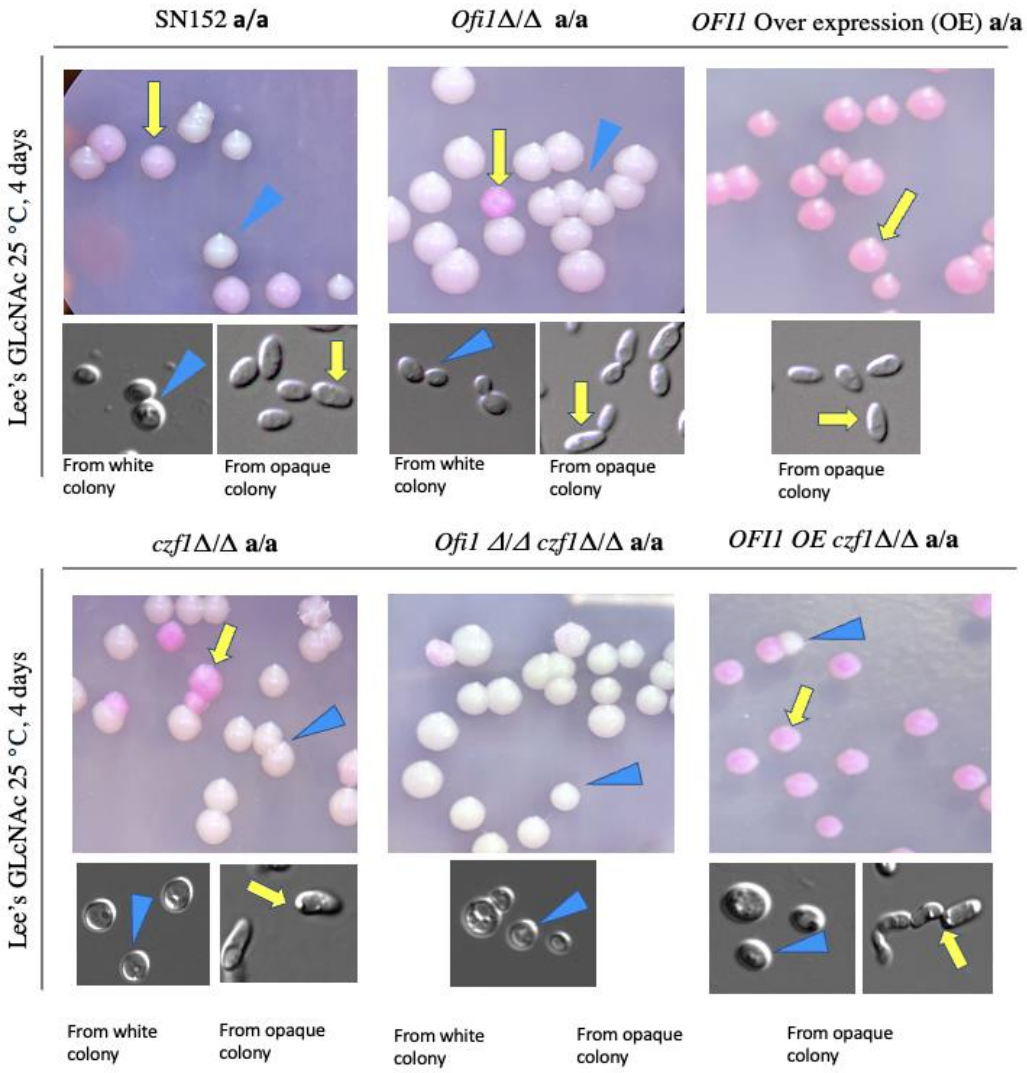


Figure 11: A) Colony morphologies of SN152 **a/a**: wild type, *ofi1*Δ/Δ **a/a**, *wor4*Δ/Δ **a/a**, *ofi1*Δ/Δ *wor4*Δ/Δ **a/a**, *OFI1* overexpression **a/a**, and *OFI1* overexpression *wor4*Δ/Δ **a/a** strains on Lee's GlcNAc medium at 25°C for 4 days, showing white and opaque colonies. B) White-to-opaque switching (W/OP) rates and total colony counts for each strain. C) Comparison of W/OP switching frequencies among strains, with data points representing mean values from three independent experiments. Significant differences in switching frequencies are marked by asterisks (****P < 0.0005), with statistical significance assessed using Fisher's exact test. Blue arrows represent white colonies and cells; yellow arrows represent opaque colonies and cells.

A)



B)

White to Opaque Switching frequency (%)

SN152 **a/a**	47.7± 0.6 (n=652)
*ofi1*Δ/Δ **a/a**	18± 6 (n = 605)
*czf1*Δ/Δ **a/a**	10.54 ± 0.68 (n= 800)
*ofi1 czf1*Δ/Δ **a/a**	1.16± 2.8 (n=762)
OFI1 OE **a/a**	100.0± 0.0 (n=500)
OFI1 OE *czf1*Δ/Δ **a/a**	97.89± 0.9 (n= 750)

C)

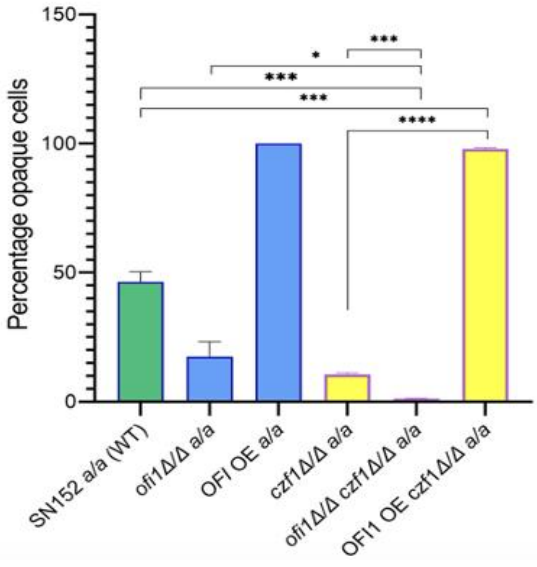


Figure 12: A) Colony morphologies of various *C. albicans* strains, including SN152 **a/a**: wild type, *ofi1*Δ/Δ **a/a**, *czf1*Δ/Δ **a/a**, *ofi1*Δ/Δ *czf1*Δ/Δ **a/a**, *OFI1* overexpression **a/a**, and *OFI1* overexpression *czf1*Δ/Δ **a/a**, cultured on Lee's GlcNAc medium at 25°C for 3 days. The distinct phenotypic variations observed highlight the differential capacity for white-to-opaque switching among the strains, with notable differences in colony morphology indicative of the opaque phenotype. B) Quantitative assessment of white-to-opaque switching (W/OP) rates and total colony counts for each strain, providing a comprehensive overview of the switching dynamics. C) Comparative analysis of W/OP switching frequencies among the strains, with data points representing mean values derived from three independent experiments. Significant differences in switching frequencies are denoted by asterisks (****$P < 0.0005$), with statistical significance determined using Fisher's exact test, underscoring the critical roles of *OFI1* and *CZF1* in regulating phenotypic transitions in *C. albicans*. Blue arrows represent white colonies and cells; yellow arrows represent opaque colonies and cells.

