

**Identification and Regulation of White-Opaque
Phenotypic Switching in *Candida tropicalis***

By

Allison Marly Porman

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by the Division of Biology and Medicine as satisfying the
dissertation requirement for the degree of Doctor of Philosophy.

Date_____

Richard Bennett, Ph.D., Advisor

Recommended to the Graduate Council

Date_____

William Fairbrother, Ph.D., Reader

Date_____

Judith Bender, Ph.D., Reader

Date_____

Mark Johnson, Ph.D., Reader

Date_____

Malcolm Whiteway, Ph.D., Reader
Concordia University

Approved by the Graduate Council

Date_____

Peter M. Weber, PhD
Dean of the Graduate School

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TABLE OF CONTENTS

TITLE PAGE.....	I
ACKNOWLEDGEMENTS	IV
TABLE OF CONTENTS.....	VI
LIST OF FIGURES	XII
LIST OF TABLES.....	XV
ABSTRACT.....	XVII

CHAPTER 1: INTRODUCTION..... 1

SECTION 1.1 OPPORTUNISTIC FUNGAL INFECTIONS

<i>1.1.a Fungi in the environment</i>	2
<i>1.1.b Pathogenesis of Candida species</i>	2
<i>1.1.c Evolution of the Candida clade</i>	3

SECTION 1.2 MATING IN FUNGI

<i>1.2.a Mating in yeast</i>	3
<i>1.2.b Mating within the Candida clade</i>	6

SECTION 1.3 MATING AND FITNESS

SECTION 1.4 MEIOSIS IN FUNGAL SPECIES

SECTION 1.5 PHENOTYPIC TRANSITIONS

<i>1.5.a A common feature of biological organisms</i>	9
<i>1.5.b Phenotypic flexibility in C. albicans</i>	10

SECTION 1.6 THE WHITE-OPAQUE PHENOTYPIC SWITCH

<i>1.6.a C. albicans white-opaque switch</i>	12
<i>1.6.b Environmental regulation of the white-opaque switch</i>	13
<i>1.6.c White-opaque switch and biofilm formation</i>	14

SECTION 1.7 REGULATORY NETWORKS

<i>1.7.a Interlocking networks in epigenetic switching</i>	16
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1.7.b Regulatory networks in <i>C. albicans</i> morphogenic switches	17
SECTION 1.8 REGULATION OF THE WHITE-OPAQUE SWITCH	
1.3.a Regulation of white-opaque switching in <i>C. albicans</i>	19
1.3.b Conservation of regulatory factors	22
SECTION 1.9 CURRENT RESEARCH	
1.9.a Introduction to <i>C. tropicalis</i>	24
SECTION 1.10 FIGURES	27
SECTION 1.11 REFERENCES	30

CHAPTER 2: A PHENOTYPIC SWITCH REGULATES SEXUAL MATING IN *CANDIDA TROPICALIS*..... 49

SECTION 2.1 ABSTRACT	50
SECTION 2.2 INTRODUCTION	51
SECTION 2.3 MATERIALS AND METHODS	54
SECTION 2.4 RESULTS	
2.4.a Identification of a phenotypic switch in <i>C. tropicalis</i>	55
2.4.b Mating in <i>C. tropicalis</i>	57
2.4.c Cell biology of mating in <i>C. tropicalis</i>	58
2.4.d Analysis of the white-opaque switch	59
2.4.e <i>C. tropicalis</i> WOR1 regulates switching and mating	60
SECTION 2.5 DISCUSSION	61
SECTION 2.6 FIGURES	66
SECTION 2.7 REFERENCES	71

CHAPTER 3: *MTL*-INDEPENDENT PHENOTYPIC SWITCHING IN *CANDIDA TROPICALIS* AND A DUAL ROLE FOR *WOR1* IN REGULATING SWITCHING AND FILAMENTATION 79

SECTION 3.1 ABSTRACT AND AUTHOR SUMMARY	80
SECTION 3.2 INTRODUCTION	82

SECTION 3.3 MATERIALS & METHODS.....	86
SECTION 3.4 RESULTS	
3.2.a <i>Comparitive analysis of C. tropicalis mating in white and opaque cells</i>	93
3.2.b <i>Phenotypic switching in C. tropicalis a/α cells</i>	95
3.2.c <i>Transcriptional profiling of C. tropicalis white and opaque states</i>	96
3.2.d <i>Mating of C. tropicalis a/α cell types</i>	98
3.2.e <i>Transcriptional regulation of C. tropicalis Switching</i>	99
3.2.f <i>WOR1 regulates filamentation and biofilm formation</i>	102
3.2.g <i>Contribution of MTL configuration to C. tropicalis fitness</i>	104
SECTION 3.5 DISCUSSION	
3.3.a <i>C. tropicalis mating and the white-opaque switch</i>	106
3.3.b <i>Profiling of C. tropicalis phenotypic states</i>	108
3.3.c <i>Evolution and function of phenotypic switching in Candida species</i>	110
3.3.d <i>A dual role for Wor1 in regulating phenotypic switching and filamentation in C. tropicalis</i>	111
SECTION 3.6 FIGURES.....	114
SECTION 3.7 REFERENCES.....	121

CHAPTER 4: TRANSCRIPTIONAL REGULATION OF *CANDIDA TROPICALIS*

WHITE-INTERMEDIATE-OPAQUE SWITCHING.....	128
SECTION 4.1 ABSTRACT	129
SECTION 4.2 INTRODUCTION	131
SECTION 4.3 MATERIALS & METHODS.....	135
SECTION 4.4 RESULTS	
4.4.a <i>A stable, third phenotypic state in C. tropicalis</i>	140
4.4.b <i>C. tropicalis cells in the intermediate phenotype undergo efficient mating</i>	141
4.4.c <i>Global gene expression in white, intermediate, and opaque cells</i>	141
4.4.d <i>Transcriptional regulation of the C. tropicalis white-opaque switch</i>	143
4.4.e <i>Identification of novel regulators of phenotypic switching in C. tropicalis.</i>	145

4.4.f Analysis of constitutive overexpression of potential white-opaque regulators in <i>C. tropicalis</i>	147
4.4.g Regulatable expression of <i>C. tropicalis</i> transcription factors by the MAL2 promoter	149
4.4.h Inheritance of ectopically induced phenotypic states.....	151
SECTION 4.5 DISCUSSION.....	153
SECTION 4.6 FIGURES AND TABLES	160
SECTION 4.7 REFERENCES.....	167
 CHAPTER 5: CONCLUSION	175
SECTION 5.1 SUMMARY & GENERAL DISCUSSION	
SECTION 5.2 FUTURE DIRECTIONS: WHITE-OPAQUE SWITCH AND THE ENVIRONMENT	176
5.2.a Aspects of the <i>C. tropicalis</i> mating cycle.....	176
5.2.b Environmental cues for white-opaque switching in <i>C. tropicalis</i>	177
5.2.c White-opaque switching and interactions with the host.....	178
SECTION 5.3 FUTURE DIRECTIONS: REGULATION OF <i>C. TROPICALIS</i> SWITCHING	
5.3.a EFG1 in <i>C. tropicalis</i>	179
5.3.b WOR1 function in <i>C. tropicalis</i>	180
5.3.c Interaction of regulatory factors & regulatory network	181
SECTION 5.4 FUTURE DIRECTIONS: FEATURES OF WHITE-OPAQUE REGULATION	
5.4.a Q-rich domains and aggregation of regulatory factors.....	183
5.4.b Epigenetic properties of white-opaque switching.....	185
SECTION 5.5 CONCLUSION.....	185
SECTION 5.6 TABLES	187
SECTION 5.7 REFERENCES.....	188

**APPENDIX A: SUPPLEMENTAL INFORMATION FOR “A PHENOTYPIC SWITCH
REGULATES SEXUAL MATING IN *CANDIDA TROPICALIS*” 191**

A.1 SUPPLEMENTAL METHODS	191
A.2 SUPPLEMENTAL DATA	
A.2.a Phloxine B staining of <i>C. tropicalis</i> and <i>C. albicans</i> white and opaque cells	197
A.2.b Mixtures of <i>C. tropicalis</i> white α and α cells do not show an efficient mating response.....	198
A.2.c Images of <i>C. tropicalis</i> and <i>C. albicans</i> white cells, opaque cells, and mating zygotes	198
A.2.d Annotation and summary of significant genes from microarrays.....	199
A.3 SUPPLEMENTAL FIGURES	202
A.4 SUPPLEMENTAL TABLES	206
A.5 REFERENCES.....	211

**APPENDIX B: SUPPLEMENTAL INFORMATION FOR “MTL-INDEPENDENT
PHENOTYPIC SWITCHING IN *CANDIDA TROPICALIS* AND A DUAL ROLE FOR
WOR1 IN REGULATING SWITCHING AND FILAMENTATION”..... 214**

B.1 SUPPLEMENTAL RESULTS	
B.1.a GO Term analysis of α/α and α/α white- and opaque-specific genes.....	214
B.1.b $\alpha 1/\alpha 2$ binding sites within the <i>WOR1</i> promoter.....	214
B.1.c Generation of an MTL marker to track MTL status during α/α mating.....	215
B.1.d Regulation of mating in <i>C. tropicalis</i> by $\alpha 2$ and $\alpha 1$ transcription factors....	215
B.1.e Comparison of white- and opaque-specific genes in <i>C. tropicalis</i> and <i>C.</i> <i>albicans</i>	216
B.1.f Comparative analysis of Δ/Δ <i>wor1</i> mutant cells with white cells, and pTDH3- <i>WOR1</i> expressing cells with opaque cells	217
B.2 SUPPLEMENTAL FIGURES	218
B.3 SUPPLEMENTAL TABLES	224
B.4 REFERENCES.....	262

APPENDIX C: SUPPLEMENTAL INFORMATION FOR “TRANSCRIPTIONAL REGULATION OF A WHITE-INTERMEDIATE-OPAQUE TRISTABLE SWITCH IN <i>CANDIDA TROPICALIS</i>”	263
C.1 SUPPLEMENTAL FIGURES	263
C.2 SUPPLEMENTAL TABLES.....	265

LIST OF FIGURES

Figure 1. Phylogeny of sequenced <i>Candida</i> and <i>Saccharomyces</i> clade species.....	27
Figure 2. <i>Mating cycles of S. cerevisiae and C. albicans</i>	28
Figure 3. Logic of mating-type circuits in <i>S. cerevisiae</i> and <i>C. albicans</i>	28
Figure 4. White and Opaque Phenotypes of <i>C. albicans</i>	29
Figure 5. Core Regulatory Network of White-Opaque switching in <i>C. albicans</i>	30
Figure 6. Phenotypic switching in <i>C. tropicalis</i>	67
Figure 7. Quantitative mating of <i>C. tropicalis</i> white and opaque cells.....	68
Figure 8. Cell microscopy of mating between <i>C. tropicalis</i> opaque a and α cells	69
Figure 9. Transcriptional profiling of white and opaque genes in <i>C. tropicalis</i>	70
Figure 10. Products of <i>C. tropicalis</i> white $\times$ white and opaque $\times$ opaque mating maintain parental phenotypes.....	114
Figure 11. <i>C. tropicalis</i> a / α cells undergo a white-opaque phenotypic switch related to that in a and α cells.....	115
Figure 12. The white-opaque switch regulates <i>C. tropicalis</i> mating in a , α , and a / α cells	116
Figure 13. <i>WOR1</i> is the master regulator of the white-opaque switch in <i>C. tropicalis</i>	117
Figure 14. <i>C. tropicalis</i> <i>WOR1</i> expression regulates filamentous growth	118
Figure 15. Biofilm formation is induced by <i>WOR1</i> overexpression in <i>C. tropicalis</i> .	119
Figure 16. Heterozygosity at the <i>MTL</i> provides a fitness advantage <i>in vivo</i>	120
Figure 17. A white-intermediate-opaque switch in <i>C. tropicalis</i>	160

Figure 18. Analysis of the intermediate state of <i>C. tropicalis</i>	161
Figure 19. <i>C. tropicalis</i> <i>wor2</i> and <i>ume6</i> mutants display distinct phenotypes	162
Figure 20. Analysis of constitutive expression of <i>C. tropicalis</i> transcription factors on the white-opaque switch.....	163
Figure 21. Analysis of phenotypes in <i>C. tropicalis</i> cells expressing transcription factors under the control of the regulatable <i>MAL2</i> promoter	164
Figure 22. Analysis of the heritability of induced phenotypic states	165
Figure A1. Mixtures of <i>C. tropicalis</i> white a and α cells do not undergo efficient mating and zygote formation	202
Figure A2. <i>C. tropicalis</i> white and opaque forms are not differentially stained with phloxine B	203
Figure A3. The products of <i>C. tropicalis</i> mating are mononuclear, tetraploid cells	203
Figure A4. Scanning electron microscope images of white cells, opaque cells, and mating zygotes from <i>C. albicans</i> and <i>C. tropicalis</i>	204
Figure A5. Analysis of GO term frequency of <i>C. tropicalis</i> white and opaque specific genes.....	205
Figure B1. Scanning Electron Micrographs of <i>MTLa/a</i> (CAY1504) and <i>MTLa/α</i> (CAY1513) white and opaque cells and RT-PCR analysis of a1 and α 2 expression	218
Figure B2. Transcriptional profiles of genes regulated by the white-opaque switch in <i>C. tropicalis</i> a , α , and a/α cell types.....	219
Figure B3. GO Term analysis of genes regulated by the white-opaque switch in <i>C.</i> <i>tropicalis</i> a and a/α cell types	221

Figure B4. Mating of <i>C. tropicalis</i> a, α , and a/ α cell types is regulated by WOR1 expression.....	222
Figure B5. Monitoring <i>MTL</i> Loss during mating of <i>C. tropicalis</i> a/ α cell types.....	223
Figure C1. Colony morphology of the white, opaque, and intermediate states.....	263
Figure C2. Switching frequencies of <i>C. tropicalis</i> deletion mutants.....	264

LIST OF TABLES

Table 1. Analysis of single cells isolated from different phenotypic states.....	166
Table 2. Prion propensity scores of white-opaque regulators generated by PAPA.....	187
Table A1. List of <i>C. tropicalis</i> strains used in Porman <i>et al.</i> 2011 PNAS.....	206
Table A2. Oligonucleotides used in Porman <i>et al.</i> 2011 PNAS.....	207
Table A3. Plasmids used in Porman <i>et al.</i> 2011 PNAS.....	210
Table B1. Clinical isolates examined in Porman <i>et al.</i> 2013 PLOS Genetics.....	224
Table B2. List of <i>C. tropicalis</i> strains used in Porman <i>et al.</i> 2013 PLOS Genetics	228
Table B3. Oligonucleotides used in Porman <i>et al.</i> 2013 PLOS Genetics.....	229
Table B4. Plasmids used in Porman <i>et al.</i> 2013 PLOS Genetics.....	231
Table B5. List of a / α white-specific genes.....	232
Table B6. List of a / α opaque-specific genes	241
Table B7. List of a / α genes down regulated in the white state relative to $\Delta/\Delta wor1$ mutants	246
Table B8. List of a / α genes up regulated in the white state relative to $\Delta/\Delta wor1$ mutants	247
Table B9. List of a / α genes down regulated in the opaque state relative to <i>WOR1</i> overexpressing strains.....	249
Table B10. List of a / α genes up regulated in the opaque state relative to <i>WOR1</i> overexpressing strains.....	257
Table B11. a2 and $\alpha 1$ genes are essential for a and α cell mating, respectively	261

Table C1. Global comparison of gene expression in white, opaque, and intermediate states	265
Table C2. White-specific gene list.....	266
Table C3. Opaque-specific gene list	270
Table C4. White-repressed gene list.....	271
Table C5. Opaque-repressed gene list	278
Table C6. Intermediate-repressed gene list.....	279
Table C7. White-specific GO terms.....	280
Table C8. Opaque-specific GO terms	282
Table C9. White-repressed GO terms.....	283
Table C10. Opaque-repressed GO terms.....	289
Table C11. Strains used in Chapter 4	290
Table C12. Plasmids used in Chapter 4	293
Table C13. Oligonucleotides used in Chapter 4.....	294

ABSTRACT

Fungal pathogens utilize a variety of strategies to adapt to environmental cues and to thrive in different host niches. This includes phenotypic switching, in which cells undergo heritable transitions between alternative phenotypic states. Such transitions are critical to the biology of *Candida* species, which are the most common human fungal pathogens, and can grow as benign commensals but have the potential to become opportunistic pathogens, particularly targeting immunocompromised hosts. Despite the importance of phenotypic transitions, there remains a limited understanding of the mechanisms underlying these transitions or how they evolved. Recent studies of phenotypic switching have revealed that complex transcriptional networks regulate switching. Investigation of these regulatory networks, including the rewiring of networks between species, can provide new insights into how such networks evolve as well as their contributions to organismal fitness.