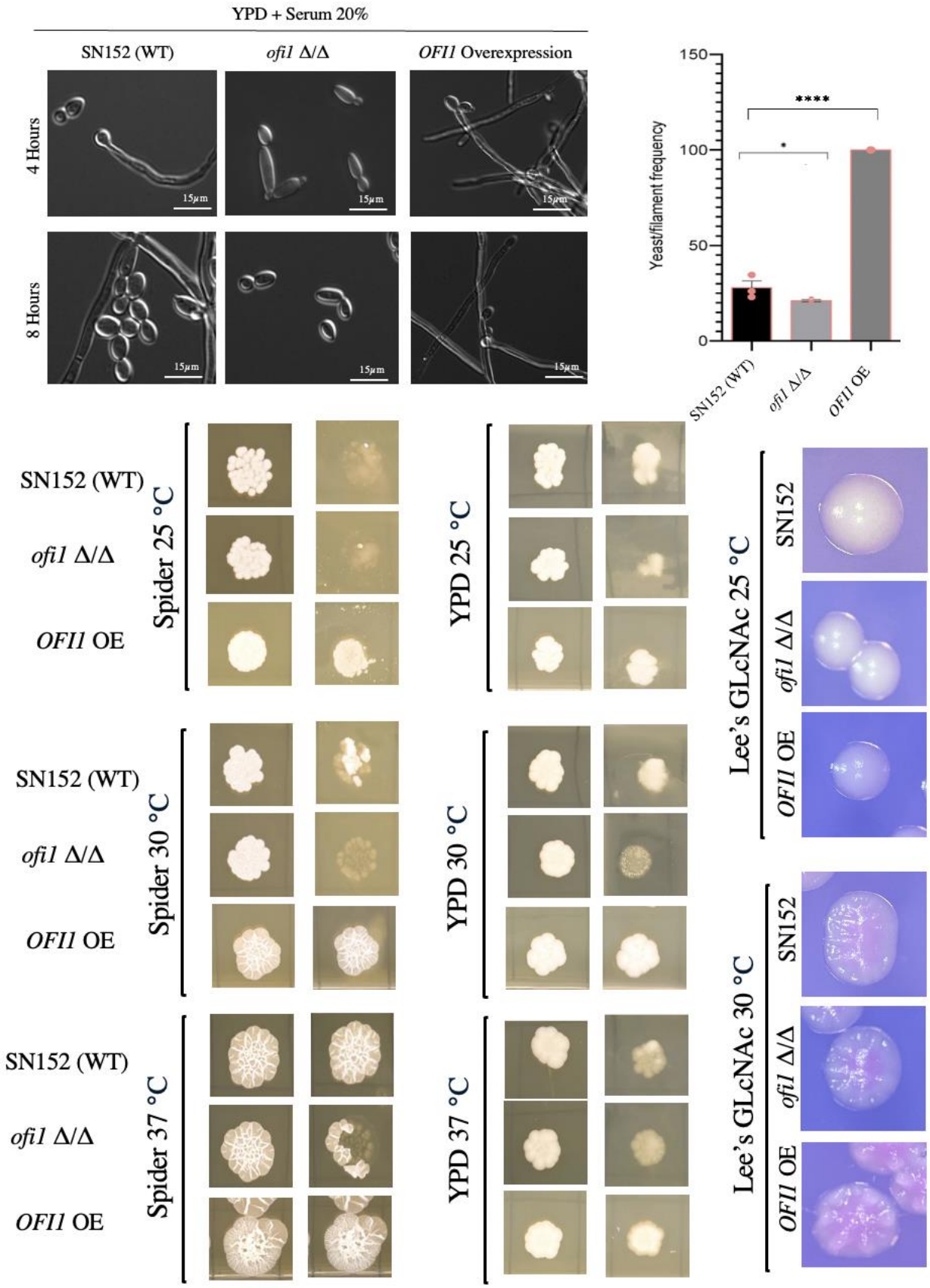

YPD + Serum 20%
SN152 (WT)
ofi1 Δ/Δ
OFI1 Overexpression
4 Hours
8 Hours
15μm
Yeast/filament frequency
150
100
50
0
****
*
SN152 (WT)
ofi1 Δ/Δ
OFI1 OE
Spider 25 °C
YPD 25 °C
Spider 30 °C
YPD 30 °C
Spider 37 °C
YPD 37 °C
Lee's GLcNAc 25 °C
Lee's GLcNAc 30 °C
SN152

Figure 13: The role of Ofi1 in filamentation under different inducing conditions. A) Microscopy images showing filamentation in *C. albicans* SN152 (control), *ofi1*Δ/Δ, and *OFI1* overexpression strains after 4 and 8 hours in filament-inducing media. B) Quantification of yeast and filamentous cells, demonstrating that *OFI1* overexpression significantly increases filamentation frequency, while *ofi1* deletion reduces it. C) Invasive growth assay on Spider and YPD solid media at 25°C, 30°C, and 37°C using 10^4 cells per spot. After incubation, colonies were washed with a stream of water for 15 seconds to assess adhesion and invasiveness, showing the impact of *OFI1* overexpression on invasive growth. BW: Before wash, AW: After. D) Colony morphology of the three strains on GlcNAc medium at 25°C and 30°C, highlighting the effect of *OFI1* overexpression on filamentous growth under this condition at 30°C. (****$P < 0.0005$)

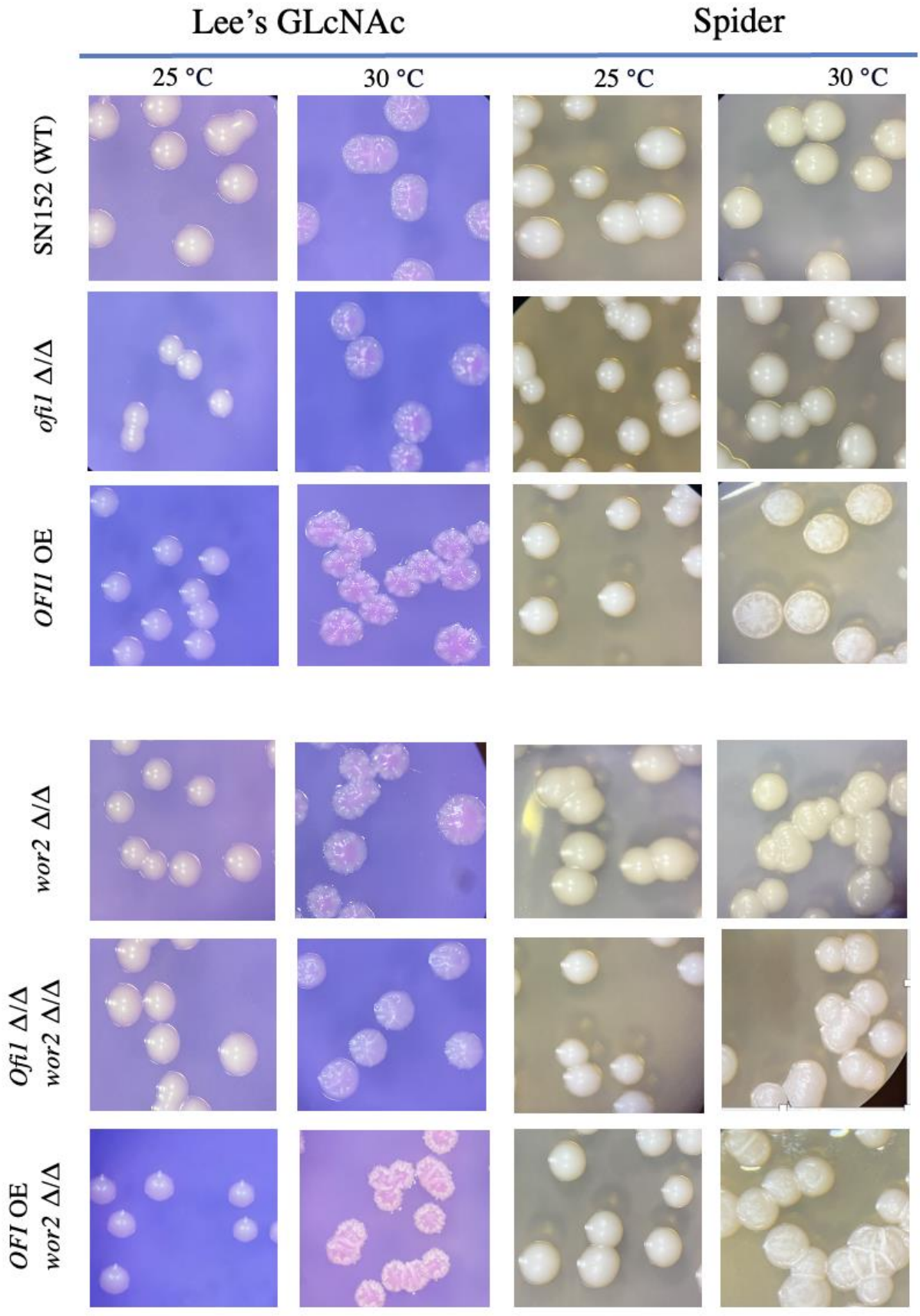

Lee's GLcNAc
Spider
25 °C
30 °C
25 °C
30 °C
SN152 (WT)
ofi1 Δ/Δ
OFI1 OE
wor2 Δ/Δ
Ofi1 Δ/Δ wor2 Δ/Δ
OFI OE wor2 Δ/Δ

Figure 14: The colony morphology of SN152: wild type, *ofi1*Δ/Δ, *OFI1* overexpression, and various double and single mutants (*ofi1*Δ/Δ *wor2*Δ/Δ, OFI1 overexpression with *wor2*Δ/Δ. Strains were spotted on Lee's GlcNAc and Spider solid medium and incubated for 4 days at 25°C and 30°C.