The work described here identifies a novel “white-opaque” phenotypic switch in *Candida tropicalis*, an opportunistic fungal pathogen in the *Candida* clade. This phenotypic switch is limited to three related *Candida* species, although its regulation is mediated by conserved transcription factors. The discovery of the *C. tropicalis* white-opaque switch is presented in Chapter 2, including the properties of the phenotypic states, the related gene expression profiles, and the regulation of sexual mating by the switch. In Chapter 3, the role of the Wor1 transcription factor in regulating opaque cell formation is presented, as well as the role of this factor in

filamentation and biofilm formation in *C. tropicalis*. In Chapter 4, a third phenotypic state, termed the intermediate state, is identified, which further distinguishes the *C. tropicalis* switch from that in other *Candida* species. Finally, this work shows that ectopic *WOR1* expression causes unstable induction of the opaque state in *C. tropicalis*, illustrating another significant difference from the related switch in *C. albicans*.

Comparison of the features identified in our studies with that of the features of the *C. albicans* white-opaque switch has revealed divergence both in upstream effectors of the switch as well as downstream outcomes. Overall, this work shows that an epigenetic switch that looks phenotypically similar can have widely different outcomes in the context of different species.

Chapter 1: Introduction

Phenotypic switching plays important roles in the fitness and development of diverse organisms. Many studies have begun to address important questions concerning the biology of phenotypic switching, including how specific phenotypes contribute to fitness, how they regulate organismal traits, and how they are controlled to enable formation of multiple stable phenotypic states. This thesis addresses these questions in relation to a newly identified phenotypic switching system in the opportunistic fungal pathogen *Candida tropicalis*.

1.1 Fungal infections

Fungi are eukaryotic organisms characterized by cell walls consisting largely of chitin and other polysaccharides, and include yeasts, molds, and mushrooms. Fungi are widespread in the environment where they play fundamental roles in environmental nutrient cycling, especially in decomposition of organic matter and in plant symbiosis. They are also prevalent as commensal species inhabiting multiple niches of animals and humans [1]. Under normal conditions with a healthy immune system, fungi are part of healthy humans' microflora. In fact, recent deep sequencing analysis of the human microbiome found the presence of fungal DNA sequences in samples from all 98 patients tested [2]. Some of the most common human commensal yeasts are members of the *Candida* clade, which inhabit the skin, gastrointestinal tract, or other mucosal surfaces [3]. However, one's own commensal organisms can become opportunistic pathogens after the host immune system has been compromised, for example in patients taking immunosuppressive drugs or those with Acquired Immunodeficiency Syndrome (AIDS) [4, 5]. *Candida* species are responsible for a variety of opportunistic diseases, including both mucosal and systemic infections [6].

Four species, *Candida albicans*, *Candida tropicalis*, *Candida parapsilosis*, and *Candida glabrata* are responsible for ~95% of infections by *Candida* species [7]. While *C. albicans* is the most frequently isolated species, infections due to *C. tropicalis*, as well as prevalence of its resistance to antifungal drugs, have been increasing worldwide, establishing it as an emerging pathogen [8]. *C. tropicalis* has been associated with nosocomial infections, especially in patients with hematologic

malignancies [7, 9]. Due to the increasing prevalence of *C. tropicalis* and the high mortality rate associated with this species, it is vital to understand the lifestyle of this fungus and the mechanisms that contribute to its virulence.

There are more than 200 *Candida* species which are hemiascomycetes, related to the model species *Saccharomyces cerevisiae*. The human *Candida* pathogens are typically diploid members of the *Candida* “CTG” clade, which translate CTG codons as serine rather than leucine [10]. Genotypic analysis of these pathogens has allowed for phylogenetic comparisons and determination of the evolutionary relationship between species [11] (Figure 1). Genome sequencing has been a major advance for the field as it facilitates comparative analyses and enables rapid genetic manipulation. Analysis of the genome sequences of *Candida* species reveals high conservation of protein coding genes among species, as well as large syntenic blocks of genomic sequence. It is also noteworthy that while the diploid *Candida* species are generally heterozygous, large stretches of homozygous regions up to 1.2 Mb are observed in both *C. albicans* and *C. tropicalis*, which could be indicative of break-induced recombination or recent passage through a parasexual cycle [11]. *C. glabrata* is also an important human fungal pathogen but resides outside the *Candida* CTG clade, and is in fact more closely related to *S. cerevisiae* than to other *Candida* pathogens.

1.2 Mating in fungi

By definition, all *Candida* species were originally thought to be obligate asexual species. The identification of a genetic mating type-like (*MTL*) locus [12]

subsequently led to the discovery that *C. albicans* has the ability to mate [13, 14]. The *C. albicans* mating pathway has been shown to have many similarities to that of the model yeast *S. cerevisiae*, including components of the pheromone secretion, processing, and response pathways [15, 16]. Specifically, in order for **a** and α cells to mate with one another, they must secrete and respond to each other's pheromones. In **a** cells, the Ste2 receptor is necessary for binding α pheromone produced by α cells and initiating a MAP Kinase (MAPK) signaling cascade. Correspondingly, the Ste3 receptor plays the reciprocal role in α cells by binding **a** pheromone. The activation of the MAPK cascade ultimately signals through the transcription factor *Cph1*, which is analogous to *Ste12* in *S. cerevisiae*. This results in the induction of specific pheromone response genes, which in turn causes the cells to produce mating projections (termed "shmoo"), which fuse to generate a zygote. This zygote then undergoes nuclear fusion, or karyogamy, to generate a tetraploid **a**/ α mating product. This process is analogous to *S. cerevisiae* mating, where two haploid **a** and α cells mate to form a diploid **a**/ α mating product (Figure 2) [17].

Regulation of mating is accomplished by transcription factors encoded at the mating-type locus. In *S. cerevisiae*, the *MAT* locus has two alleles (or idiomorphs), *MAT^a* and *MAT α* , each of which encodes unique sequence-specific DNA binding proteins which identifies a cell as an **a**, α , or **a**/ α . These factors control expression of haploid-specific genes (hsgs), genes that are expressed in **a** and α cells but not **a**/ α cells [18] (Figure 3). Although hsgs vary between species, core hsgs include components of the MAPK pathway and other downstream mating genes [19]. These genes are repressed in **a**/ α cells by a transcriptional regulatory complex formed

between **a**1, expressed from the *MATa* locus, and α 2, produced by the *MAT α* locus [20, 21]. An analogous process occurs in *C. albicans* **a**/ α cells, however the molecular details differ between species in terms of regulation of the **a**- and α -specific genes (**asgs** and α sgs, respectively) [21, 22]. *S. cerevisiae* encodes three transcription factors: **a**1 at the *MATa* locus, and α 1 and α 2 at the *MAT α* locus. In *S. cerevisiae*, **asgs** are constitutively expressed in **a** cells, but are negatively regulated in α and **a**/ α cells by the α 2 transcription factor. α sgs are induced by α 1 in α cells, while α 1 is itself repressed by the **a**1/ α 2 complex in **a**/ α cells [18, 23] (Figure 3). In *C. albicans*, α sgs are similarly induced by α 1 in α cells, while there is an additional regulator, **a**2, at the mating-type-like locus (*MTL*) in **a** cells, which positively regulates **asg** expression [12, 22] (Figure 3). There is also another level of regulation in *C. albicans*, as induction of a phenotypic switch is required for efficient mating [24], which will be discussed in Section 1.5.

In general, *C. albicans* is heterothallic, and mating occurs between **a** and α cells. **a** cells express the conserved *BAR1* gene which produces an aspartyl protease that degrades MF α , the canonical α pheromone produced by α cells [25]. Expression of *BAR1* in *S. cerevisiae* is thought to sharpen the pheromone gradient between **a** and α cells, thereby enabling cells to find mates of the opposite mating type [26]. Bar1 also prevents overstimulation by α pheromone, and can help **a** cells recover from pheromone-induced cell cycle arrest following an unsuccessful mating event [25].

In *C. albicans*, deletion of *BAR1* in **a** cells was found to activate autocrine α pheromone signaling, resulting in homothallic mating whereby **a** cells mate with **a**

cells [27]. This mechanism was due to **a** cells secreting low amounts of α pheromone (in addition to **a** pheromone); in the absence of Bar1, levels of α pheromone accumulated resulting in activation of the mating response and same-sex mating. Mating can also occur between wild type **a** cells when exogenous pheromone is provided from α cells, or by addition of synthetic α pheromone [27, 28]. These results demonstrate that while heterothallic mating can occur between cells of opposite mating types, it is also possible to undergo sexual reproduction in a unisexual population.

Less is known about sexual reproduction in other members of the *Candida* clade, although several species appear limited in their ability to undergo sexual cycles, which may be a mechanism to maximize virulence [29]. Of the four diploid *Candida* species, only *C. albicans* and, more recently, *C. tropicalis* have been shown to have a mating cycle [30, 31]. *C. parapsilosis* has never been observed to mate, and isolates sampled contain only an *MTLa* idiomorph at the *MTL* locus, which is also a pseudogene, suggestive of a deterioration of the *MTL* locus in this species. [32]. *Lodderomyces enlogisporus*, a related yeast, has been reported to undergo homothallism, or self-mating, based upon the formation of ascospores [33]. Among the haploid *Candida* species, *Debbaromyces hansenii* has also been observed to be homothallic [34], while *C. lusitaniae* and *C. guilliermondii* are heterothallic and have a complete sexual cycle [35].

Comparative analysis reveals that *C. tropicalis* has a conserved *MTL* locus similar to that of *C. albicans*, both in terms of gene content and synteny [11]. Despite

genomic and population evidence for mating in *C. tropicalis*, this species had never been observed to undergo sexual mating prior to the work presented here.

1.3 Mating and Fitness

Mating in fungi has implications for fitness and virulence in pathogenic species. Although sexual cycles can be beneficial for generating diversity and transferring genetic information, mating cycles can also have associated fitness costs. In *S. cerevisiae*, it has been shown that reduction of mating pheromone production can increase asexual viability by up to 50%, illustrating the high metabolic cost of the mating process [36]. A pheromone-induced death pathway has also been demonstrated in *C. albicans*, where, depending on the clinical isolate, 20-70% of mating competent opaque cells die upon exposure to pheromone, while the chaste white state survives [37]. On the other hand, mating can alter the virulence properties of pathogenic fungi. For example, in the basidiomycete *Cryptococcus neoformans*, which causes meningoencephalitis and has both heterothallic and homothallic mating, sex is essential for pathogenesis since the infectious particles of *C. neoformans* are its sexual spores [38-40]. Additionally, same-sex mating in the related sibling species *Cryptococcus gattii* has been implicated in generating a highly virulent isolate responsible for a recent outbreak of this species [41, 42]. A complete sexual program was also recently identified in an airborne fungal pathogen, *Aspergillus fumigatus* [43].

Genetic information contained at the *MTL* locus has also been shown to alter virulence in some *C. albicans* strains. Naturally occurring **a**/ α cells tend to be more

virulent than naturally occurring **a** and α cells, and it has been shown that strains are less virulent and less competitive after homozygosis of the *MTL* or of the entire chromosome 5, which harbors the *MTL* [44]. Some hypothesize that maintenance of the *C. albicans* mating cycle is due to the competitive advantage of **a**/ α strains over *MTL* homozygotes.

1.4 Meiosis in fungal species

Species with complete sexual cycles undergo a meiotic process to return to the mating competent state. Under stressful or nutrient limiting conditions, *S. cerevisiae* diploid **a**/ α cells undergo meiosis to generate a tetrad spore with 4 haploid cells enclosed inside of an ascus (Figure 2). The pathogen *C. lusitaniae* has also been shown to have a complete sexual cycle despite its lack of a key meiotic regulator from *S. cerevisiae*, *IME1* [11, 35]. This species has been shown to have coupled sexual cycle; regulation of mating has fused with the regulation of meiosis, contributing to its preference to exist in the haploid state [45].

In the case of *C. albicans*, a conventional meiosis has not been identified despite the fact that this species contains many conserved genes associated with the meiotic program [46]. Instead, *C. albicans* has a unique process to complete its mating cycle, termed the parasexual cycle, in which tetraploid cells undergo concerted chromosome loss to transition from the tetraploid state to the diploid or near-diploid state [30] (Figure 2). This process is induced in response to nutritional cues such as growth on sorbitol or *S. cerevisiae* pre-sporulation medium. If diploid strains generated from this cycle inherit the proper *MTL* configuration (become **a** or

α cells through loss of the opposite locus or homozygosis of the *MTL*), they are also mating competent, completing the parasexual cycle [30] (Figure 2). The parasexual cycle has been shown to generate recombinant progeny with distinct genotypes from that of the parental strains [47]. These strains can undergo recombination between homologous chromosomes as well as gene conversion, and this requires the conserved Spo11 protein (which is also essential in meiotic recombination). The parasexual cycle also contributes to phenotypic variation, as many distinct colony morphologies are generated from the process [47]. Due to the genetic and phenotypic variation generated by this cycle, the parasexual process has important implications for strain fitness and evolution of *C. albicans* isolates.

1.5 Phenotypic transitions

Besides genetic alterations that lead to phenotypic variation, epigenetic phenotypic transitions are also ubiquitous in biological systems. These transitions play prominent roles in development of different cell types in multicellular organisms, as well as in stress tolerance and niche adaptation in microorganisms. Phenotypic changes underlie the progression of disease states such as cancer, in which genetic or epigenetic reprogramming alters gene expression to allow expansion and metastasis of tumors [48]. During development, epigenetic modifications accompany cellular differentiation to give rise to the many different cell types of the organism and ensure fidelity in tissue-specific gene expression [49].

In single-celled organisms, being able to adopt many different phenotypes in response to the environment is important for organismal fitness, as it allows the

organism to survive in multiple niches and respond to different stresses by altering gene expression. Phenotypic variation is also a mechanism to generate diversity in mainly clonal populations. For example, in bacterial cell populations, some cells transition from actively growing to a “persister” phenotype, which is metabolically dormant [50]. When exposed to stresses such as antibiotics, these persister cells are able to survive and, once the stress is removed, regenerate cells with a wild type phenotype that are able to repopulate the niche [51]. Another important transition in microorganisms is that between planktonic growth and growth as a multicellular biofilm. Bacteria are able to switch from growing freely as single cells to being adherent to a surface and to other bacterial cells. In this biofilm state, cells secrete an extrapolymeric matrix composed of polysaccharides, nucleic acids, protein, lipids, and signaling molecules, which encloses the biofilm [52]. This switch requires the regulation of hundreds of genes and increases the prevalence of persister cells and antibiotic tolerance [53, 54].

Morphological plasticity has been shown to play key roles in the lifestyle of several fungal pathogens. *Cryptococcus neoformans* is able to reversibly switch between several different colony phenotypes, and these phenotypes differ in virulence [55, 56]. The phenotypic switch alters the cell wall of the pathogen, which in turn influences the inflammatory response of the host [55]. Certain therapeutic treatments may favor more harmful switch variants, emphasizing the clinical importance of studying phenotypic switching in pathogenic fungi in the context of infection [57].

In *C. albicans*, there are multiple phenotypic switches that have been shown to play important roles in virulence and commensalism, affecting features such as host invasion, dissemination, niche specificity, and immune interactions. The switch between the budding yeast form and filamentous forms, including hyphae or pseudohyphae, is one of the most well studied transitions in *C. albicans* [58]. Hyphal forms have been shown to be important for adherence to and invasion of host tissues, which can cause extensive epithelial damage [59]. This transition is induced in response to a variety of stimuli that may act through the cyclic AMP-dependent pathway and the Efg1 transcription factor, or via a mitogen-activated protein kinase (MAPK) pathway that activates the Cph1 transcription factor [60, 61]. Several hyphal-specific genes have been identified including *ALS3*, *HWP1*, *RBT1*, and *ECE1*, which encode cell wall associated proteins [62]. *ALS3* is a member of an agglutinin-like family and has been implicated in the adhesion of *C. albicans* to the host cell surface [63]. *HWP1* can serve as a target for mammalian transglutaminases which forms attachments between *C. albicans* and host epithelial cells [64]. These genes have been shown to be necessary for full virulence of *C. albicans* in systemic mouse models, suggesting host tissue adherence and invasion by hyphal formation are major virulence factors.

Filamentation is also an important step in biofilm development of *C. albicans* [65, 66]. Like bacteria, *C. albicans* can attach to surfaces and grow as multicellular biofilm communities that have important clinical implications for patients with indwelling medical devices; mortality of device-associated infections approaches 40% [67, 68]. Like bacterial biofilms, *C. albicans* biofilm formation also increases

resistance to antimicrobial drugs, possibly due to increased expression of drug efflux pumps, generation of persister cells, production of extracellular matrix, or a combination of these and other features [69-72]. Biofilm development involves adherence of yeast cells to a surface, growth of attached yeast cells into a thin layer, maturation of the biofilm through development of pseudohyphae and hyphae, excretion of extracellular matrix, and lastly, the dispersal of yeast cells from the biofilm, which can lead to colonization of other niches [73, 74]. A mature biofilm, which develops within 24-48 hours after initiation, consists of a thin layer of yeast cells anchoring a thick upper layer of yeast and hyphae to a biotic or abiotic surface [75, 76]. Like other epigenetic switches, this process regulates the expression of many genes, including those shown to play direct roles in different steps of biofilm development [77].