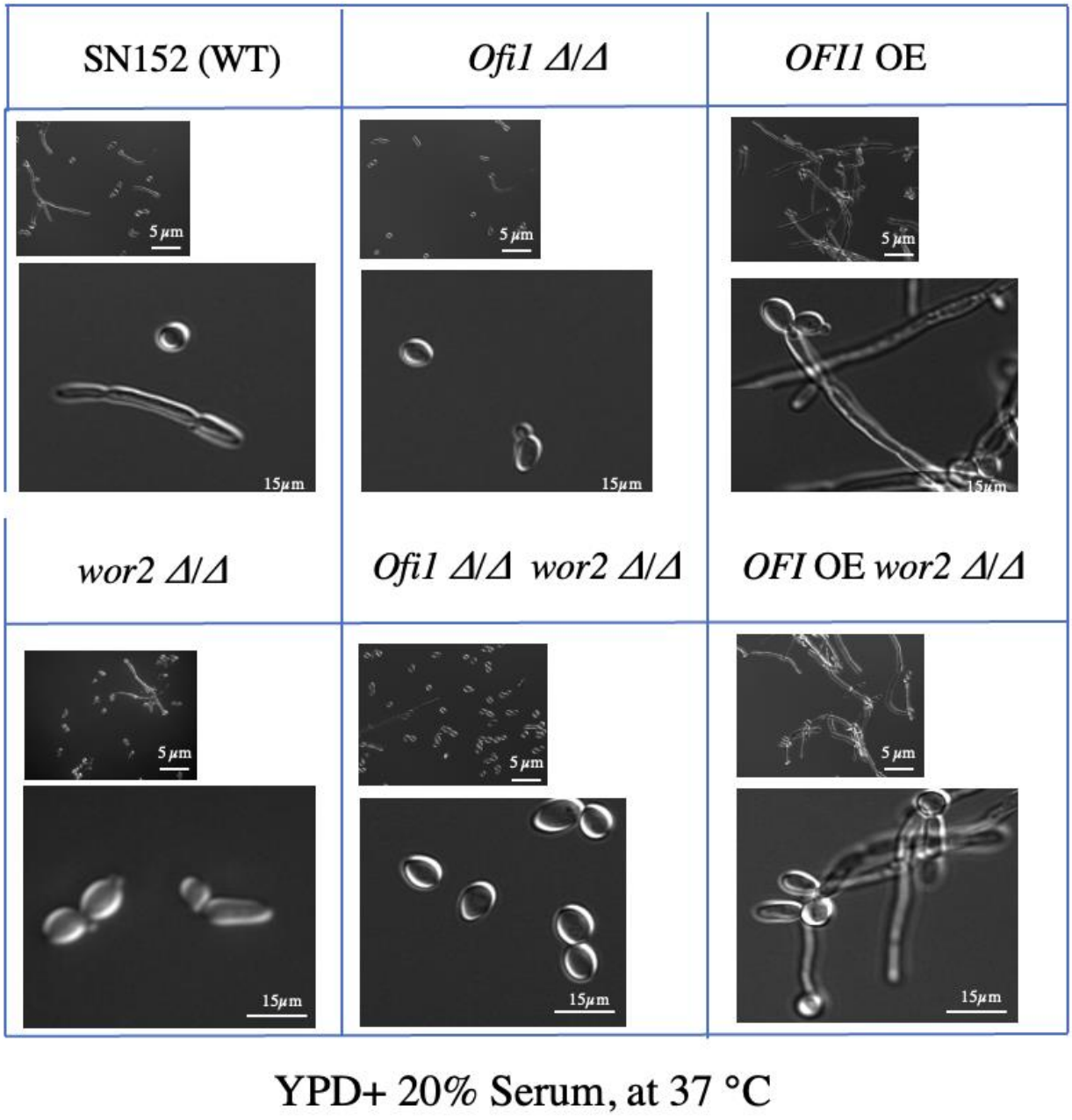


Figure 15: The cellular morphology of the tested strains (SN152: wild type, *ofi1*Δ/Δ, *OFI1* overexpression, *ofi1*Δ/Δ *wor2*Δ/Δ, *OFI1* overexpression with *wor2*Δ/Δ) was visualized using differential interference contrast (DIC) microscopy after growth in YPD + 20% serum at 37°C for 8 hours.

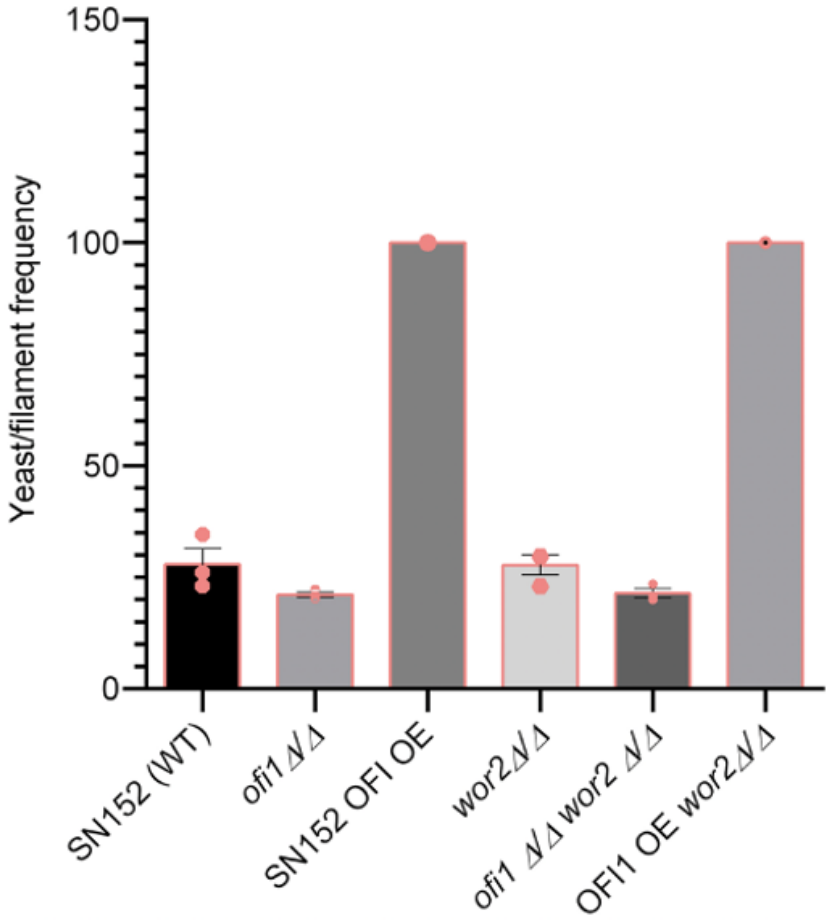


Figure 16: *wor2*Δ/Δ does not affect the yeast-to-filament transition. Overexpression of *OFI1* shown with or without *wor2* increases the filamentation up to 100% compared to 26% of SN152: wild type on liquid YPD+ 20% serum at 37°C.