1.6 The white-opaque phenotypic switch

A particularly well-studied phenotypic switch in *C. albicans*, and the focus of this work, is the white-opaque switch. Discovered in 1987, *C. albicans* cells were shown to reversibly switch between a white phenotype, consisting of round cells with a smooth surface texture that form shiny, dome-shaped colonies, to an opaque phenotype in which cells are more elongated, have a pimpled surface texture, and form flatter, darker colonies that are stained with the dye phloxine B [78, 79] (Figure 4). However, it was not until 2002 that white-opaque switching was shown to regulate mating in *C. albicans*. Unlike *S. cerevisiae*, which can mate at high levels in its natural yeast form, *C. albicans* requires a switch from the white state to the

opaque state in order to undergo efficient mating, as the opaque state mates about a million times more efficiently than the white state [24] (Figure 2). Additionally, only strains that are homozygous at the *MTL* (**a** or α) undergo switching at high frequencies, restricting the switch to strains that are mating competent [24, 80]. As described in Section 2, a heterodimer forms between *C. albicans* **a**1 and α 2 homeodomain proteins which acts as a transcriptional repressor and inhibits switching to the opaque state [24]. This heterodimer binds the promoter of the *White-Opaque Regulator 1* (*WOR1*) gene, which blocks its expression and formation of the opaque state [21, 22, 81]. The role of *WOR1* in regulating the white-opaque switch will be discussed in more detail in Section 1.8.

The *C. albicans* white-opaque switch is an epigenetic switch that is regulated by a number of environmental signals. This includes a variety of stress conditions such as genotoxic and oxidative stress, as well as by mutations that cause a reduction in growth rate [82]. This may be due to the accumulation of the Wor1 protein (due to changes in the cell cycle) to levels that induce the switch to opaque. Several other cues that cells might be exposed to within the host have been shown to induce white-to-opaque switching, including anaerobic conditions, N-acetylglucosamine, and CO₂ [83-85]. Conversely, high temperature is a potent inducer of opaque-to-white switching, and induction of the white state occurs within 5 hours of incubation at 37°C [86]. Moreover, Xie et al. found that many natural **a**/ α strains are able to undergo the switch to opaque when exposed to both N-acetylglucosamine and CO₂, demonstrating that **a**/ α cells are able to undergo the switch to opaque under certain environmental conditions [87].

C. albicans white and opaque cells not only differ in mating ability, but also in aspects related to metabolism, pathogenesis, and commensalism. The white-opaque switch regulates the expression of approximately 1000 genes, many of which are metabolism genes, indicating fundamental differences in metabolism between the two states [22, 88, 89]. White cells have been shown to be more virulent than opaque cells in a systemic mouse model, while opaque cells are more fit in a cutaneous model of infection [90, 91]. The white-opaque switch also regulates interaction with immune cells, as some immune cells preferentially phagocytose white cells compared to opaque cells [92, 93]. It has recently shown that an opaque-like state in α/α cells that is regulated by *WOR1* increases fitness in a model of murine gastrointestinal colonization [94].

There also exists a link between the programs regulating white-opaque switching and filamentation in *C. albicans*. While white cells filament in response to serum, neutral pH, CO₂, and embedded conditions [59], opaque cells are refractory to filamentation under these conditions. However, other environmental stimuli have been established as inducers of opaque filamentation, including sorbitol and nutrient-poor media, which do not induce filamentation in white cells [95]. Temperature also differentially regulates filamentation in white cells versus opaque cells: white cells tend to filament more under higher temperatures, with 37°C being the optimal temperature for filamentous growth, while opaque cell filamentation is most efficient at room temperature [95]. The distinct environmental cues that induce filamentation in white and opaque cells illustrate the differential regulation, expression, and metabolism of these two states.

The white-opaque switch also has important consequences for biofilm formation, and can even drive the formation of specialized biofilms. While conventional biofilm formation occurs in response to certain environmental cues such as serum [96], white cells that are *MTL* homozygous are also capable of forming biofilms in response to mating pheromone [97]. These have been termed “sexual biofilms” and are mediated by the canonical MAPK pheromone response pathway, including the terminal transcription factor *CPH1* [98]. It has also been shown that a hyperactive allele of Ste11, the MAP kinase kinase kinase in the pheromone response pathway, induces *CPH1*-dependent biofilm formation [99]. Interestingly, expression of this allele also induces the switch to opaque, leading to the hypothesis that pheromone signaling may induce the switch to a mating competent state under some conditions that have not yet been tested [99].

Recently, another phenotype, termed the “gray” state, was identified in *C. albicans*. This state has a distinct colony morphology from white and opaque, appearing dark but shiny, and cells are elongated but smaller than both white and opaque cells [100]. Gray cells are able to undergo mating ~1000-fold more efficiently than the white state, but ~1000-fold less efficiently than the opaque state, and exhibit differential virulence in infection models [100]. White, opaque, and gray phenotypes are not the only phenotypic variants that have been observed in *C. albicans*; it has been noted that this species can switch between multiple different colony morphologies [101]. Still, the white-opaque switch is considered bistable, as switching between the two states is heritable and no stable intermediate state has

been observed, likely due to autoregulatory feedback loops regulating the switch [102]. This will be addressed further in the next sections.

1.7 Regulatory networks

Transcription factors are sequence-specific DNA binding proteins that regulate expression of a set of target genes and play important roles in the control of many developmental pathways. Often, they regulate a group of target genes that function together to carry out a specific function in the cell. Hence, the study of transcription regulators and their regulatory networks can provide mechanistic insight into biological processes. Many phenotypic switches are regulated by multiple interlocking transcriptional feedback loops, generating regulatory networks, even in simple biological systems. For *Escherichia coli*, one of the best characterized free-living organisms, computational analyses have been performed to provide an overview of all the transcriptional regulatory interactions [103, 104]. Networks in this organism act to regulate metabolism as well as morphological transitions including those discussed in Section 1. It has been shown that a common network motif in *E. coli* is the feed forward loop, where one transcription factor, X, positively regulates another transcription factor Y, both of which positively regulate an operon Z [105]. Several of these feed forward loops can be combined into more complex networks. This network structure occurs much more frequently in *E. coli* networks than in randomized networks, suggesting that it is a common feature of biological regulatory networks [105].

Another example of a regulatory network occurs in human embryonic stem (ES) cells, which require several core transcription factors, including Oct4, Sox2, and Nanog, to maintain pluripotency and for normal development [106-109]. These factors have been shown to co-regulate expression of many genes, including repression of many developmental transcription factors. These factors also activate expression of each other to form multiple positive feed forward loops, which has been proposed to stabilize the pluripotent ES state while retaining the ability to react to differentiation signals [110]. The core transcription factors in ES cells act at “super-enhancers”, or large transcriptional enhancers that drive expression of genes that define cell identity [111]. High levels of binding of components of the transcriptional apparatus, including mediator complex and RNA Pol II, as well as “open chromatin” marks on histones including H3K4 methylation, H3K56 acetylation, and H3K9 acetylation, can be used to identify super-enhancers [111, 112]. This highlights the important role of epigenetics in developmental transitions and cell identity. The transcription factors that identify cell state are often highly expressed and regulated by themselves and other state-identifying transcription factors, forming tightly regulated interlocking transcriptional networks that can readily respond to environmental changes or differentiation signals.

In *C. albicans*, complex regulatory networks also regulate morphogenesis. The switch to hyphal forms is regulated by two key transcription factors, *CPH1* and *EFG1*, and mutants lacking these factors fail to generate hyphae or exhibit virulence in a mouse model of infection [113]. This is not only due to the block in hyphal formation, as deletion of *HGC1*, the hyphal-specific G1 cyclin also required for

hyphal formation, retains 30% lethality [114]. *EFG1* also regulates expression of other genes, many of which have been shown to have roles in virulence, cell wall formation, and metabolism [115-117]. Hyphal induction also causes expression of many virulence genes, and the co-regulation of hyphae formation and virulence genes suggests that hyphal morphogenesis is a major virulence contributor in *C. albicans* [118]. The hyphal regulatory network also involves other transcription factors, including the repressors *TUP1*, *NRG1*, and *RFG1*, deletion of which causes de-repressed hyphal growth [119-121]. Studies of these regulatory proteins and others have demonstrated that numerous transcription factors regulate genes in the hyphal regulon [118]. By having these genes under the control of multiple pathways and transcription factors, *C. albicans* can adapt to different host niches and produce infection in a wide range of tissues. The complexity and flexibility in regulation of morphogenesis and virulence allows the response to complex environmental signals at the appropriate time.

An interlocking transcriptional network also controls biofilm development in *C. albicans*. This network consists of 6 core regulatory factors that act in an interwoven network to regulate expression of over 1,000 target genes [122]. Two or more transcriptional regulators bind the majority of target genes in the network, including the regulators themselves. Additionally, each of the 6 transcriptional regulators positively regulates the expression of the other regulators in the network, including its own expression, thus creating multiple positive feedback loops for biofilm formation [122]. Complex circuitry such as this is common in other systems, as well, including pseudohyphal growth in *S. cerevisiae* and embryonic

stem cells in mammals described above [112, 123]. The prevalence of core sets of master transcription regulators that function together to regulate each other and a large set of target genes suggests that this is a common feature of biological processes or developmental transitions that must respond to a variety of complex environmental stimuli.

1.8 Regulation of the white-opaque switch

The white-opaque switch is another example of a phenotypic transition that is regulated by interlocking transcriptional feedback loops. There is an interwoven network of 6 core regulatory factors: *Wor1*, *Wor2*, *Wor3*, *Czf1*, *Efg1*, and *Ahr1* [102, 124, 125] (Figure 5). *Wor1* functions as the master regulator of the opaque state; deletion of *WOR1* blocks formation of the opaque phenotype, and induction of this gene drives opaque formation [81, 124]. *Efg1*, also an essential regulator of hyphal and biofilm development, is necessary for the formation of the white state; strains lacking *Efg1* form opaque cells at high frequency, while induction of *Efg1* forces opaque cells to return to the white state [102, 126, 127].

The core regulatory circuit in opaque cells is complex and highly interactive. Of the six regulators, *WOR1*, *WOR2*, *WOR3*, *CZF1*, and *EFG1* upstream regions are bound by all six of the switch regulators, and the *AHR1* upstream region is bound by *Wor1*, *Wor2*, *Wor3*, and *Ahr1* itself [102, 125]. Induction and maintenance of the opaque phase by *WOR1* is due to a network of positive feedback loops, including: (1) auto-induction of the *WOR1* gene by *Wor1*, (2) induction of *WOR2*, *WOR3*, and *CZF1* by *Wor1*, (3) induction of *WOR1* by *Wor2* and *Wor3*, and (4) repression of the

white-regulator *EFG1* by Czf1 and Wor1 [102, 124, 125] (Figure 5A). Wor2 is an opaque-specific transcription factor and required for heritable maintenance of the opaque state. Czf1 is also opaque-enriched and is important for induction of the switch and modulation of switching frequency, but not for maintenance of the opaque phase. Another opaque-specific regulator, Wor3, was also recently identified, and induction of this factor also causes an *en masse* conversion to the opaque state similarly to Wor1 [128].

Efg1, Ahr1, and Czf1 can be detected binding to DNA in white cells, while Wor1, Wor2, and Wor3 do not produce detectable enrichment in ChIP-chip experiments [125]. The core regulatory circuit consists of Efg1 binding to its own promoter and to that of *WOR1* and *WOR2*, Ahr1 binding its own promoter and to those of *EFG1* and *WOR2*, and Czf1 binding to its own promoter and upstream of *WOR2* and *EFG1* [125]. Here, *EFG1* directly represses *WOR1*, and Efg1 and Ahr1 cooperatively repress *WOR2* to prevent white cells from switching to opaque [102, 125, 129, 130]. Czf1 plays an opposing role, as it both represses *EFG1* and induces expression of *WOR2* to promote formation of the opaque cell type [102, 125] (Figure 5B). One or more of these factors binds to 179 gene promoters, and Efg1 and Ahr1 function as both repressors of the opaque state (~77 genes) and activators of the white state (~18 genes). This analysis suggests that Efg1 is the major controller of the white state, as its expression represses *WOR1* and *WOR2* and promotes the expression of the white-specific program, including additional downstream regulators. Ahr1 is also important for the white state, although it does not regulate as many genes as Efg1. The main role of Czf1 is to modulate the switching rate by

repression of *EFG1* and activation of *WOR2*. Consistent with this, white-to-opaque switching decreases 10-fold when *Czf1* is deleted [102, 131].

In addition to the regulatory transcription factors listed above, general regulation of chromatin structure through histone and DNA modifications has been implicated in regulation of the white-opaque switch. It has been shown that histone deacetylase inhibitors, including trichostatin-A and nicotinamide, as well as deletion of the histone deacetylase *HDA1*, increases switching to the opaque state [132, 133]. On the other hand, deletion of the deacetylase gene *RPD3* results in decreased switching frequency in both directions [134]. It has also been shown that *RTT109*, encoding the histone acetyltransferase for H3K56, is required for maintenance of the opaque phase, while partial deletion of *HST3*, the H3K56 deacetylase, leads to increased opaque formation [133]. More evidence for the role of chromatin regulation of the white-opaque switch comes from Hnisz et al., who demonstrated the role of the Set3/Hos2 histone deacetylase complex in regulation of the switch, depending on the methylation status of histone H3 [135]. Nucleosome assembly factors Caf-1 and Hir also play roles in switch regulation [136]. Interestingly, overexpression of *WOR1* can bypass some of these chromatin defects, suggesting that the mechanism of action is at the *WOR1* locus.

Components of the conserved mediator complex, which recruits transcriptional machinery to activating regulators, have also been shown to play a fundamental role in regulating the white-opaque switch, as deletion of the Med3 subunit destabilizes switching even in the presence of *Wor1* [137]. These results emphasize the important role of transitions in chromatin state, or activation or

deactivation of certain genes through chromatin modification, in reversible white-opaque switching, and provide another level of complexity to the networks of transcription factors regulating the white-opaque transition of *C. albicans*.

There also seems to be additional regulation at the level of transcription, and analysis by Tuch et al. demonstrated that the switch regulates nearly three times as many transcripts than was previously reported [22, 88, 89]. These newly identified transcripts included many non-coding sequences as well as those encoding short proteins [89]. Moreover, 5' and 3' UTRs of mRNAs in the circuit are unusually long and 5' UTRs often have differential lengths between white and opaque cells. Thus, UTRs may play important regulatory roles, and the use of alternative promoters is common between cell types [89]. Comparison of the properties of the Wor1 circuit with the Oct4 circuit in embryonic stem cells reveals striking similarities, which may suggest that these features are general hallmarks of heritable differentiation states in eukaryotes [89].

The white-opaque switch is thought to have evolved recently, as only *C. albicans*, its sister species *C. dubliniensis*, and, as presented here, *C. tropicalis* have been observed to switch to the opaque state [31, 138]. The 6 regulators of the *C. albicans* white-opaque regulatory circuit are conserved to varying degrees among other *Candida* species. For Wor1 and Efg1, there is evidence for an early origin prior to divergence from *S. cerevisiae*, as many ascomycetes and other fungi share close orthologs to these genes. In fact, orthologs of Wor1 have been shown to play diverse roles in fungal morphological transitions, including Mit1 in *S. cerevisiae*, which regulates pseudohyphal formation, and Ryp1, a regulator of the yeast-

mycelium transition in *Histoplasma capsulatum* [139, 140]. This suggests that Wor1 orthologs are conserved regulators of morphological transitions in fungi. The Wor1 DNA binding region consists of a 300 amino acid region termed the WOPR box that is distinct from other classes of DNA binding proteins [141].

Efg1 is also a major regulator of morphogenesis in fungal species, including important roles in *C. albicans* in white-opaque switching, filamentation, and biofilm formation [61, 65, 126]. Efg1 and its orthologs are members of the APSES family of DNA-binding proteins whose binding domain is related to a basic helix-loop-helix domain that is conserved in many viruses [142]. In the closely related species *C. parapsilosis*, Efg1 has been shown regulate pseudohyphal growth and biofilm formation [143]. Its two co-orthologs in *S. cerevisiae*, Phd1 and Sok1 (arising from the whole genome duplication), are both involved in the regulation of pseudohyphal growth [144-146]. Other Efg1 orthologs include StuA in *Aspergillus nidulans*, Asm-1 in *Neurospora crassa*, and Ust1 in *Ustilago maydis*, which all regulate yeast-hyphal transitions [147-149]. In other species, APSES proteins regulate additional processes such as mating or conidiation rather than dimorphism, suggesting that Efg1 only regulates hyphal formation in a subset of species [150].