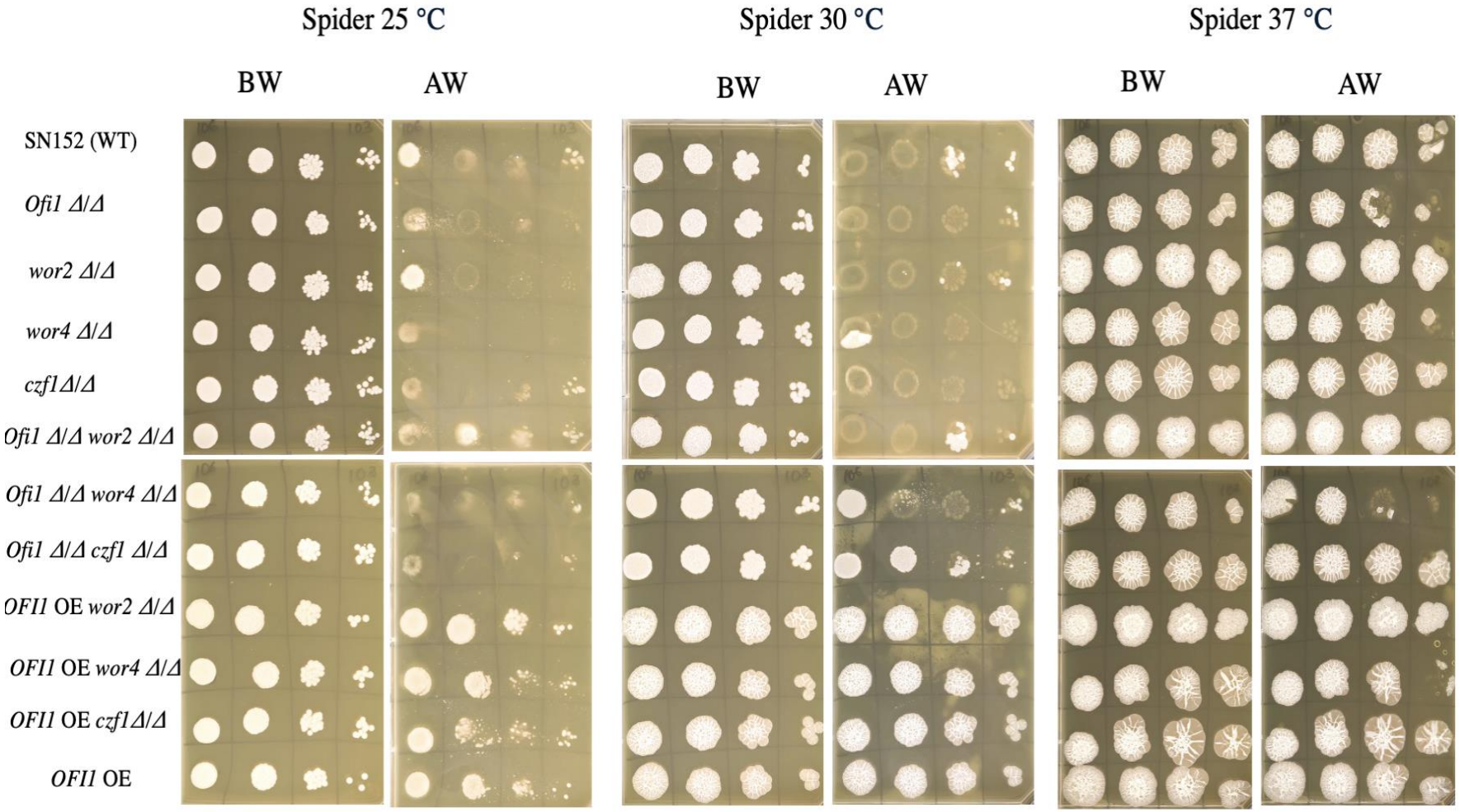

Spider 25 °C
Spider 30 °C
Spider 37 °C
BW
AW
BW
AW
BW
AW
SN152 (WT)
Ofi1 Δ/Δ
wor2 Δ/Δ
wor4 Δ/Δ
czf1Δ/Δ
Ofi1 Δ/Δ wor2 Δ/Δ
Ofi1 Δ/Δ wor4 Δ/Δ
Ofi1 Δ/Δ czf1 Δ/Δ
OFI1 OE wor2 Δ/Δ
OFI1 OE wor4 Δ/Δ
OFI1 OE czf1Δ/Δ
OFI1 OE

Figure 17: Invasive growth assay of *C. albicans* mutants on Spider media at different temperatures. Strains were spotted in 10-fold serial dilutions (10^7 to 10^3 cells) and incubated at 25°C, 30°C, and 37°C. Plates were washed after 15 seconds to assess adhesion and invasion. Most single deletion mutants were washed off at 25°C and 30°C, except for *wor2*Δ/Δ, which retained invasion at the highest concentration. At 37°C, *ofi1*Δ/Δ was the only strain that completely washed off, indicating its essential role in invasive growth.

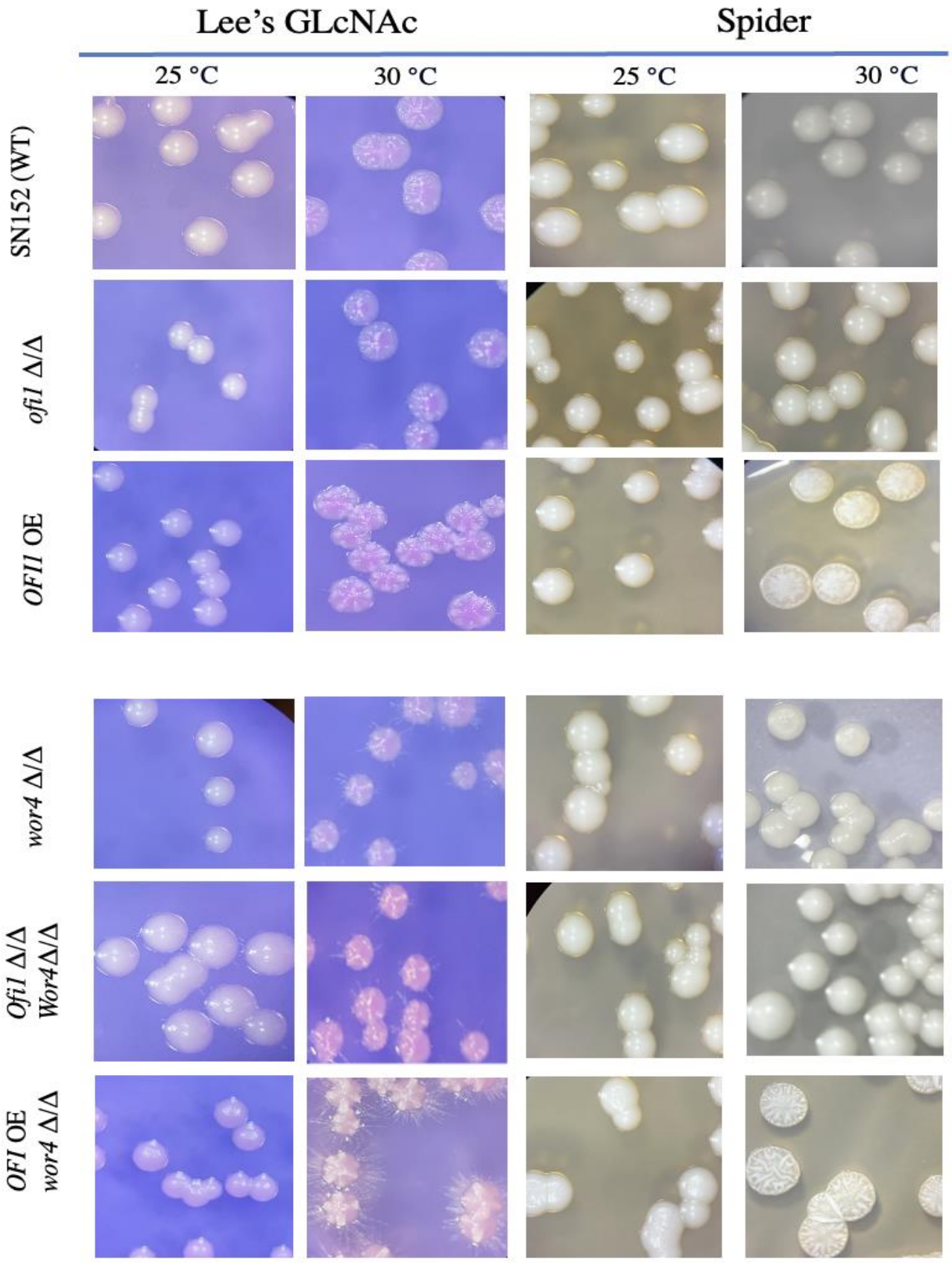

Lee's GLcNAc
Spider
25 °C
30 °C
25 °C
30 °C
SN152 (WT)
ofi1 Δ/Δ
OFI1 OE
wor4 Δ/Δ
Ofi1 Δ/Δ Wor4Δ/Δ
OFI OE wor4 Δ/Δ

Figure 18: Colony morphologies of SN152: wild type, *ofi1*Δ/Δ, *OFI1* overexpression, and various single and double mutants (*ofi1*Δ/Δ *wor4*Δ/Δ, *wor4*Δ/Δ) were observed on Lee's GlcNAc and Spider solid medium after 4 days of incubation at 25°C and 30°C.

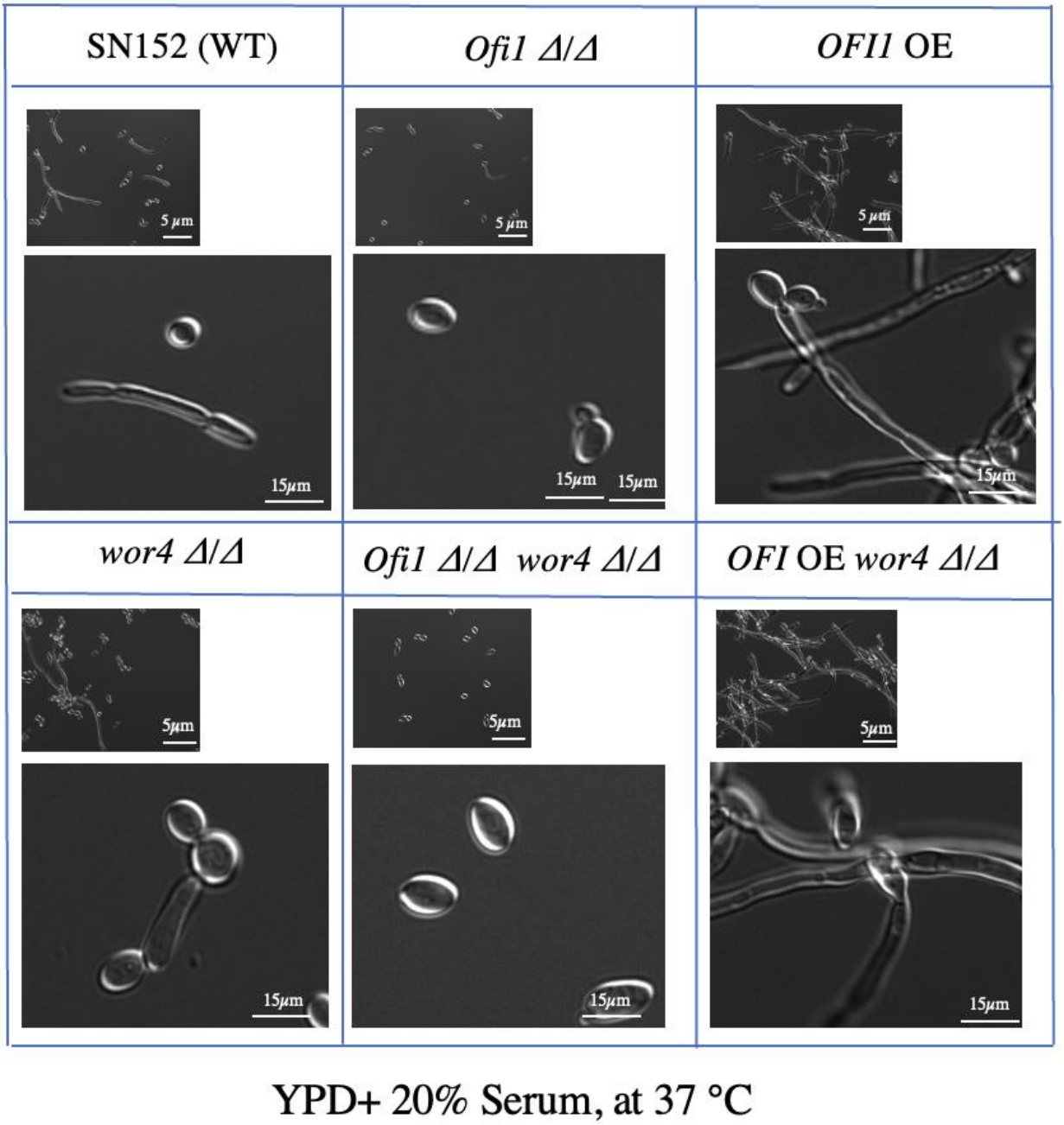


Figure 19: Cellular morphology of strains (SN152: wild type, *ofi1*Δ/Δ, *OFI1* overexpression, and *ofi1*Δ/Δ *wor4*Δ/Δ, *wor4*Δ/Δ), grown in YPD + 20% serum at 37°C for 8 hours was visualized using differential interference contrast (DIC) microscopy.

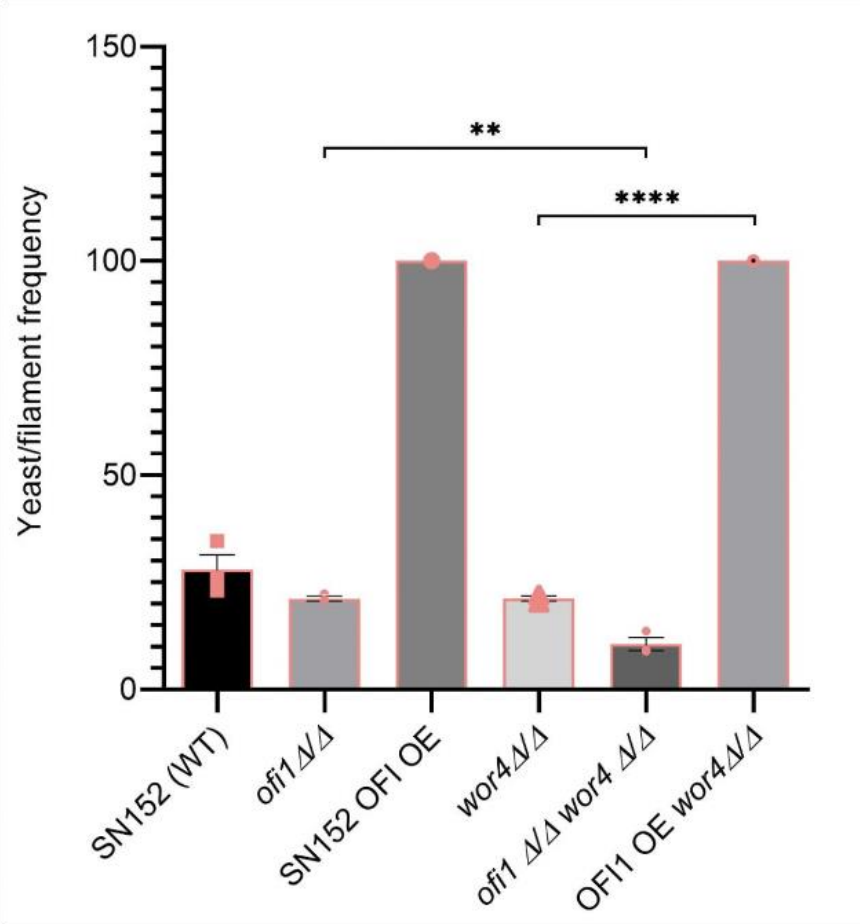


Figure 20: Counting the yeast and filamentous cells in the *wor4*Δ/Δ related constructs. *OFI1* overexpression with or without *WOR4* increases the filamentation significantly to 100%. Deletion of *wor4*Δ/Δ in *ofi1*Δ/Δ background reduces the filamentation. Statistical significance was determined using Fisher's exact test. (****P < 0.0005)

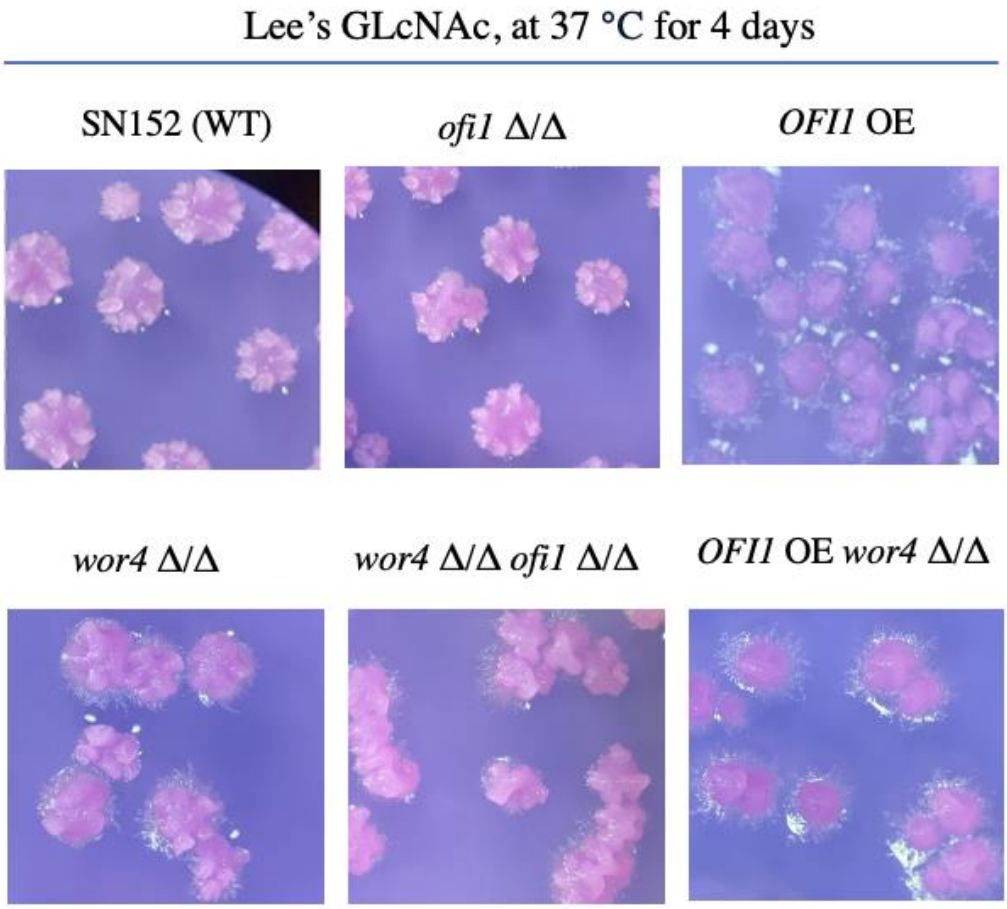


Figure 21: Colony morphology analysis of strains grown on Lee's GlcNAc medium at 37°C for 4 days. *wor4*Δ/Δ strains, with or without *OFI1* overexpression, formed fuzzy colonies even at 37°C, indicating that *wor4*Δ/Δ induces filamentation under these conditions, unlike wild type and *ofi1*Δ/Δ strains.