Despite the high conservation of Efg1, the published genome sequence of *C. tropicalis* does not contain an ortholog of this gene. A related APSES transcription factor, Efh1, is present, but this represents a different clade from Efg1 [116]. Paralogs falling in the Efg1 clade are highly conserved among all ascomycetes, whereas Efh1 is unique to the *Candida* CTG clade and its paralogs are more divergent between species, suggestive of rapid evolution of this gene [143]. The

function of Efh1 remains unclear, although deletion mutants in *C. albicans* form lateral hyphae and are more filamentous in hypoxic conditions [116, 151]. Efg1 and Efh1 share few overlapping targets in *C. albicans*, indicative of the divergent functions of these two transcription factors [116]. While *C. tropicalis* contains Efh1, the loss of Efg1 in this species is peculiar considering its high level of conservation among other fungal species and may have important consequences for the regulation of morphogenesis, including white-opaque switching [126].

There is little evidence for an early origin of the zinc finger proteins Wor2, Czf1, and Ahr1, as homologs of these factors are only found in the CTG clade. These proteins could have been recent innovations created through duplication and divergence, or have diverged so much that their true orthologs can no longer be detected. Wor3 has a 200 amino acid conserved region that contains 16 conserved cysteine residues grouped in 8 “CxxC” motifs. This gene is conserved in the CTG clade as well as the species *Cyberlindnera jadinii* and *Wickerhamomyces anomalus*, which diverged prior to the split between *S. cerevisiae* and *K. lactis*, suggesting that Wor3 was lost in this common ancestor [128].

1.9 Current research

In this work, I show for the first time that *C. tropicalis* is competent for white-opaque switching and sexual mating. This was surprising as the white-opaque switch was thought to be limited to *C. albicans* and its sister species *C. dubliniensis* [89, 138]. Like *C. albicans*, white-opaque switching regulates the expression of ~1000 genes in *C. tropicalis*, and these genes are largely species-specific although

there is significant overlap with switch-regulated genes in *C. albicans* [31]. The environmental inputs influencing induction of the switch are distinct between the two species, as many of the conditions tested that induce the switch in *C. albicans* do not do so in *C. tropicalis*. Switch regulation by the *MTL* also appears to be absent in *C. tropicalis*, since **a**/ α mating products derived from opaque **a** and α parents maintain the opaque phenotype, and natural **a**/ α isolates are able to switch to opaque at similar frequencies as **a** and α cells. *Wor1* has a key role in opaque formation in both *C. albicans* and *C. tropicalis*, although I demonstrate that it also has a role in filamentation and biofilm formation in *C. tropicalis*. Finally, there is also divergence between *C. albicans* and *C. tropicalis* in terms of maintenance of white and opaque states, which includes the presence of a third phenotypic state in *C. tropicalis* that appears intermediate to that of white and opaque cells, both in terms of phenotype and gene expression. Other potential regulatory factors, including those implicated in *C. albicans* regulation as well as DNA binding proteins identified as differentially regulated in *C. tropicalis* white and opaque cells, are tested for their role in regulation of the switch. We identify *WOR1*, *WOR3*, and CTRG_03621 as inducers of the opaque and intermediate states, while *WOR2* and *NDT802* contribute to white cell formation. However, artificial induction of the opaque state by *Wor1* induction appears to be unstable in *C. tropicalis*, possibly due to the lack of positive feedback by *Wor1* on its own promoter, loss of induction of another positive regulator that is necessary for stability, or expression of a white-specific factor that prevents stable opaque phase maintenance. Future experiments will test these

possibilities as well as the interaction of the regulatory factors identified within the white-opaque regulatory circuit of *C. tropicalis*.

Overall, this work contributes to our knowledge about human fungal pathogens and the phenotypic switches that regulate their virulence. It also provides an excellent model for studying evolution of a transcriptional regulatory network of a phenotypic switch in two closely related species. Because the functional outputs and environmental regulation of white-opaque switching in these species appear to be so divergent, it begs the question as to why this switch would be conserved in these species. The key function of the white-opaque switch in regulating mating seems to be one of the few conserved features of this switch, possibly emphasizing the importance of mating regulation in pathogenic fungi. Still, other work demonstrates roles for white-opaque switching in commensalism and immune cell interaction in both species, suggesting complex and diverse functions for the switch within the host. This work provides a foundation for the examination of white-opaque switching in *C. tropicalis* and other species, evolution of its regulation and environmental impacts, and its role within the human host in commensalism and virulence.

1.10 Figures

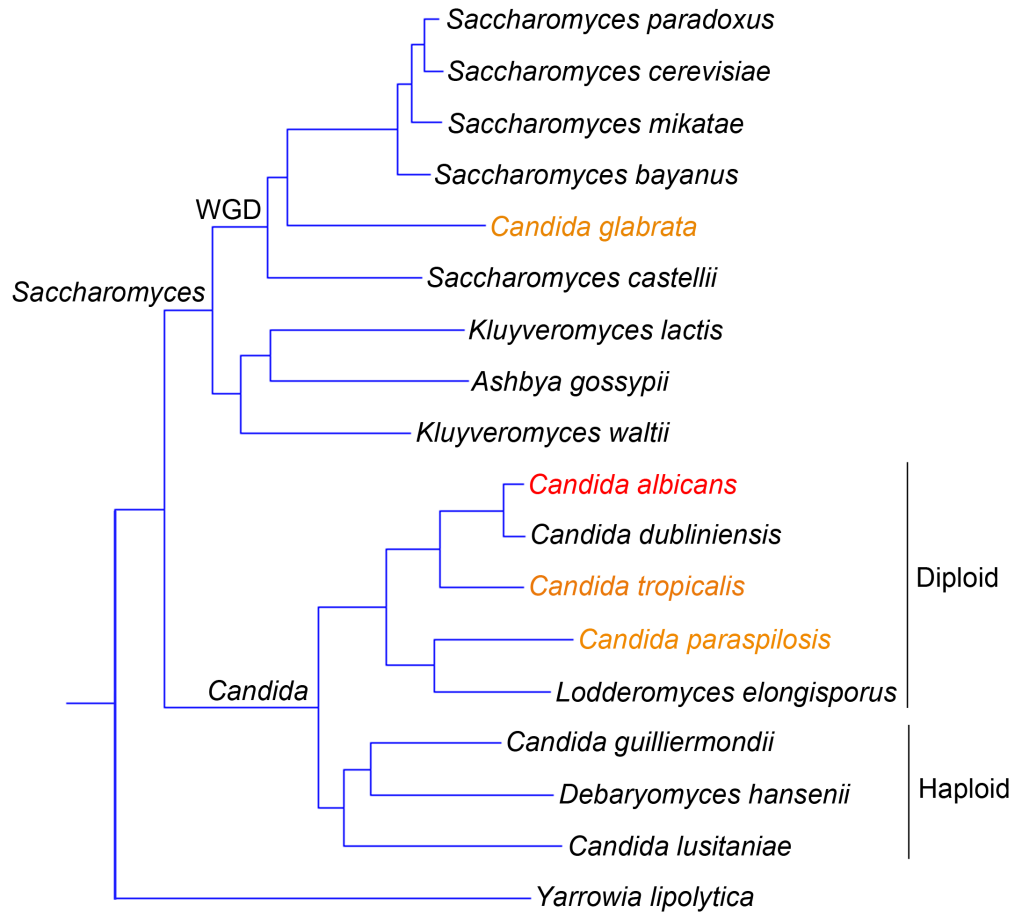


Figure 1. Phylogeny of sequenced *Candida* and *Saccharomyces* clade species. Adapted from [11]. Pathogenic species are shown in red (most prevalent) or orange (less prevalent).

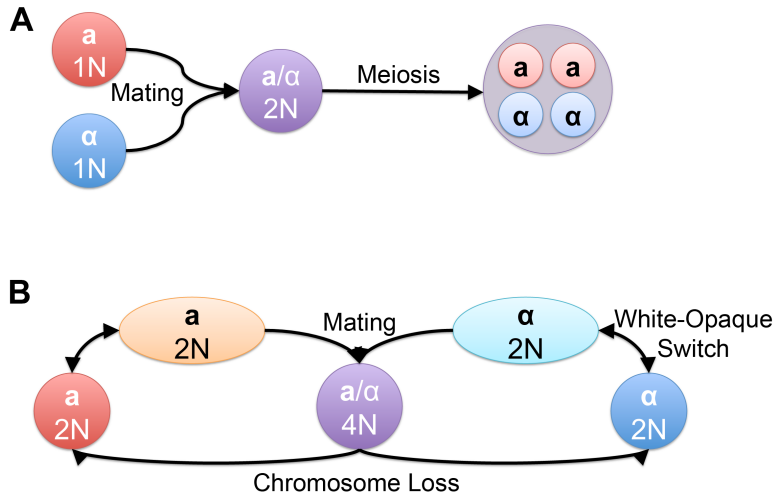


Figure 2. Mating cycles of *S. cerevisiae* and *C. albicans*. A) *S. cerevisiae* haploid **a** and **α** cells fuse to generate a diploid **a/α** cell, which can undergo meiosis to generate a tetrad spore and return to the haploid state. B) *C. albicans* diploid **a** and **α** cells switch from white to opaque prior to efficient fusion occurs. The tetraploid **a/α** mating product undergoes chromosome loss to return to the diploid (or near diploid) state.

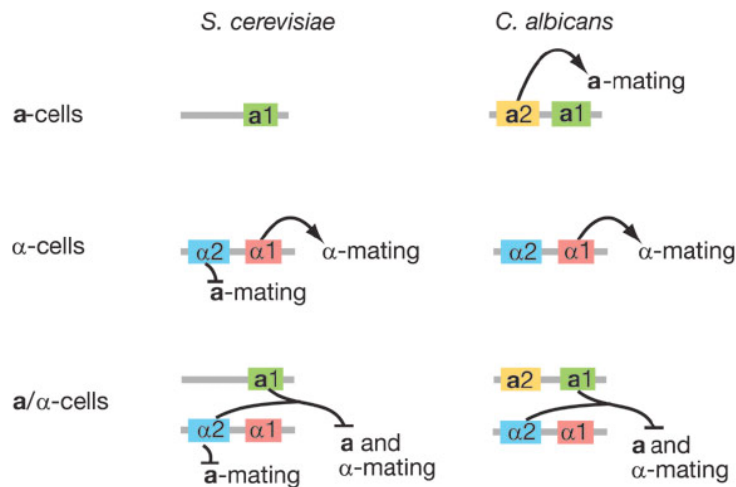


Figure 3. Logic of mating-type circuits in *S. cerevisiae* and *C. albicans*. Adopted from [152]. Reproduced with permission from Nature.

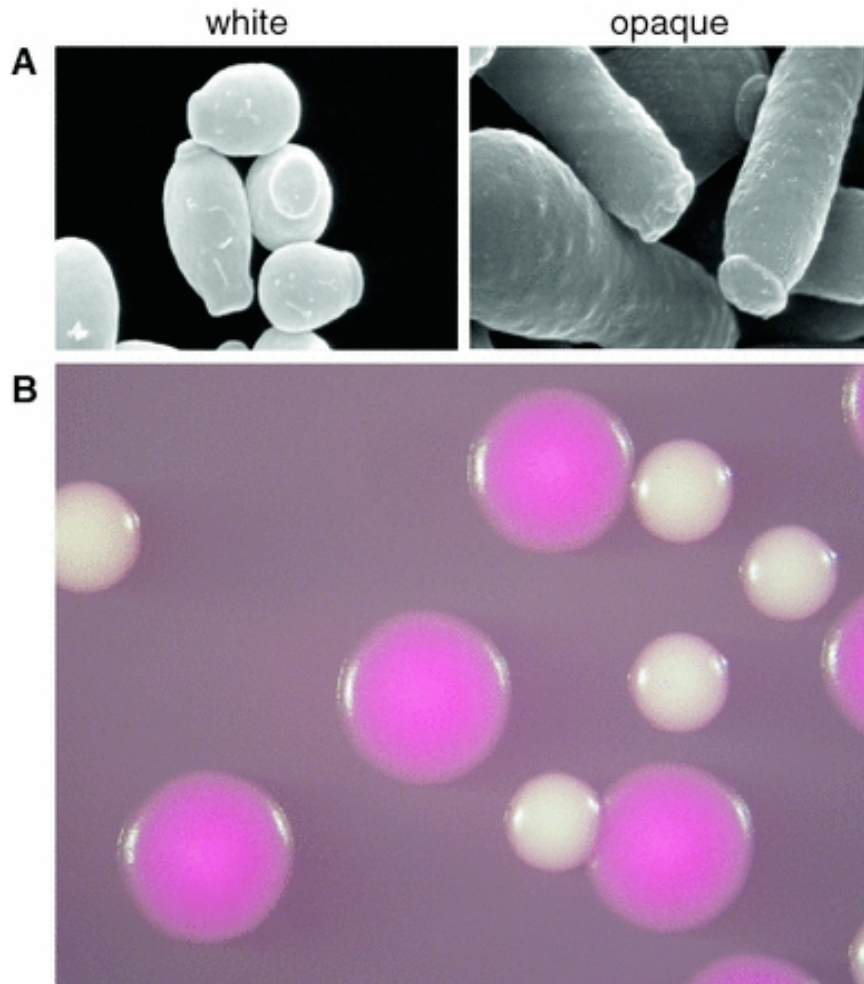


Figure 4. White and Opaque Phenotypes of *C. albicans*. Adopted from [153]. Reproduced with permission from Medical Microbiology and Immunology. **(A)** Scanning electron micrographs of white and opaque cells. **(B)** White and opaque colonies grown on media containing the dye phloxine B.

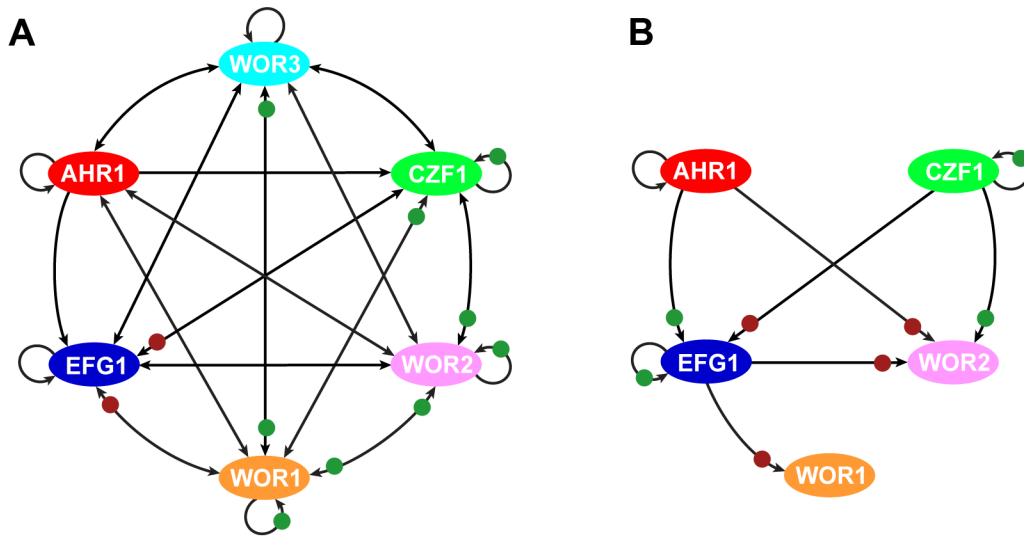


Figure 5. Core Regulatory Network of White-Opaque switching in *C. albicans*. Adopted from [125]. Reproduced with permission from Wiley. Dark green circles denote known positive regulation, while dark red circles denote known repression. Arrows denote direct binding of transcription factor. **(A)** Opaque cell regulatory circuit. **(B)** White cell regulatory circuit.

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Chapter 2: Discovery of a Phenotypic Switch Regulating Sexual Mating in the Opportunistic Fungal Pathogen *Candida tropicalis*

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Supplemental material that accompanies this paper is located in **Appendix A**.

AMP and KA performed phenotypic switching and mating experiments. AMP performed microarray analysis. AMP constructed *C. tropicalis* strains with the help of RJB and KA with PCRs and plasmid construction. KA performed FACs analysis and RT-PCR. RJB performed fluorescence microscopy. MPH performed scanning electron microscopy experiments.