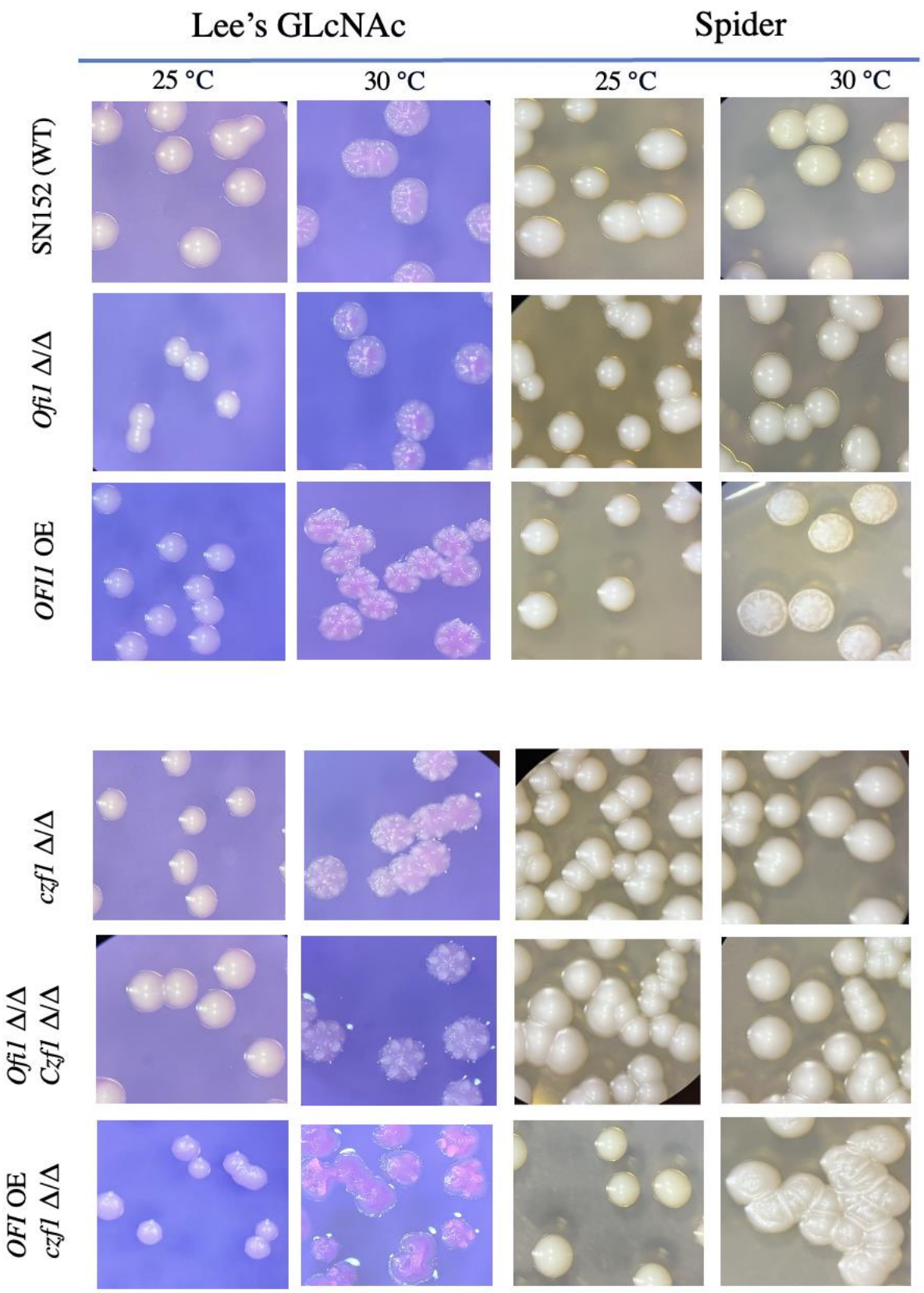

Lee's GLcNAc
Spider
25 °C
30 °C
25 °C
30 °C
SN152 (WT)
Ofi1 Δ/Δ
OFI1 OE
czf1 Δ/Δ
Ofi1 Δ/Δ Czf1 Δ/Δ
OFI OE czf1 Δ/Δ

Figure 22: Filamentation and invasive growth assays of *CZF1* and *OFI1* strains. Colony morphology of wild type: SN152, *czf1*Δ/Δ, *ofi1*Δ/Δ, *OFI1* overexpression with and without *CZF1*, and respective double mutants on Lee's GlcNAc and Spider solid medium at 25°C and 30°C after 4 days.

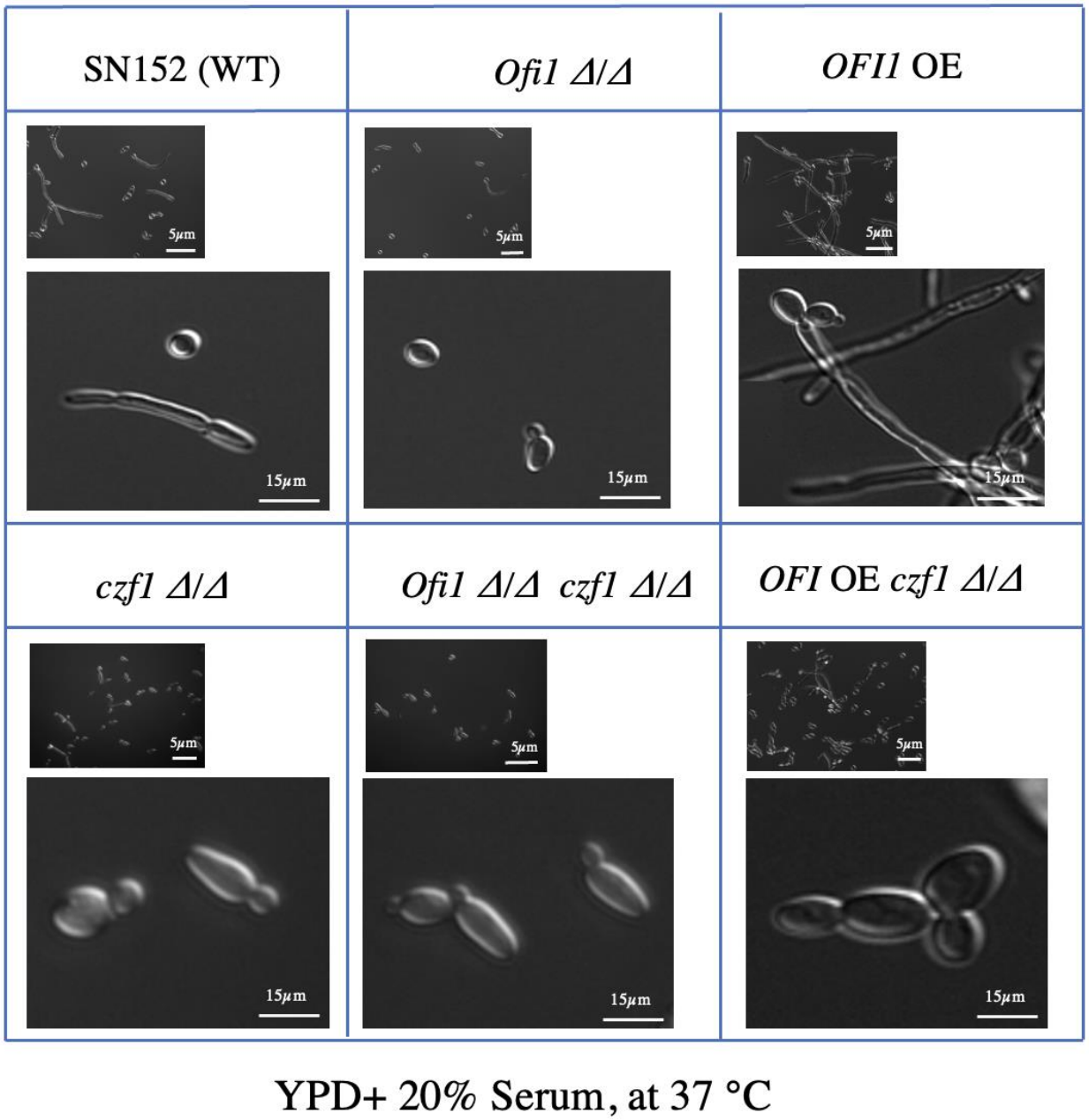


Figure 23: Cellular morphology of strains (SN152: wild type, *ofi1*Δ/Δ, *czf1*Δ/Δ, *ofi1*Δ/Δ *czf1*Δ/Δ, *OFI1* overexpression with or without *czf1*) in YPD + 20% serum at 37°C after 8 hours, observed by DIC microscopy.