Allison M. Porman¹, Kevin Alby^{1,2}, Matthew P. Hidakawa¹, and Richard J. Bennett^{1,3}

¹ Molecular Microbiology and Immunology Department, Brown University,
Providence RI 02912

² Current Address: UNC Hospitals, McLendon Clinical Laboratories, Chapel Hill, NC
27514

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2.1 Abstract

Sexual reproduction can promote genetic diversity in eukaryotes, and yet many pathogenic fungi have been labeled as obligate asexual species. It is becoming increasingly clear, however, that cryptic sexual programs may exist in some species, and that efficient mating requires the necessary developmental switch to be triggered. In this study we investigate *Candida tropicalis*, an important human fungal pathogen that has previously been reported to be asexual [2]. Significantly, we demonstrate that *C. tropicalis* utilizes a novel phenotypic switch to regulate a cryptic program of sexual mating. Thus, diploid **a** and α cells must undergo a developmental transition to the mating-competent form, and only then does efficient cell-cell conjugation take place resulting in the formation of stable **a/a** tetraploids. We show that both the phenotypic switch and sexual mating are

dependent on the conserved transcriptional regulator Wor1, which is regulated by temperature in other fungal species. In contrast, *C. tropicalis* mating occurs efficiently at both 25°C and 37°C, suggesting that it could occur in the mammalian host and have direct consequences for the outcome of an infection. Transcriptional profiling further reveals that approximately 400 genes are differentially expressed between the two phenotypic states, including the regulatory factor Wor1. Taken together, our results demonstrate that *C. tropicalis* has a previously unrecognized sexual program, and that entry to this program is controlled via a Wor1-mediated, meta-stable switch. These observations have direct implications for the regulation and evolution of cryptic sexual programs in related fungal pathogens.

2.2 Introduction

The prevalence of sexual reproduction amongst eukaryotic organisms indicates that sex brings significant benefits despite the costs associated with the sexual lifestyle (1, 2). Benefits include increased rates of recombination and accelerated adaptation, which may be particularly advantageous during exposure to hostile environments (3, 4). Sexual reproduction may also help organisms maintain a fitness advantage against coevolving pathogens, a tenet of the Red Queen hypothesis (5). In this model, sexual outcrossing is necessary if an organism is to generate sufficient genetic variation to outpace infectious agents. Recent experiments have provided support for this hypothesis, as populations of

Caenorhabditis elegans are better suited to survive bacterial infection if able to undergo sexual outbreeding (6, 7).

Many fungal species have also been investigated for their ability to undergo sexual reproduction, and the yeast *Saccharomyces cerevisiae* has served as an excellent model system for studies into sexual development. Fungal sex in this hemiascomycete species is regulated by genes encoded at the mating-type (*MAT*) locus. *MATa*-containing cells are able to mate with *MATa*-containing cells to generate *MATa/a* mating products. *MATa/a* cells are unable to mate but are competent to undergo meiosis under conditions of nutrient starvation.

Transcription factors encoded at the *MAT* locus regulate the characteristics of the three cell types, *a*, *a*, and *a/a*, that together define the sexual program of *S. cerevisiae* and many related fungi (8, 9).

Genome sequencing has revealed the presence of mating-type-like (*MTL*) loci in other hemiascomycete species, including those with apparently obligate asexual lifestyles (10, 11). This is best exemplified by *Candida* species, which were originally classified as yeasts that propagate by asexual budding and form hyphae or pseudohyphae, yet lack sexual cycles and ascospore production (12). The *Candida* genus is now recognized as containing a large number (>150) of diverse species due to its broad classification (12). Many *Candida* species are medically relevant, and together represent the most common cause of opportunistic fungal infections in humans (13). While complete sexual cycles have been described for several *Candida* species, the most prevalent human pathogens either lack sexual mating or have restricted sexual cycles, which may indicate that limiting sexual reproduction can

benefit fungal pathogenesis (14-16). In particular, rare sexual reproduction could allow propagation of a well-adapted clonal lineage in the host, while retaining the ability to undergo sexual recombination in response to stressful environments. Further understanding of the lifestyles of pathogenic fungi is therefore important for establishing how mating and virulence have evolved in these species.

The *Candida* species most commonly encountered in the clinic are *Candida albicans*, *Candida tropicalis*, *Candida parapsilosis*, and *Candida glabrata*, which together are responsible for more than 85% of invasive infections (13). Strikingly, of these species, only *C. albicans* has been shown to undergo sexual mating (17, 18), and entry to the sexual program is regulated by a unique phenotypic switch (19). Thus, *C. albicans* α and α cells must undergo a heritable switch from the white form to the opaque form to be mating competent. The phenotypic switch is regulated by the Wor1 transcription factor, as high expression of this factor is necessary and sufficient for formation of the opaque state (20-23). Homologs of Wor1 are found across the entire fungal lineage (24), although the white-opaque switch is thought to be unique to *C. albicans* (and its sister species *C. dubliniensis*), and has not been described in any other hemiascomycete species (25-27). In contrast to *C. albicans*, sexual mating has never been observed in *C. tropicalis*, *C. parapsilosis*, or *C. glabrata*, despite extensive efforts to find laboratory conditions conducive to mating (28-32). Sequence analysis reveals that 'asexual' *Candida* species contain *MAT/MTL* loci resembling those of sexual species, and they encode many of the genes necessary for mating and meiosis (10, 11, 31). It is therefore an open question whether these

species have lost the ability to undergo sex, or whether the developmental signals necessary for mating have yet to be uncovered (28, 33).

In this study, we address the potential for diploid *C. tropicalis* strains to undergo a cryptic sexual program. Surprisingly, our experiments reveal that *C. tropicalis* exhibits a novel form of white-opaque phenotypic switching, and that this switch regulates an extant program of sexual reproduction. Thus, only cells in the opaque state undergo efficient cell-cell conjugation. Mating occurs efficiently at 37°C, making it likely that sexual recombination can occur during colonization and infection of the mammalian host. We further show that the phenotypic switch in *C. tropicalis* is regulated by the conserved transcription factor, Wor1. However, transcriptional regulation of the *C. tropicalis* phenotypic switch shows fundamental differences to that of the white-opaque switch in *C. albicans*, which may account for the distinct switching characteristics of the two species. We discuss these findings as they reveal the regulation of the hitherto undiscovered sexual program in *C. tropicalis*, and propose that cryptic sexual cycles remain to be discovered in related *Candida* pathogens.

2.3 Materials and Methods

A complete description of all materials and methods can be found in **Appendix A**.

Media. Media was prepared as described previously (34, 35). Yeast extract peptone dextrose (YPD) plates containing 200 µg/ml nourseothricin (NAT) was used for selection of strains that were resistant to nourseothricin (*SAT^R* strains)(36).

Mating Assays. Quantitative mating analyses of *C. tropicalis* strains were performed essentially as described for *C. albicans* (19). Briefly, His⁻ and Arg⁻ mating strains in the white or opaque phase were taken from plates and resuspended in water, and approximately 2×10^7 cells of each strain mixed and pipetted onto 0.8-µm-pore-size nitrocellulose filters. Filters were grown on the surface of Spider media for 1 day at room temperature. Cells were collected from the filters and plated at different dilutions onto His⁻ Arg⁻ media to select for mating products and onto His⁻ and Arg⁻ plates to monitor each parent population. The limiting parent was used to calculate mating frequencies as follows: mating efficiency = conjugants / (limiting parent + conjugants) = the greater of (Arg⁻ His⁻) / Arg⁻ or (Arg⁻ His⁻) / His⁻. Cell ploidies were determined by flow cytometry as described previously (17). PCR to determine mating type was performed using primers 47/48 directed at *MTLa2* and primers 49/50 directed at *MTLα2*.

RT PCR. RNA was isolated from approximately 2×10^8 cells plated onto Spider media for 4 h. Cells were removed from the media and RNA was isolated using the

RibopureTM-Yeast Kit (Ambion). RNA was treated with Turbo DNase (Ambion), and 500 ng of RNA was used for subsequent cDNA generation using the GoScriptTM enzyme (Promega). PCR was then performed using gene specific primers listed in Table A2.

2.4 Results

2.4.a Identification of a phenotypic switch in *C. tropicalis*

To address the potential for sexual mating in *C. tropicalis*, we first generated isogenic *MTLa* and *MTL α* strains from the diploid **a**/ α parental strain ATCC34139 (see Materials and Methods). Mixing of **a** and α cells on a variety of media did not, however, result in either the formation of polarized mating projections or mating zygotes, as typically seen during mating of many hemiascomycete species (Figure A1). We subsequently examined whether *C. tropicalis* **a** and α strains may undergo phenotypic switching, in which there is a heritable change either in the cell or colony phenotype. A phenotypic switch between white and opaque states was previously shown to regulate the sexual program in *Candida albicans*, as efficient cell-cell conjugation is dependent on cells being in the opaque state in this species (19, 37).

C. tropicalis strains were analyzed under a variety of *in vitro* culture conditions and occasional sectoring of colonies was observed, indicative of phenotypic switching (Figure 6A). Cells from sectoried colonies were restreaked and found to represent a stable, heritable state distinct from that of the parental cells.

Analysis of cells from the two phenotypic states also revealed differences at the cellular level. Cells from the lighter-colored parental colonies appeared round and had a smooth cell surface, while cells from the darker-colored colonies were elongated and exhibited a spotted cell surface (Figure 6B-E and Figure A3). Cells in both states appeared to be meta-stable; cell types were heritable for multiple generations, with stochastic switching observed between states. On average, we observed white-to-opaque switching at a frequency of ~0.3% in **a** strains and ~1% in α strains, while no white-opaque switching was seen in the **a/a** parental strain. Due to the superficial similarities between the *C. tropicalis* phenotypes and those previously described in *C. albicans*, we have termed the light and dark states of *C. tropicalis* white and opaque, respectively. It should be noted, however, that several characteristic features of *C. albicans* opaque cells are not seen in *C. tropicalis*, such as red staining with the chemical phloxine B (38) (Figure A2), and switching of opaque cells *en masse* to white cells at 37°C (39, 40) (Figure 6F).

2.4.b Mating in *C. tropicalis*

To test for *C. tropicalis* mating in both the white and opaque states, we constructed auxotrophic **a** and α strains defective in histidine ($\Delta his1/\Delta his1$) or arginine ($\Delta arg4/\Delta arg4$) metabolism. Successful mating events could subsequently be monitored by selection for His⁺ Arg⁺ prototrophs. As shown in Figure 7A, mating between **a** and α cells was observed using both white and opaque forms of the species, but the frequency of mating increased more than 100-fold when both partners were in the opaque form. When white cells were mated with opaque cells,

the mating efficiency was intermediate to that of white-white and opaque-opaque crosses (Figure 7A). These results indicate that mating is most efficient when cells are in the opaque state in both mating-types. Same-sex mating was not detected between different auxotrophic strains, indicating that mating takes place specifically between **a** and α partners. Surprisingly, mating in *C. tropicalis* was also highly efficient at 37°C, whereas the instability of *C. albicans* opaque cells prevents mating at this temperature *in vitro* (Figure 7B). PCR analysis of the *MTL* locus confirmed that **a/a** cells were generated from **a** and α parental strains (Figure 7C). In addition, DAPI (4',6'-diamidino-2-phenylindole) staining and flow cytometry indicated that the majority of mating products were mononuclear, tetraploid cells (Figure 7D and Figure A3).

Genetic analysis of *C. tropicalis* mating was used to address the specificity of cell-cell conjugation. In hemiascomycete yeast, *STE2* encodes the receptor for a pheromone, while *STE3* encodes the receptor for **a** pheromone (41). Loss of *STE2* therefore prevents mating of **a** cells while loss of *STE3* prevents mating of α cells. Gene deletions of *STE2* or *STE3* were generated in auxotrophic *C. tropicalis* strains, and subsequently tested for the ability to undergo mating. Deletion of *STE2* in **a** cells or *STE3* in α cells resulted in a complete block in *C. tropicalis* mating, while reintegration of the deleted gene successfully restored mating competency (Figure 7E). These results confirm that **a**/ α cells are products of cell-cell conjugation and that mating is dependent on the canonical pheromone signaling pathways conserved amongst hemiascomycetes.

2.4.c Cell Biology of Mating in *C. tropicalis*

To visualize the process of mating in *C. tropicalis* we utilized several complementary microscopy techniques. First, the two mating types were differentially labeled with FITC-ConA and Rhodamine-ConA, which labels the cell walls of *Candida* cells green and red, respectively (42). Labeled cells were then co-incubated on Spider medium to induce mating and subsequently analyzed for cell-cell conjugants. Within several hours of co-incubation, mixtures of opaque **a** and α cells showed polarized projections, indicative of cell “shmooing” in response to pheromone. In addition, mating zygotes were readily observed, in which **a** and α cells had fused and daughter cells started to bud from the center of the fusant cell (Figure 8A). Similar experiments using white **a** and α cells showed only rare fusant cells, confirming that opaque cells are necessary for efficient cell-cell conjugation.

The process of nuclear fusion (karyogamy) during *C. tropicalis* mating was examined by generating strains with fluorescently labeled nuclei. Histone proteins fused to GFP or mCherry demonstrated that nuclei from opaque **a** and α cells had undergone fusion within a subset of mating zygotes, as revealed by the presence of a single nucleus showing red/green fluorescence (Figure 8B). In addition, scanning electron micrographs of zygote cells were taken that provide high resolution images of mating cells (Figure 8C and Figure A4). Taken together, these experiments demonstrate that even in the absence of genetic selection, mating between opaque **a** and α cells leads to the formation of mononuclear, tetraploid *C. tropicalis* mating products.

2.4.d Analysis of the *C. tropicalis* white-opaque switch

To compare the transcriptional profiles of *C. tropicalis* white and opaque cells, custom microarrays were hybridized with cDNA derived from both cell states. These experiments revealed that distinct sets of genes are expressed in the two phenotypic states, with 250 genes upregulated in white cells and 146 genes upregulated in opaque cells (Figure 9A and Tables A4 and A5). Comparison with *C. albicans* white-opaque cell types (40, 43-46) revealed that most phase-specific genes were species-specific. Thus, of the 134 opaque-specific genes in *C. tropicalis* that have homologs in *C. albicans*, only 25 (19%) were opaque-specific in both species. In fact, 15 genes that were expressed in the opaque state in *C. tropicalis* were white-specific genes in *C. albicans* (Table A5). Conversely, 218 of the *C. tropicalis* white genes have *C. albicans* counterparts, and of these 62 (28%) were white-specific in *C. albicans* (Figure 9C and Appendix A). Again, a number of genes were expressed in the opposite cell type in the other species, as 31 white-specific genes in *C. tropicalis* were opaque-specific in *C. albicans* (Table A4). Despite apparent large-scale differences in gene expression, analysis of white- and opaque-specific genes in *C. tropicalis* and *C. albicans* indicated that there was a statistically significant overlap of phase-specific genes between the two species (Appendix A).

2.4.e *C. tropicalis* *Wor1* regulates phenotypic switching and mating

One notable gene found to be upregulated in opaque cells of both *C. tropicalis* and *C. albicans* was *WOR1*. *WOR1* is the master regulator of the white-opaque phenotypic switch in *C. albicans*, and its expression is both necessary and sufficient

for formation of the opaque state (20-23). Expression of *WOR1* is low in *C. albicans* white cells but is high in opaque cells. We show here that *C. tropicalis* *WOR1* is also preferentially expressed (>5-fold) in the opaque form (Figure 9A). Upregulation of the *WOR1* gene in *C. tropicalis* opaque cells was verified using RT-PCR (Figure 9B). To analyze the potential role of Wor1 in *C. tropicalis* switching, *WOR1* was deleted in **a** and α strain backgrounds. Significantly, no detectable switching to the opaque form was observed in $\Delta wor1/\Delta wor1$ strains, indicating that Wor1 function is critical for promoting opaque cell formation in both *C. tropicalis* and *C. albicans*.

The role of *WOR1* in regulating *C. tropicalis* mating was investigated by deletion of the *WOR1* gene in auxotrophic **a** and α strain backgrounds. Strikingly, crossing of these strains revealed that mating was essentially abolished between $\Delta wor1$ **a** and $\Delta wor1$ α cells (limit of detection $<10^{-7}$). Mating between a $\Delta wor1$ white strain and a wildtype white strain was also extremely low (in the range of 10^{-5} to 10^{-6}). Reintroduction of the *WOR1* gene into $\Delta wor1$ strains restored mating ability, as *WOR1* complemented strains mated with wildtype opaque strains at 2.7% efficiency, confirming the key role of this gene in mating. These results establish that deletion of *WOR1* eliminates even the low frequency mating exhibited by white cells. Two models could account for this result. First, *WOR1* is required for white cells to switch to opaque, and these switched cells then undergo mating. Alternatively, Wor1 expression in white cells could be directly relevant for the appreciable mating seen in these cell types.

Wor1 regulation of the *C. albicans* white-opaque switch involves interlocking transcriptional feedback loops with three additional factors, Czf1, Wor2, and Efg1.

Czf1 and Wor2 promote formation of the opaque state, while Efg1 promotes formation of the white state (23). Surprisingly, expression of *C. tropicalis* *CZF1* and *WOR2* did not change significantly between white and opaque cells. In addition, the *EFG1* gene is absent from *C. tropicalis*, and expression levels of a related APSES transcription factor, *EFH1* (47), were similar between white and opaque forms. In *C. albicans* the Mcm1-Ahr1 complex also regulates the white-opaque switch, yet the motif recognized by this complex is absent from other *Candida* species including *C. tropicalis* (27, 48, 49). Taken together, these findings establish that the transcriptional circuitry regulating the phenotypic switch in *C. tropicalis* is fundamentally distinct from that regulating the white-opaque switch in *C. albicans*.