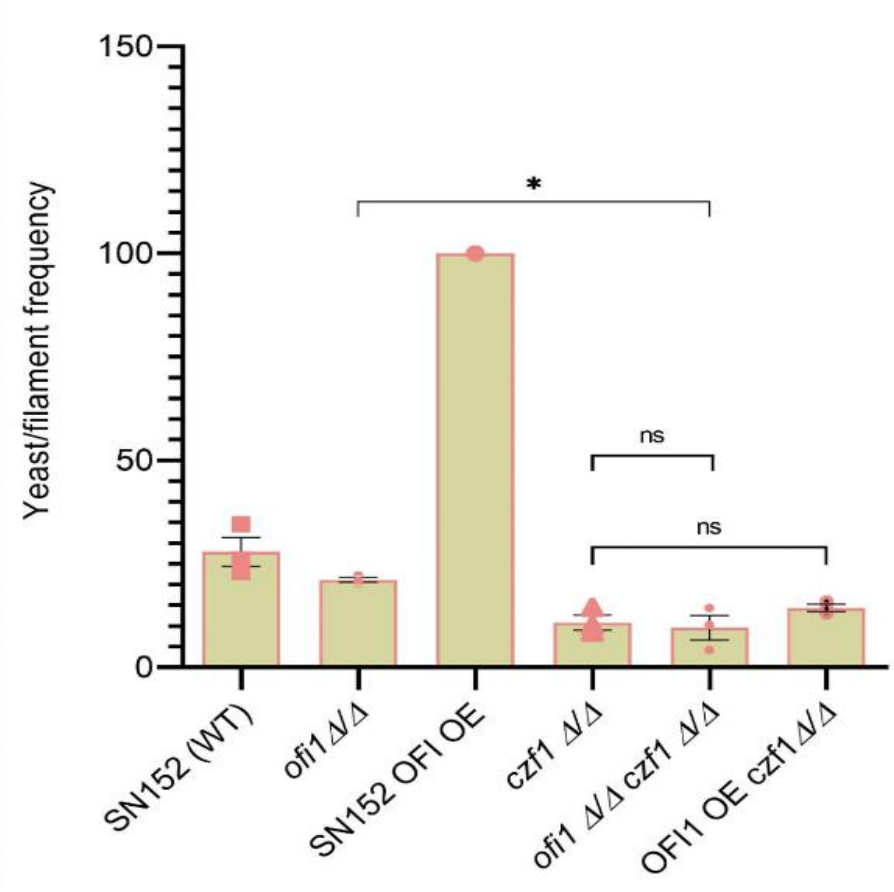


Figure 24: Overexpression of *OFI1* failed to promote filamentation in the absence of *czf1*. Deleting *ofi1* in the *czf1* mutant had no major difference compared to the *czf1* mutant alone. However, the double deletion showed much less filamentation compared to the *ofi1*Δ/Δ strain.

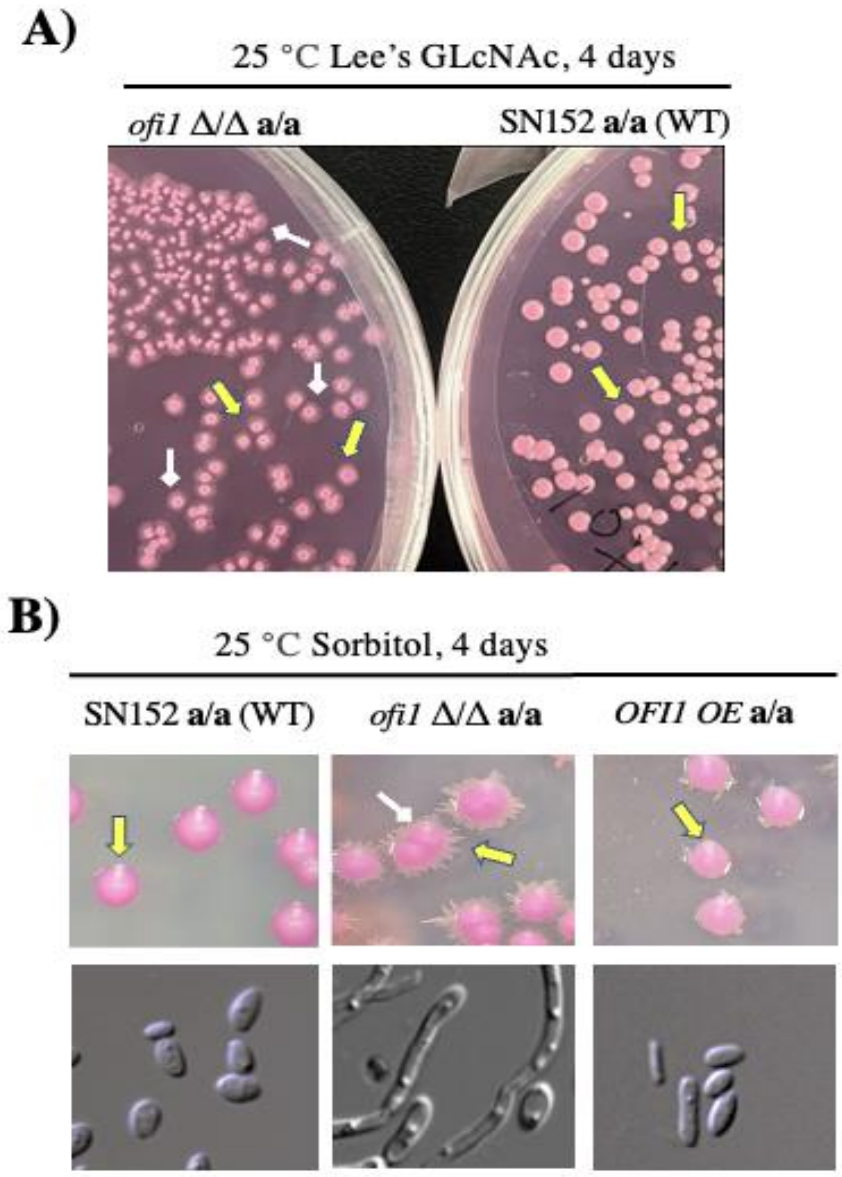


Figure 25: The possible role of *OFI1* in the opaque-to-filament transition. A) Colony morphology of *ofi1*Δ/Δ and SN152 control opaque colonies on Lee's GlcNAc plates after 4 days at 25°C, showing differences in filamentation potential. B) Filamentation assay of opaque cells from SN152 (wild-type control), *ofi1*Δ/Δ, and *OFI1* overexpression strains on sorbitol medium at 25°C, the optimal condition for inducing hyphal growth from opaque cells. Microscopy images show cellular morphology under these conditions.

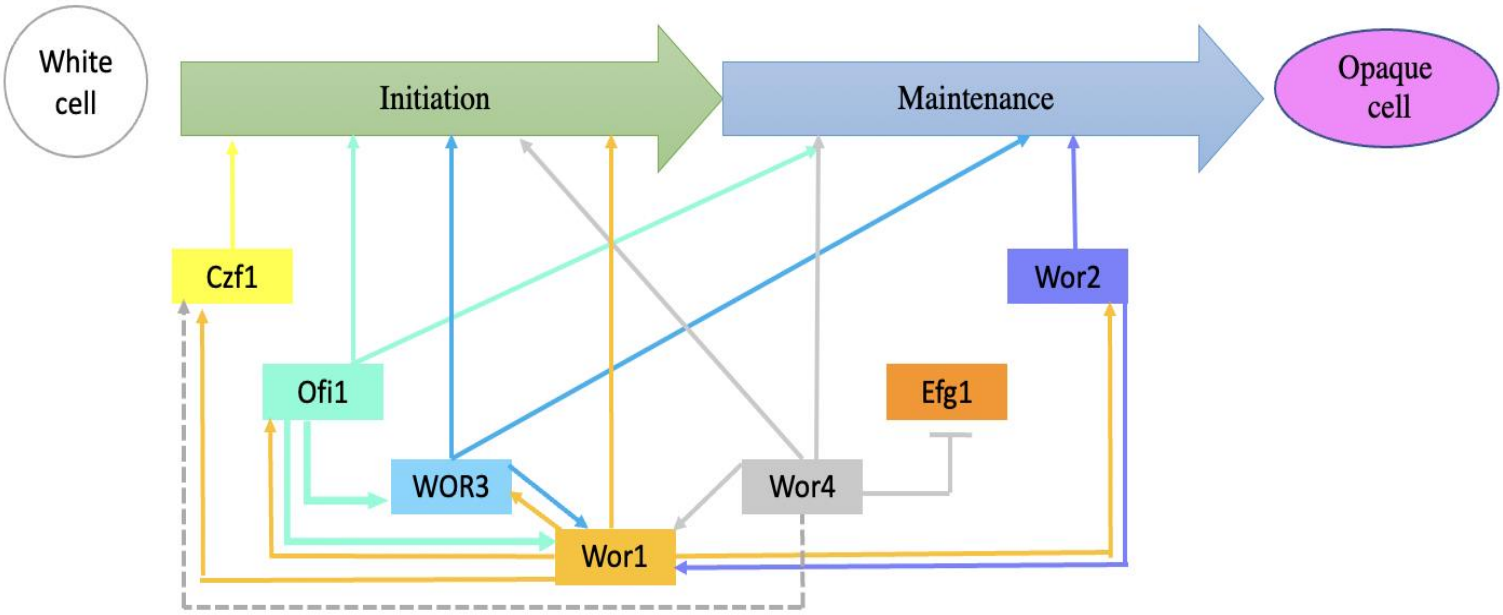


Figure 26*:* Schematic representation of the white-to-opaque regulatory pathway in *C. albicans*. The pathway involves two main steps: (1) Initiation — the transition from white-to-opaque cells, and (2) Maintenance — stabilizing cells in the opaque phase. Key TFs regulating this process are shown, along with the proposed position of Ofi1 within the regulatory network, highlighting its potential role in both initiating and maintaining the opaque state.

Table S1: Primers used in this study

Name	Sequence	Source
OFI1-sg-up	atttgGCAGTGAAAAGTCCTATTGGg	This study
OFI1-sg-down	aaaacCCAATAGGACTTTTCACTGCc	This study
WOR2-sg-up	atttgCCACAACAACAACAACTCCAg	This study
WOR2-sg-down	aaaacTGGAGTTGTTGTTGTTGTGGc	This study
WOR4-sg-up	atttgACAAGAAATCCTAGATCCAAg	This study
WOR4-sg-down	aaaacTTGGATCTAGGATTTCTTGTc	This study
CZF1-sg-up	atttgACAATCTGTAGGTTACCTAGg	This study
CZF1-sg-down	aaaacCTAGGTAACCTACAGATTGTc	This study
ARG4-Repair-F *OFI1*	TATATTGGAGATCCCCACTTACAAAAGAACACTCG AGTTTTTATATTACGTATTCAAATAAATCGTTCAAT TGAGAA/ TCCAATACCTCGCCAGAAC	This study
ARG4-Repair-R *OFI1*	GAAACTAAAGATGTTGGTTAAACCAGGAAAGGAC AATAGTTTGAGGATTGCCAATTTATTAGAACTTTTT AAAA/ CTCACTATAGGGAGACCG	This study
ARG4-Repair-F *WOR2*	GAAGCTAATTACAAACAATATAATTAATTTTTGCA GTTTATTCCCAAATTACATATACACATAAATTGACT AACATCAGC/ TCCAATACCTCGCCAGAAC	This study
ARG4-Repair-R *WOR2*	GAGTATAGTAGAACAAAATAATGTGTATATAGATA ATAAATCCAATATATTCATACTTTTGATGTTTAATG ATACTAAGA/ CTCACTATAGGGAGACCG	This study

ARG4-Repair-F *WOR4*	TATTTTCAAGAATAATTTGTTAGTATATAATTGAGA GAGAAACTATTTTTGCAGCTTGAGGTATATGAAAT TAGTAAAAT/ TCCAATACCTCGCCAGAAC	This study
ARG4-Repair-R *WOR4*	AATGCGTATATTGTACAAATTAATTTTCTAAAATAA ATCTATATGAATACAACAGAGGATCATTCAAATCC TCATTTCAT/ CTCACTATAGGGAGACCG	This study
ARG4-Repair-F *CZF1*	TTCGATTAACAAACATTCAAACGAAAACTATCTGG TATTTAATTTTCCATTTTCAACATCAAGTGTTTAAA TATCAACA/ TCCAATACCTCGCCAGAAC	This study
ARG4-Repair-R *CZF1*	ACACTTAACATAATACAAGAGTGGTGATTTACCTT TTTATTGAGTTTTTTTTAAACGATATCCCTCCAACA CAGAGAAGC/ CTCACTATAGGGAGACCG	This study
MTL **a**1-F	TTGAAGCGTGAGAGGCAGGAG	This study
MTL **a**1-R	GAATGAGAAAGAAGGAACCCAAAC	This study
MTL **a**2-F	TCCAAAGCTGCAGTCAAGTT	This study
MTL **a**2-R	GCTGAATAGGAAACCACGGA	This study
MTL α1-F	TTCGAGTACATTCTGGTCGC	This study
MTL α1-R	CGGGTACAATTGAGGATGTTTACA	This study
MTL α2-F	TTCACATCTGGAGGCACTCT	This study
MTL α2-R	TTGGCTGCTTTGCTATTAACA	This study
Ex-*OFI1*-F	CCGATTAGAGCTGGAGACTTC	This study
Ex-*OFI1*-R	GACACTGATTTGGTTTTGAACC	This study
In-*OFI1*-F	ACTTTCCTTAACAGCGAGTACC	This study
In-*OFI1*-R	CTCAAACAGCTGTTATCCACG	This study
In-ARG4-F	CTGTGGGTCGTGTACTTTTGA	This study
In-ARG4-R	CTGCTAATGAACGTCGATTAGG	This study
ACT1 P-In-F	GCAAGTATATCTGGCAAACCTG	This study
HindIII-*OFI1*-F	AAGCTTGCCGCCACCATGAATCTGAATTCTAATGA TGAGC	This study
MluI-*OFI1*-R	GTATATATCACAATGGATCAGACGCGTATTGTAGA CTTTG	This study

Xmal-*ACT1*-F	tccccccgggGTAGCCTCTTACATAAGCAAG	This study
BamHI-*OFI1*-R	GTATATATCACAATGGATCAGgatccgcg	This study

Table S2: Strains constructed for this study

Strains	Mating type	Genotype	Source
SN152	**a**/α	*arg4*/*arg4 leu2*/*leu2 his1*/*his1*	Lab stock
SN152	**a**/**a**	*arg4*/*arg4 leu2*/*leu2 his1*/*his1*	This study
ofi1 Δ/Δ	**a**/α and **a**/**a**	*ofi1*::*LUE*/*ofi1*::*HIS3*	This study
ofi1 Δ/Δ	**a**/α and **a**/**a**	*ofi1*::*ARG4*/*ofi1*::*ARG4*	This study
wor2 Δ/Δ	**a**/α and **a**/**a**	*wor2*::*ARG4*/*wor2*::*ARG4*	This study
wor4 Δ/Δ	**a**/α and **a**/**a**	*wor4*::*ARG4*/*wor4*::*ARG4*	This study
czf1 Δ/Δ	**a**/α and **a**/**a**	*czf1*::*ARG4*/*czf1*::*ARG4*	This study
ofi1 Δ/Δ *wor2* Δ/Δ	**a**/α and **a**/**a**	*ofi1*::*LUE*/*ofi1*::*HIS3*, *wor2*::*ARG4*/*wor2*::*ARG4*	This study
ofi1 Δ/Δ *wor4* Δ/Δ	**a**/α and **a**/**a**	*ofi1*::*LUE*/*ofi1*::*HIS3*, *wor4*::*ARG4*/*wor4*::*ARG4*	This study
ofi1 Δ/Δ *czf1* Δ/Δ	**a**/α and **a**/**a**	*ofi1*::*LUE*/*ofi1*::*HIS3*, *czf1*::*ARG4*/*czf1*::*ARG4*	This study
OFI1 Over-Expression (OE)	**a**/α and **a**/**a**	SN152 + p*ACT1-OFI1*	This study
OFI1 OE *wor2*Δ/Δ	**a**/α and **a**/**a**	p*ACT1-OFI1*, *wor2*::*ARG4*/*wor2*::*ARG4*	This study
OFI1 OE *wor4* Δ/Δ	**a**/α and **a**/**a**	p*ACT1-OFI1*, *wor4*::*ARG4*/*wor4*::*ARG4*	This study
OFI1 OE *czf1* Δ/Δ	**a**/α and **a**/**a**	p*ACT1-OFI1*, *czf1*::*ARG4*/*czf1*::*ARG4*	This study

Table S3: Plasmids used for this study

Strains	Description	Source
Cip*ACT1*-CYC	Complementation plasmid	(Tebbji et al., 2020)
Cip*ACT1-OFI1*-CYC	Complementation plasmid	This study

pV1093	Solo system CaCas9/sgRNA entry expression vector, contains NatR gene, and 2kb targeting arms for the upstream and downstream of ENO1 coding region.	Lab stock
pV1093-*ACT1* p- *OFI1*	Complementation plasmid	This study
pFA-*ARG4*	Disruption cassette	Gola et al., (2003)
pV1093-Ofi1-sgRNA	Complementation plasmid	This study
pV1093-Wor2-sgRNA	Complementation plasmid	This study
pV1093-Wor4-sgRNA	Complementation plasmid	This study
pV1093-Czf1-sgRNA	Complementation plasmid	This study

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