2.5 Discussion

In this paper, we demonstrate that *Candida tropicalis*, one of the most prevalent human fungal pathogens, has a hitherto unrecognized sexual program, and that sexual reproduction is regulated by a novel phenotypic switch. These findings provide additional support for the theory that many 'asexual' fungal pathogens are actually sexual species when the appropriate conditions can be established. In the case of *C. tropicalis*, diploid *MTLa* and *MTL α* cells must undergo a developmental transition from the white form to the opaque form before efficient cell conjugation can take place. Following the formation of mating zygotes, karyogamy can ensue between parental nuclei resulting in stable, mononuclear, tetraploid **a**/ α cells. This discovery has important ramifications for the lifestyle of this pathogenic fungus, as it indicates that recombinant forms of the species can be generated via cell conjugation. *C. tropicalis* mating is efficient even at 37°C, making it likely that strains can undergo sexual exchange during colonization and infection of the mammalian host. In support of mating in natural populations, an analysis of clinical isolates has revealed that the population structure of *C. tropicalis* is consistent with it being a sexually active species (50). The discovery of sexual reproduction is significant, as mating could potentially result in recombinant strains with increased virulence. This has been reported in other human fungal pathogens such as *Cryptococcus gattii*, which is responsible for an ongoing outbreak in the Pacific Northwest of the United States and Canada (51-53).

The discovery that a phenotypic switch regulates *C. tropicalis* mating indicates that this mechanism is used to control sexual reproduction in multiple

pathogenic *Candida* species. Previous studies established that *C. albicans*, the most common human fungal pathogen, undergoes a cryptic sexual program that is controlled by a white-opaque phenotypic switch (19). In this system, only cells in the opaque state are competent for efficient mating. It was originally thought that this switch was unique to *C. albicans* (and its closely related sister species *C. dubliniensis*), in part because a similar switch had not been observed in any other hemiascomycete yeast (26). The results described here now require revision of this model, as they indicate that a white-opaque switch also exists in *C. tropicalis* and likewise acts to regulate the program of sexual reproduction.

Despite physical similarities between white-opaque switching in *C. albicans* and *C. tropicalis*, transcriptional regulation of the switch is significantly different in the two lineages. For example, white-opaque switching in *C. albicans* is regulated by the Mcm1-Ahr1 complex, yet the motif recognized by this complex is absent from species other than *C. albicans*/*C. dubliniensis* (27, 48, 49). In addition, the Efg1 transcription factor that is essential for formation of white cells in *C. albicans*, and is conserved in most hemiascomycete yeast, has been lost from the *C. tropicalis* genome (10). While the current data does not allow us to formally distinguish between a model for convergent or divergent evolution of the white-opaque switch in *C. albicans* and *C. tropicalis*, our data is consistent with the ancestor of these species evolving a *WOR1*-regulated switch, which has since undergone transcriptional rewiring events since these two species diverged. Further analysis is now required to fully define how the white-opaque switching mechanisms evolved and the means by which they regulate sexual reproduction.

The most notable aspect of white-opaque switching shared between *C. tropicalis* and *C. albicans* is regulation by Wor1. Wor1 is the master regulator of white-opaque switching in *C. albicans* and its expression is both necessary and sufficient for formation of the opaque state (20-23). Interestingly, Wor1 is also recognized as being the founding member of a family of DNA binding proteins that are conserved across the fungal lineage (24). Wor1 orthologs have been studied in distantly related fungi including *Histoplasma capsulatum*, where the Ryp1 protein regulates a morphologic switch (54, 55), and *Fusarium oxysporum*, where Sge1 is necessary for parasitic growth in the plant (56). Curiously, *C. albicans* Wor1 and *H. capsulatum* Ryp1 are associated with temperature-induced changes in cell fate. In the case of *C. albicans*, opaque cells are unstable at 37°C, as Wor1 expression is compromised and cells rapidly convert *en masse* to the white state (39, 40). In contrast, *C. tropicalis* opaque cells are stable at 37°C, thereby allowing efficient mating at the body temperature of the mammalian host. Differences in the transcriptional regulation of the switch may account, at least in part, for the differential stability of the opaque states in *C. albicans* and *C. tropicalis*. Deletion of Ahr1 (also called Zcf37) in *C. albicans* increased the temperature stability of opaque cells (49), and it is therefore possible that the loss of this regulatory factor in *C. tropicalis* contributes to the increased thermal stability of the opaque state in this species.

While several studies have begun to dissect the mechanism of phenotypic switching, it remains an open question as to the precise role of this switch in *Candida* biology (57-59). One hypothesis is that white and opaque cells evolved

differential responses to pheromone in order to promote mating in the host. This model is based on the observation that whereas *C. albicans* opaque cells are the mating-competent form, white cells can also respond to sexual pheromones resulting in biofilm formation (60, 61). The resulting biofilm matrix allows stable pheromone gradients to be generated between opaque cells, thereby potentially enhancing mating in the host. Biofilms are also an important first step in medical device-associated infections, making an understanding of these processes important for fungal pathogenesis (62, 63). The discovery of a white-opaque switch in *C. tropicalis* will now allow further testing of this model, and will reveal if biofilm formation is also regulated by pheromone signaling and white-opaque switching in this species.

Finally, it is becoming apparent that highly specialized sexual cycles are the norm rather than the exception for human fungal pathogens. Discovering the developmental signals necessary for activation of cryptic sexual programs is therefore a key goal for the field. The results presented here establish Wor1 as a critical regulator of sexual activity in *Candida* species, as Wor1 is required for phenotypic switching and sexual mating in both *C. albicans* and *C. tropicalis*. This result was particularly striking in *C. tropicalis*, where the loss of Wor1 further reduced the mating efficiency of white cells by several orders of magnitude. It will therefore be revealing to test if engineered expression of Wor1 can promote mating in related 'asexual' species such as *C. parapsilosis* (64), as we predict that cryptic sexual programs remain to be discovered in related human fungal pathogens.

2.6 Figures

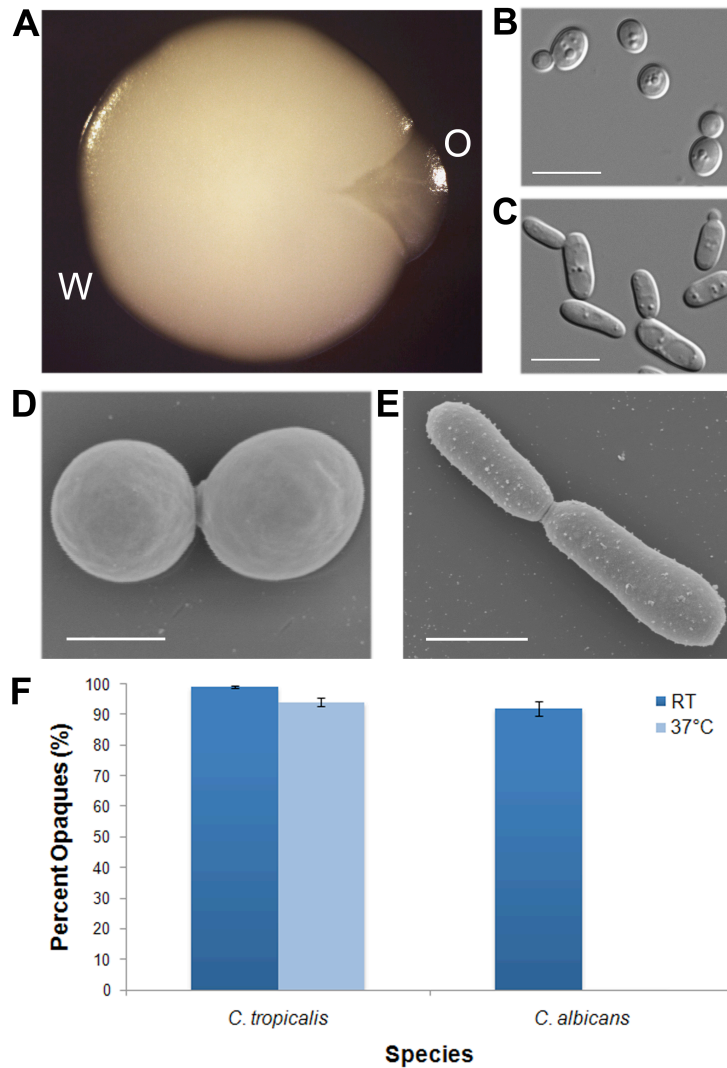


Figure 6. Phenotypic switching in *C. tropicalis*. Colony and cellular morphologies of *C. tropicalis* ‘white’ and ‘opaque’ states. **(A)** A white colony of *C. tropicalis* (CAY1504) that has a sector in which cells have switched to opaque. **(B, C)** Cells from white colonies of *C. tropicalis* exhibit a characteristic round shape (B), while cells from opaque colonies exhibit an elongated cell shape (C). Scale bar, 10 μ m. **(D, E)** Scanning electron micrographs of white and opaque cells. White cells (CAY1504) exhibit a smooth cell surface, while opaque cells (CAY2275) typically exhibit a spotted cell surface. D, scale bar, 2.5 μ m; E, scale bar, 5 μ m. **(F)** Comparison of opaque stability in *C. tropicalis* (CAY2054) and *C. albicans* (RBY1179). Cells were grown for 1 day on YPD medium at room temperature or at 37°C and re-plated and grown at room temperature to determine their phenotype.

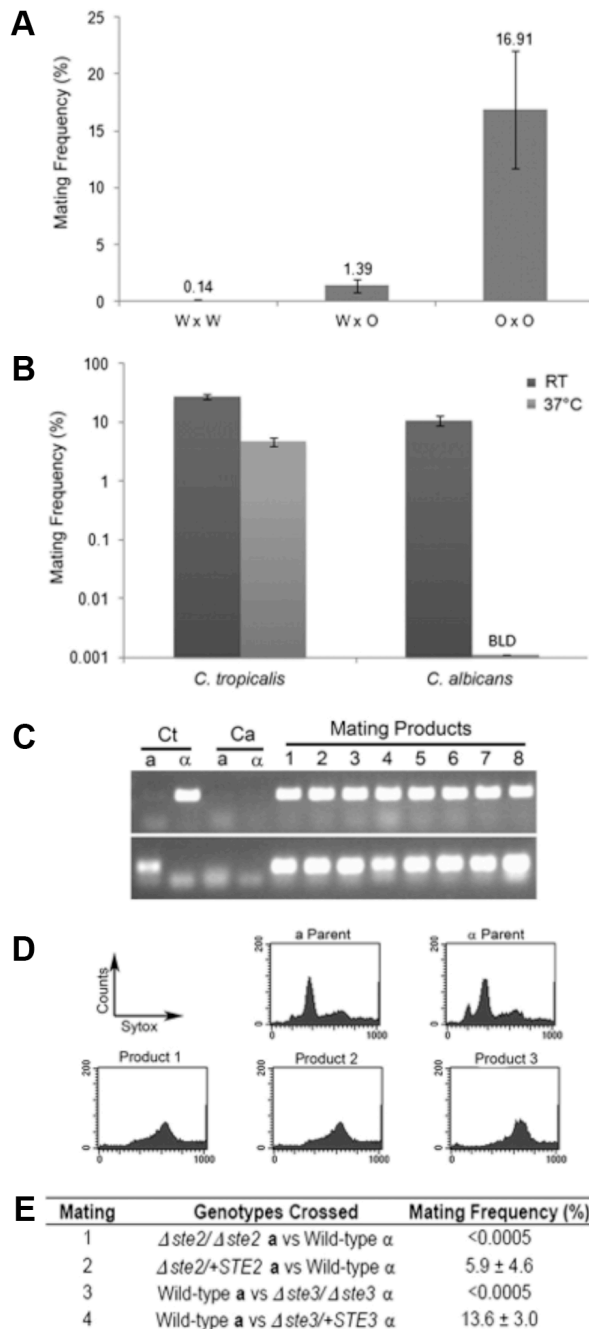


Figure 7. Quantitative mating of *C. tropicalis* white and opaque cells. (A) Mating of *C. tropicalis* strains at room temperature. White (W) or opaque (O) versions of auxotrophic strains were co-incubated on Spider medium for 1 day and cells subsequently plated on selective media to determine the efficiency of mating. Data shows mating between a/a (CAY1503 or CAY1504) and α/α (CAY1505) strains. (B) Mating of *C. tropicalis* and *C. albicans* opaque cells at room temperature and 37°C. Mating mixes were incubated on Spider medium for 1 day and plated to selective media to determine the mating frequency. *C. albicans* strains used were RBY1119, RBY1179 (65). BLD = below limit of detection (0.0005%). Error bars represent standard error. (C) PCR confirms that *C. tropicalis* mating products are a/a cells. Top panel, PCR of the *MTL α 2* gene, lower panel, PCR of the *MTLa2* gene. (D) Flow cytometric analysis confirms that *C. tropicalis* mating products are tetraploid. (E) Table of mating frequencies for different mating crosses. Crosses 2 and 4 utilize *Dste2* and *Dste3* mutant strains (CAY2200/CAY2202 and CAY2244/CAY2245, respectively) with a wild-type copy of the receptor added back to the native

locus (CAY2246/CAY2247 and CAY2285, respectively). Values are listed as frequency $\pm$ standard deviation.

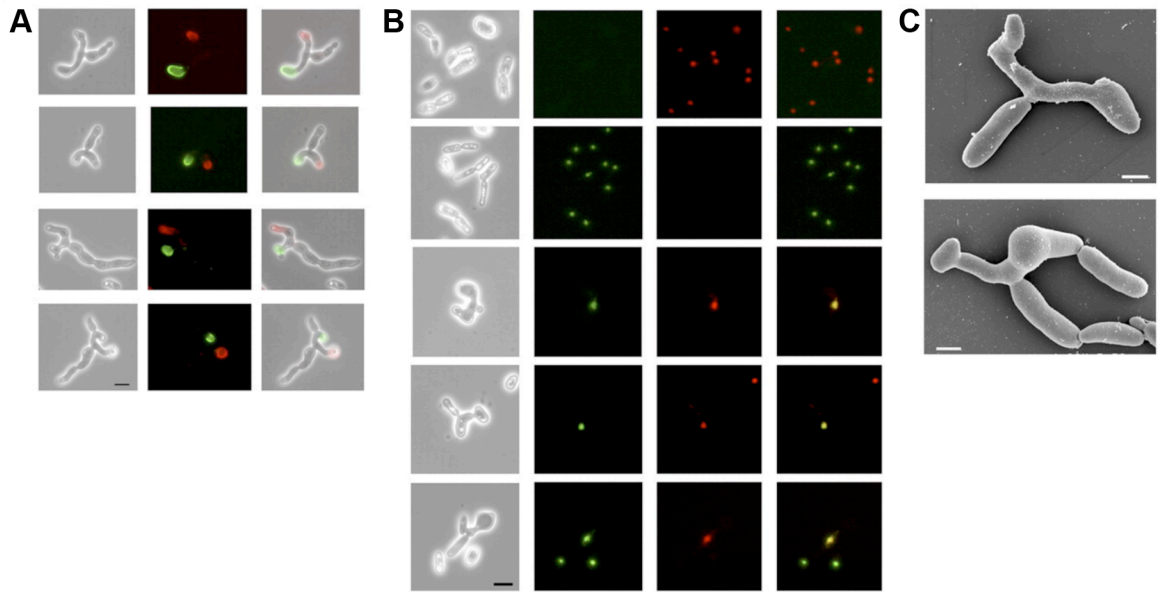


Figure 8. Cell microscopy of mating between *C. tropicalis* opaque *a* and α cells. **(A)** Mating zygotes formed by fusion between opaque *a* and α cells. *MTLa* and *MTL α* cells were incubated with FITC-conA or rhodamine-conA to label cell walls green or red, respectively. Labeled cells were co-incubated on Spider medium for 8 h and zygote cells identified by microscopy. Scale bar, 10 μ m. **(B)** Nuclear fusion during mating between opaque *a* and α cells. Nuclei in parental *MTLa* and *MTL α* cells were labeled by expression of a histone-mCherry or histone-GFP fusion protein, respectively. Nuclear-labeled strains were co-incubated on Spider medium for 5-24 h and zygotes identified by microscopy. Scale bar, 10 μ m. **(C)** Scanning electron micrographs of *C. tropicalis* zygotes. Scale bar, 4 μ m.

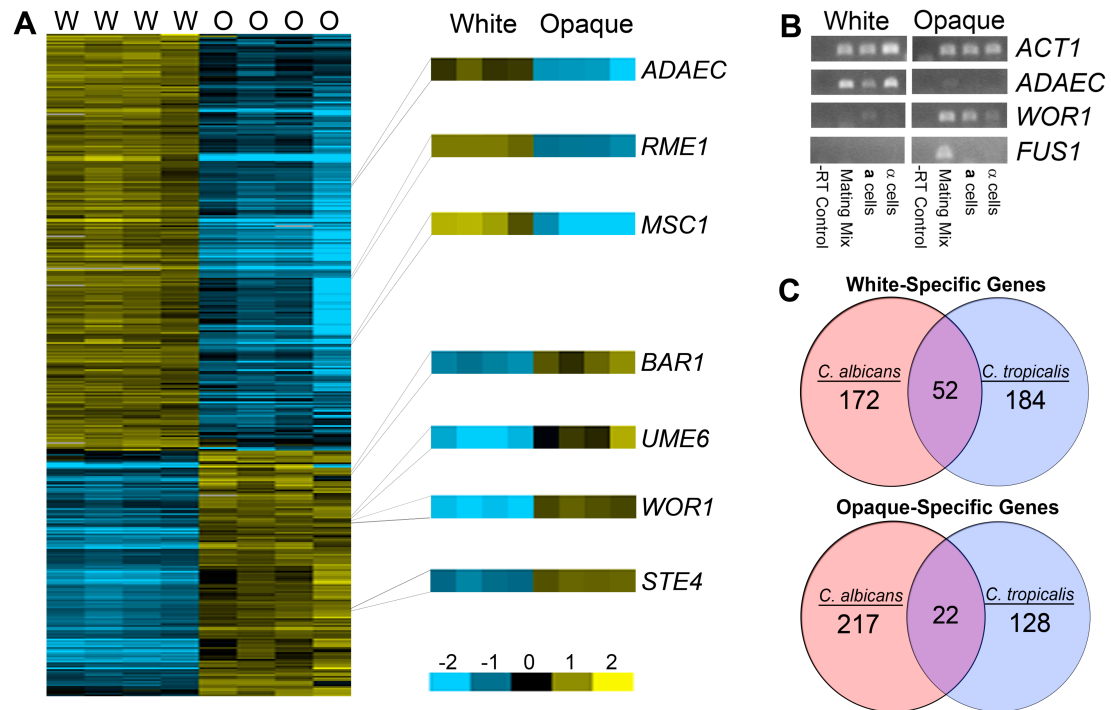


Figure 9. Transcriptional profiling of white and opaque genes in *C. tropicalis*.

(A) Gene expression differences between *C. tropicalis* white and opaque cells. cDNA was prepared from white and opaque cells of RBY1504 (**a/a**) in four independent experiments, and hybridized against a universal reference (cDNA from all 8 samples). Upregulated genes are shown in yellow and downregulated genes are shown in blue. A full list of phase-specific genes is shown in Tables S4 and S5.

(B) RT-PCR showing expression of a white-specific gene (*ADAEC*) and an opaque-specific gene (*WOR1*). The *FUS1* gene is only expressed only in mating mixes of opaque **a** and α cells (bottom panel), and is a mating-specific gene in *C. albicans* and *S. cerevisiae*. **(C)** Comparison of white-specific and opaque-specific genes between *C. tropicalis* and *C. albicans*. Data for *C. albicans* was obtained from references (40, 43-46).

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Chapter 3: *MTL*-Independent Phenotypic Switching in *Candida tropicalis* and a Dual Role for Wor1 in Regulating Switching and Filamentation

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Supplemental material that accompanies this paper is located in **Appendix B**.

AMP performed strain construction, phenotypic switching assays, quantitative mating assays, microscopy, and microarray analysis. SKJ performed biofilm experiments and microarrays. MPH performed virulence experiments. NW contributed to strain construction and phenotypic switching assays.

Allison M. Porman¹, Matthew P. Hirakawa^{1#}, Stephen K. Jones^{1#}, Na Wang¹, and
Richard J. Bennett^{1*}

¹Department of Microbiology and Immunology, Brown University, Providence, RI, 02912, USA.

These authors contributed equally to this work

*Correspondence: Richard_Bennett@brown.edu

3.1 Abstract

Phenotypic switching allows for rapid transitions between alternative cell states, and is important in pathogenic fungi for colonization and infection of different host niches. In *Candida albicans*, the white-opaque phenotypic switch plays a central role in regulating the program of sexual mating as well as interactions with the mammalian host. White-opaque switching is controlled by genes encoded at the *MTL* (mating-type-like) locus which ensures that only **a** or α cells can switch from the white state to the mating-competent opaque state, while **a**/ α cells are refractory to switching. Here, we show that the related pathogen *C. tropicalis* undergoes white-opaque switching in all three cell types (**a**, α , and **a**/ α), and thus switching is independent of *MTL* control. We also demonstrate that *C. tropicalis* white cells are themselves mating-competent, albeit at a lower efficiency than opaque cells. Transcriptional profiling of *C. tropicalis* white and opaque cells reveals significant overlap between switch-regulated genes in *MTL* homozygous and *MTL* heterozygous cells, although twice as many genes are white-opaque regulated in **a**/ α cells as in **a** cells. In *C. albicans*, the transcription factor Wor1 is the master regulator of the white-opaque switch and we show that Wor1 also regulates switching in *C. tropicalis*; deletion of *WOR1* locks **a**, α , and **a**/ α cells in the white state, while *WOR1* overexpression induces these cells to adopt the opaque state. Furthermore, we show that *WOR1* overexpression promotes both filamentous growth and biofilm formation in *C. tropicalis*, independent of the white-opaque switch. These results demonstrate an expanded role for *C. tropicalis* Wor1, including the regulation of processes necessary for infection of the mammalian host.

We discuss these findings in light of the ancestral role of Wor1 as a transcriptional regulator of the transition between yeast form and filamentous growth.

Author Summary

The white-opaque phenotypic switch has been extensively characterized in the human fungal pathogen *Candida albicans*, where it plays a central role in regulating entry into sexual reproduction. This epigenetic switch is strictly regulated by the *MTL* locus so that only **a** or α cell types can switch to the opaque state, whereas **a**/ α cells are locked in the white state. In contrast, we show that in the related pathogen *Candida tropicalis*, white cells are capable of sexual mating and that the white-opaque switch is independent of *MTL* control. Thus, *MTLa*, α , and **a**/ α cells all undergo reversible switching between white and opaque states. Despite these differences, switching in both *C. tropicalis* and *C. albicans* is dependent on the expression of the Wor1 transcription factor. This factor is conserved amongst fungal ascomycetes, and in several species acts as a master regulator of the yeast-to-filament transition. We show that in addition to regulating the white-opaque switch in *C. tropicalis*, Wor1 expression also promotes filamentation and biofilm formation in this species. We therefore propose that *C. tropicalis* Wor1 has retained the ancestral role of this family of transcription factors, while also gaining control over the more recently evolved white-opaque phenotypic switch.

3.2 Introduction

The incidence of opportunistic fungal infections has increased in recent years as a result of immunosuppressive diseases such as AIDS, as well as the use of immunosuppressive drugs in modern medical practices [1]. *Candida* species are typically harmless commensals of humans but are also important fungal pathogens, responsible for both systemic and mucosal opportunistic infections [2]. Most clinically relevant species belong to the *Candida* clade of hemiascomycete yeasts, which diverged from the model yeast *Saccharomyces cerevisiae* 300-600 million years ago [3,4]. Three *Candida* clade pathogens, *Candida albicans*, *Candida dubliniensis*, and *Candida tropicalis*, have been shown to undergo an epigenetic switch between distinct ‘white’ and ‘opaque’ states [5-8], and this phenotypic switch plays a crucial role in modulating behavior. The white-opaque switch has been extensively studied in *C. albicans*, where the two states differ in metabolic preferences [9], environmental responses [10-14], interactions with host immune cells [15,16], and the ability to undergo sexual reproduction [17,18].

C. albicans and *C. dubliniensis* are closely related species that can undergo productive mating with one another [7]. While *C. albicans* represents the most commonly isolated *Candida* species in the clinic, *C. dubliniensis* is rarely found in infections, which may reflect the more limited ability of this species to undergo filamentation [2,19]. *C. tropicalis* is also a prevalent human pathogen, particularly in individuals with neutropenia or hematologic malignancies [2], and shows a similar overall genome structure to that of *C. albicans*, including synteny at the mating-type-like (*MTL*) locus [20]. *C. albicans* and *C. tropicalis* both contain more than 6,000

protein-coding genes, and analysis of the 5,254 orthologs shared between the two species indicates an average protein sequence identity of ~70% [20]. However, relatively little is known about the biology of *C. tropicalis* compared to that of the model species *C. albicans*, including the factors that promote pathogenesis in the mammalian host.

In all three *Candida* species, white and opaque forms are distinguished by differences in cell shape, colony morphology, and gene expression profiles. White cells are generally round and give rise to smooth, shiny colonies, while opaque cells are elongated and produce duller, darker colonies [5,6]. The transcriptional profiles of white and opaque forms are also significantly different, with one-sixth of the transcriptome regulated by the switch in *C. albicans* [9,21,22]. Furthermore, white and opaque forms exhibit differences in the propensity to undergo filamentous growth; *C. albicans* white cells are induced to filament in response to multiple environmental stimuli while opaque cells do not generally undergo filamentation [23,24].

The white-opaque switch plays a particularly prominent role in regulating mating, as only cells in the opaque state undergo efficient conjugation [5,18]. Switching is regulated by *WOR1* such that loss of this gene prevents formation of the opaque state while, in *C. albicans*, *WOR1* overexpression drives cells into the opaque state [5,25-27]. Genes encoded at the *MTL* locus control white-opaque switching in *C. albicans*; only **a** or α cells switch to the opaque state as a complex between *MTLa1* and *MTLa2* proteins blocks **a**/ α cell switching due to repression of *WOR1* [18,21,26]. An analysis of 220 clinical isolates of *C. albicans* further demonstrated that only *MTL*

homozygous (**a/a** or α/α) strains could form stable opaque cells at a detectable frequency [17]. *MTL* regulation ensures that opaque formation only occurs in cells that have the potential to undergo mating, which can take place between *MTL α* and *MTL α* cells or via same-sex mating of these cell types [18,28].

In addition to Wor1, three other transcription factors, Czf1, Wor2, and Efg1, regulate the *C. albicans* white-opaque switch via a network of positive and negative feedback loops [29]. While Wor1 is the master regulator of the opaque state, Czf1 and Wor2 also play positive roles in promoting formation of opaque cells, while Efg1 antagonizes opaque formation and promotes switching to the white state [29]. Surprisingly, the mechanism regulating white-opaque switching in *C. tropicalis* appears distinct from that in *C. albicans*; while switching is dependent on Wor1 in both species, the three associated network transcription factors are not white-opaque regulated in *C. tropicalis* [5]. It is therefore likely that significant differences exist between the mechanisms regulating phenotypic switching in *C. albicans* and *C. tropicalis* [5].

The white-opaque switch is thought to have evolved relatively recently in the *Candida* clade, probably just prior to the divergence of *C. albicans*/*C. dubliniensis* and *C. tropicalis* [5,8]. Although this phenotypic switch is limited to within the *Candida* clade, the master transcriptional regulator Wor1 is conserved across the ascomycete lineage [30]. In *S. cerevisiae*, the Wor1 homolog Mit1 acts as part of a transcriptional network to regulate pseudohyphal formation [31], while in the more distantly related ascomycete *Histoplasma capsulatum*, the Wor1 homolog Ryp1 is a master regulator of the transition between yeast and mycelial forms [32]. It has

therefore been proposed that the ancestral role of Wor1 was the transcriptional regulation of morphological changes, including the control of filamentous growth [31].

In this study, we investigated the white-opaque transition in *C. tropicalis* and uncovered several features that further distinguish it from the analogous switch in *C. albicans*. We first demonstrate that a stable white-opaque switch can occur in *C. tropicalis* **a**/ α cells, indicating that the **a**1/ α 2 complex does not repress switching in this species. Transcriptional profiling reveals that genes regulated by the white-opaque switch in **a**/ α cells show a significant overlap with those regulated by the switch in **a** or α cell types. However, many white-opaque regulated genes are unique to *MTL* heterozygous or *MTL* homozygous cells, indicating that the *MTL* configuration significantly impacts gene expression in the two phenotypic states. Despite these transcriptional differences, the phenotypic switch in all three cell types (**a**, α , and **a**/ $\alpha\alpha\alpha$) is dependent on the master regulator, Wor1; deletion of Wor1 blocked white-to-opaque switching while overexpression of Wor1 forced cells into the opaque state. We further show that Wor1 promotes filamentous growth in *C. tropicalis*, as elevated *WOR1* expression led to a significant increase in both filamentous growth and biofilm formation. These studies provide new insights into the evolution of Wor1 homologs as conserved regulators of filamentation and epigenetic switching.

3.3 Materials & Methods

Ethics Statement. This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals as defined by the National Institutes of Health (PHS Assurance #A3284-01). Animal protocols were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of Brown University. All animals were housed in a centralized and AAALAC-accredited research animal facility that is fully staffed with trained husbandry, technical, and veterinary personnel.

Media. Yeast extract peptone dextrose medium (YPD), synthetic complete dextrose medium (SCD), and Spider medium were made as described previously [34,52]. YPD plates containing 200 µg/ml nourseothricin (NAT) were used for selection of strains that were resistant to nourseothricin (Werner Bioagents, Jena, Germany) as previously described [53]. Lee's media containing 12.5 g/L N-acetylglucosamine (Alpha Acera) was used for switching assays, and Lee's media containing 1.25% glucose was used for biofilm assays. [12,54].

Plasmids and Strains. *C. tropicalis* strains used in this study are listed in Tables S1 and S2. Gene deletions were constructed by using the *SAT1* flipper strategy as described [55]. Strains were transformed with 1–4 µg of DNA by using a modified electroporation protocol [5]. To delete *WOR1*, *HIS1*, *ARG4*, *MTLa2*, and *MTLa1* genes using the *SAT1*-flipper method, oligonucleotides were used to amplify ~900 bp of the 5' and 3' homologous flanks of each gene. The resulting PCR products were

digested with restriction enzymes noted in Table S3 and cloned into the plasmid pSFS2a [55]. The resulting plasmids were digested with restriction enzymes as noted in Table S4 to liberate cassettes containing the 5' and 3' gene flanks as well as the *SAT1* selectable marker and used for transformation. Correct genomic integration of transformant colonies was confirmed by PCR of the 5' and 3' junctions (for oligonucleotides, see Table S3). The *SAT1* marker was recycled by growing transformants on maltose media at room temperature or 30°C and subsequent replica patching to both YPD and YPD+NAT plates. Alternatively, the *SAT1* marker was recycled by growing cells in liquid YEP + 2% maltose at room temperature for ~2 days and selected by plating to YPD+ low NAT (10 µg/mL), as previously described [55]. The transformation process was repeated to delete the remaining copy of the gene, and loss of the ORF was confirmed by PCR. To overexpress *WOR1*, fusion PCRs were performed to create a *pTDH3-WOR1* construct, which was cloned into pSFS2a. The plasmid was linearized in the *TDH3* promoter with *SmaI* and transformed. Correct genomic integration was confirmed by PCR. To insert a *SAT1* marker next to the *MTL*, PCR was performed to amplify a sequence immediately upstream of the *MTL*, which was cloned into pSFS2a. The plasmid was linearized within this sequence with *EcoRI* and transformed into *C. tropicalis*. Correct genomic integration next to the *MTLa* or *MTLα* locus was confirmed by PCR.

Mating assays. Quantitative mating assays between *C. tropicalis* strains were performed as described previously [5,18]. In brief, *C. tropicalis* cells were taken from Spider plates that had been grown at room temperature for 1-2 days and

resuspended in water. Approximately 1×10^7 cells of each strain was mixed and pipetted onto 0.8- μm pore-size nitrocellulose filters and grown on the surface of Spider medium for 1-3 days at room temperature. Cells were collected from the filters and plated at different dilutions onto His⁻ Arg⁻ media to select for mating products and onto His⁻ and Arg⁻ plates to monitor each parent population. The limiting parent was used to calculate mating frequencies as follows: mating efficiency = conjugants/(limiting parent + conjugants) = the greater of (Arg⁻ His⁻)/Arg⁻ or (Arg⁻ His⁻)/His⁻. Statistical significance was determined using a Student's T-test.

Phenotypic Switching Assays. White phase cells were inoculated into liquid SCD medium and incubated at room temperature overnight. Cells from this culture were diluted to 0.1 OD₆₀₀ and incubated at 37°C for 5 days. Cultures were diluted in water and plated onto Spider medium or Lee's medium containing N-acetylglucosamine at a concentration of ~100 colonies per plate. Colonies were examined for opaque sectors after growth at room temperature for 7-10 days.

RT-PCR. RNA was isolated from cells grown in Spider liquid at 0.8-1.0 OD₆₀₀ using the Ribopure-Yeast Kit (Ambion). RNA was treated with Turbo DNaseI (Ambion), and 2 μg of RNA used for subsequent cDNA generation using the GoScript enzyme (Promega). qRT-PCR was then performed by using the gene specific primers listed in Table S3 with the SYBR® Green Kit (Applied Biosystems) ran on an Applied Biosystems 7300 Real-Time PCR System.

Microarrays. Cells were harvested after being grown to OD₆₀₀ 1.0-1.2 in Spider medium at room temperature. Cells were collected, flash frozen, and stored at -80°C. For microarrays performed on biofilms, cells were collected after the two-day incubation detailed in the biofilm method. Total RNA was extracted from cell pellets using the RiboPure-Yeast Kit protocol (Ambion). RNA was treated with Turbo DNaseI (Ambion) to eliminate DNA contamination and re-extracted with phenol/chloroform. Aminoallyl-labeled cDNA synthesis and hybridization to custom Agilent *C. tropicalis* microarrays was previously described by Porman *et al.* [5]. Arrays were scanned on a GenePix 4000 scanner (Axon Instruments), data quantified using GENEPIX PRO version 3.0 and normalized using Goulphar (<http://transcriptome.ens.fr/goulphar>). Data analysis was performed as previously described [5]. GO term analysis was facilitated by CGD (<http://candidagenome.org>) and Princeton University's Generic GO Term Mapper (<http://go.princeton.edu/cgi-bin/GOTermMapper>). Array data is available from GEO (accession numbers GSE40179, GSE42517 and GSE43267).

Microscopy. Digital images of colonies were collected using a Zeiss Stemi 2000-C microscope using an Infinity 2 digital camera and Infinity Analyzer software (Lumenera Corporation, Ottawa, Canada). Differential interference contrast (DIC) of cells were captured using a Zeiss Inverted Microscope (Axio Observer. Z1) fitted with an AxioCam HR. Images were processed with AxioVision Rel. 4.8 (Zeiss, Germany).

For electron micrographs, cells were resuspended in water and attached to poly-L-lysine coated-coverslips. Samples were fixed with 2.5% (w/v) glutaraldehyde in 0.1 M Na-cacodylate buffer, pH 7.4 at 4°C, and washed with 0.1 M Na-cacodylate buffer, pH 7.4. Samples were then treated with 1% aqueous osmium tetroxide in 0.1 M Na-Cacodylate buffer, pH 7.4, at 25°C for 90 minutes, and washed with 0.1 M Na-Cacodylate buffer, pH 7.4. Cells were gradually dehydrated using a gradient ethanol series, dried in a critical point dryer, and coated with 20 nm gold palladium (60:40) in an Emitech K550 sputter coater. Images were captured with a Hitachi S-2700 scanning electronic microscope with Quartz PCI software.

To quantify filamentation from colonies grown on Spider medium, cells were removed from the center of patches and counted for the fraction of filamentous cells. At least 4 fields of view and 400 cells were counted for each data point. Statistical significance was determined using a Student's T-test.

Adherence Assays. Cultures were inoculated in 3 ml of Spider medium then incubated at 25°C overnight. 2 ODs of cells were spun down and resuspended in 1 ml of Lee's + Glucose medium and transferred to the well of a 12-well polystyrene plate. Plates were incubated at 25°C for 1-2 days without shaking, then decanted. Each well was washed 3 times with 1 ml of water to remove non-adherent cells. Plates were imaged using a Chemidoc XRS+ with Image Lab software (Bio-Rad). Adherent cells were scraped off the plastic surface and resuspended in 1 ml of water and optical density determined. For significant differences between data sets, each was tested for normal distribution. A one-way ANOVA was performed on OD₆₀₀

results. Nine representative microscope fields were counted for each condition to determine the fraction of filamentous cells. Statistical significance was determined using a Mann-Whitney pair-wise test due to non-parametric datasets.

Crystal Violet Staining. Samples were prepared similarly to the adherence assays. Following the 3 washes of the biofilm, 12-well plates were decanted and left to dry for 45 minutes, and subsequently stained with 385 μ L of 0.4% aqueous crystal violet per well for 45 minutes. Each well was washed 3 times with 1 mL of water, then destained with 700 μ L of 95% ethanol. Finally, 100 μ L of each destain solution was transferred to a 96-well plate and diluted 10-fold. Optical density was read at 595 nm using a BioTek Synergy HT plate reader and statistics performed similar to the adherence assays.

XTT Reduction Assay. Samples were prepared similarly to the adherence assays. Following the 3 washes of the biofilm the plates were decanted, then 315 μ L of 1 mg/mL XTT (2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide) and 35 μ L of 360 μ g/mL phenazine methosulfate were added to each well. The plates were incubated at room temperature for 20 minutes, then 100 μ L of solution from each well was transferred to a 96-well plate. Optical density was read at 450 nm using a BioTek Synergy HT plate reader and statistics performed as for the adherence assays.

Analysis of C. tropicalis Fitness in vivo. Female BALB/c mice (18-20g, Charles River

Laboratories) were made neutropenic by intraperitoneal injection of 200 μ g anti-Gr-1 mAb (clone: RB6-8C5, BioXCell, West Lebanon, NH) one day prior to infection with *C. tropicalis*. *C. tropicalis* strains were grown overnight in Spider medium at 30°C, diluted to 0.2 OD₆₀₀ in fresh Spider medium and grown at 30°C to log phase. Cells were collected and washed three times in sterile phosphate-buffered saline (PBS). Mice were infected with a mixture of two *C. tropicalis* strains in a 50:50 ratio with a total inoculum of $\sim 1.0 \times 10^6$ colony forming units (CFUs) by injection into the tail vein. Each *C. tropicalis* mixture included one isolate auxotrophic for histidine (His) and one isolate auxotrophic for arginine (Arg) biosynthesis. Dilutions of the inoculum were plated onto SCD medium lacking either histidine or arginine to confirm initial cell concentrations. Mice were euthanized 72 hours after infection and kidney and brains isolated. Organs were homogenized through a 70 μ m filter and dilutions of the organ suspensions plated onto SCD medium lacking His or Arg. CFUs were counted on each plate to quantify the relative abundance of each strain. Mice where no cells were recovered after infection were excluded from analyses. Statistical significance was determined using a Student's T-test.

3.2 Results

3.2.a Comparative Analysis of Mating in *C. tropicalis* White and Opaque Cells

A key feature of the *C. albicans* white-opaque switch is that **a**/ α cells are locked in the white state due to repression of *WOR1* transcription by the **a1**/ α 2 heterodimer. A direct consequence of this control is that **a**/ α mating products formed between opaque **a** and α cells are typically white colonies [18]. Rare opaque colonies (<5%) were observed in *C. albicans* mating products, but in each case these colonies had undergone loss of **a1** or α 2, thereby relieving repression of *WOR1* and allowing propagation of the opaque state [18]. In *C. tropicalis*, the white-opaque switch also regulates mating, although the difference in mating efficiency between white and opaque forms is less striking than that in *C. albicans*. Whereas *C. albicans* opaque cells mate a million times more efficiently than white cells [18], mating of *C. tropicalis* opaque cells is only about a hundred times greater than that of white cells [5].

These observations led us to hypothesize that *C. tropicalis* white cells may be capable of appreciable mating frequencies, even without switching to the opaque state. Close inspection of *C. tropicalis* **a**/ α mating products revealed that colonies generated by mating white **a** and α cells were distinct from those formed by mating opaque cells. This is illustrated in Figure 10A, where a colony formed from mating between white **a** and α cells exhibited a shiny, smooth appearance, while the product of mating between opaque cells was consistently duller and darker. Examination of the cells from these colonies revealed that the products of white $\times$ white mating were round, resembling classical white cells, while the products of

opaque × opaque mating were elongated, reminiscent of opaque cells (Figure 10B). PCR analysis of the mating products confirmed that **a1** and $\alpha 2$ were still present in the products formed from mating white or opaque cells (Figure 10C). The **a1** and $\alpha 2$ loci were also sequenced and shown to exactly match the published gene sequences [20], indicating that these genes were not mutated in the *C. tropicalis* strains used for these experiments. Together, these results indicate that the *C. tropicalis* white-opaque switch is not under strict regulation by the *MTL* locus. Thus, unlike *C. albicans*, mating of *C. tropicalis* opaque **a** and α cells (and formation of the **a1**/ $\alpha 2$ complex) does not force cells back to the white form. Instead, *C. tropicalis* **a**/ α mating products inherit the phenotype of their parental cells and can even stably propagate in the opaque form.

These experiments reveal another important aspect of *C. tropicalis* biology, as they establish that white cells can mate without switching to the opaque form. Our observation that the products of white and opaque cell mating are distinguishable, and that white × white mating generates white mating products, indicates that *C. tropicalis* white cells can directly undergo conjugation with one another. In support of the fact that both white and opaque cells can mate, we also observed structural differences in zygotes formed between white cells and those formed between opaque cells. Zygotes formed from white cells were generally smaller and contained rounder cells than zygotes formed from opaque cells (Figure 10D). We conclude that *C. tropicalis* white cells are competent for mating, albeit at a lower efficiency than that of opaque cells.

To complete this analysis, we also examined the products of mating in crosses between white and opaque cells. Interestingly, the products of these mixed crosses were predominantly opaque (data not shown). Since Wor1 expression is essential for induction of the opaque form, we surmise that Wor1 protein from the parental opaque cell is present at sufficient levels in the zygote to result in stable propagation of the opaque state in these mating products.

3.2.b Phenotypic Switching in C. tropicalis a/α Cells

A previous study by Xie *et al.* indicated that **a/α** cells of *C. tropicalis* were able to form opaque cells based on similar cell morphologies to **a/a** and **α/α** opaque cell types [8]. To address whether **a/α** cells could directly switch to the opaque state, cells were grown on medium containing N-acetylglucosamine, as this was previously shown to induce *C. tropicalis* switching [8]. Rare colony switching was observed with darker sectors forming at the edge of **a/α** colonies, similar to that in conventional white-to-opaque sectoring of **a** and **α** colonies (Figure 11A). Cells from the **a/α** sectors were elongated rather than spherical, a characteristic feature of opaque cells [5]. Additionally, white **a/α** cells had a smooth surface while opaque **a/α** cells had an uneven or pimpled surface, and thus resembled conventional white and opaque cells (Figure B1A and [33]). Once formed, diploid opaque **a/α** cells were stably maintained in the opaque state, similar to tetraploid opaque **a/α** mating products (Figure 10A and data not shown). The frequency of white-to-opaque switching in **a/a** and **a/α** cells varied widely from experiment to experiment (0-7%

switching), but the difference in switching between strains was not statistically significant.

As the **a1**/ α 2 complex acts to repress white-to-opaque switching in *C. albicans*, we also examined whether the **a1** and α 2 genes were expressed in *C. tropicalis* opaque **a**/ α cells. Reverse transcription (RT)-PCR was performed on each cell type and revealed that **a1** and α 2 genes were actively expressed in **a**/ α cells regardless of phenotype (Figure B1B). This result indicates that the white-to-opaque switch in *C. tropicalis* appears independent of **a1** and α 2 function.

We further examined 8 additional *C. tropicalis* clinical **a**/ α strains for their ability to undergo the white-opaque switch. Opaque formation was observed in 2 of these strains, indicating that other **a**/ α strains are able to undergo the white-opaque switch (Table S1). The presence of the **a1** and α 2 genes in switching strains was confirmed by PCR. These genes were sequenced from opaque cells derived from one of the three clinical **a**/ α strains and also found to match the published sequence (data not shown). Our results therefore establish that *C. tropicalis* white-opaque switching occurs independent of *MTL* control and that multiple **a**/ α strains can switch to a stable opaque state.

3.2.c Transcriptional Profiling of *C. tropicalis* White and Opaque States

To ascertain the relationship between genes regulated by the white-opaque switch in *MTL* heterozygous **a**/ α cells with those in *MTL* homozygous **a/a** or α/α cells, transcriptional profiling was performed on cells from both morphological states in each cell type (Figure 11B). SAM (Statistical Analysis of Microarrays) was

used to determine gene expression changes that were significant for white-opaque regulated genes in **a** and **a/α** cells (see Materials and Methods and Tables S5 and S6). Expression profiles of **a/α** white and opaque forms showed significant overlap with the corresponding white- and opaque-specific genes in **a** cells (Figure 11B). In particular, of the 120 genes significantly upregulated in white **a** cells, 73 of these genes were also upregulated in white **a/α** cells, while of the 129 genes upregulated in opaque **a** cells, 22 of these genes were also upregulated in opaque **a/α** cells (Figure B2). These results establish that *C. tropicalis* **a/α** cells undergo a white-opaque switch related to that in *MTL* homozygous cells [5], and that there is significant overlap between white-opaque genes regulated in *MTL* homozygous and heterozygous cell types ($p < 1e-150$ and $p < 1e-20$ for white- and opaque-specific genes, respectively).

Despite significant overlap between white-opaque regulated genes in the different cell types, a large number of switch-regulated genes were unique to either *MTL* heterozygous or *MTL* homozygous cells. This was particularly striking in **a/α** cells, where the expression of 549 genes was significantly different between white and opaque forms, while only 249 genes were white-opaque regulated in **a** cells (Figure 11B, 2C and Figure B2). Many of the additional white-opaque regulated genes in **a/α** cells were involved in translation, biosynthetic processes, or gene expression based on GO term analysis (Text S1 and Figure B3). This indicates that the switch in **a/α** cells regulates additional cellular processes to those in **a** or **α** cells, and these differences could have important implications for the role of the switch in *MTL* heterozygous strains.

Conversely, white and opaque **a** and α cells also differentially expressed genes that were not white-opaque regulated in **a**/ α cells. For example, the mating-related genes *STE4* and *BAR1* were expressed at higher levels in opaque *MTL* homozygous cells than in white cells, but were not differentially expressed between white and opaque states in *MTL* heterozygous cells (Figure 11C). Taken together, these results indicate that *MTL* homozygous and *MTL* heterozygous cells share related white-opaque expression profiles, but that the genes regulated by this switch are also influenced by the configuration of the *MTL* locus.

3.2.e Mating of *C. tropicalis* **a**/ α Cell Types

A key role of the white-opaque switch in *Candida* species is the regulation of sexual reproduction [5,18]. Given that *C. tropicalis* **a**/ α cells undergo the white-to-opaque switch, we tested whether these cell types undergo productive mating. We found that **a**/ α cells could not mate with other **a**/ α cells, and that same-sex mating of **a** or α cells was also not observed (data not shown), in agreement with previous studies [5]. However, low levels of mating were obtained between **a**/ α cells and either **a** or α cells as partners. The frequency of this **a**/ α cell mating was 10^3 to 10^5 -fold lower than conventional mating between **a** and α cells (Figure 12 and Figure B4). Mating could be detected using both white and opaque **a**/ α cells, and although **a**/ α cell mating was higher in the opaque state, this difference was not significant.

C. albicans **a**/ α cells from the SC5314 background do not undergo mating with other cell types ([18] and data not shown). However, overexpression of *WOR1* in these cell types can override **a**/ α control, forcing them to adopt the opaque state

and promoting low frequency mating with **a** or α cells [25]. These studies indicated that productive mating was likely due to loss of *MTL* genes, allowing *C. albicans* **a**/ α cells to mate as **a** or α cells [25]. We similarly suggest that low level mating of *C. tropicalis* **a**/ α cells is due to *MTL* instability, as loss of *MTL* genes was frequently detected in the mating products from **a**/ α crosses (see Text S1 and Figure B5). In addition, genetic recombination at the *MTL* was observed in a subset of mating products, indicating that homozygosis of the *MTL* could promote **a**/ α mating (Figure B5). However, regardless of the mechanism involved, mating of **a**/ α cells occurred at a very low frequency compared to conventional mating between **a** and α cells.

3.2.e Transcriptional Regulation of *C. tropicalis* Phenotypic Switching

WOR1 is the master transcriptional regulator of the white-opaque switch in both *C. tropicalis* [5] and *C. albicans* [25-27], and its expression is therefore critical for opaque cell formation. To further investigate the role of *WOR1*, we constructed $\Delta wor1/\Delta wor1$ and *WOR1* overexpression strains in *C. tropicalis* **a**, α , and **a**/ α backgrounds. Initially, a *pACT1-WOR1* construct was used to overexpress *WOR1*. However, unlike in *C. albicans*, this construct failed to induce switching to opaque in *C. tropicalis* (data not shown and [8]). Instead, an alternative promoter, *TDH3*, was utilized for *WOR1* expression and found to be sufficient to induce opaque formation in *C. tropicalis* **a**, α , and **a**/ α cells (Figure 13A). Thus, *WOR1* expression is both necessary and sufficient for formation of the opaque state in *C. tropicalis*.

Previously, we showed that *wor1* mutant **a** or α cells do not undergo detectable mating in *C. tropicalis* [5]. Mating assays were also performed on *WOR1*

overexpression strains and these strains exhibited increased mating between **a** and α cells (~70% mating) relative to wild-type opaque strains (~15% mating, Figure B4). This result further establishes the key role of *Wor1* in directing expression of genes necessary for efficient conjugation.

Transcriptional profiling was also performed on *wor1* mutant and *WOR1* overexpression **a**/ α strains, and profiles compared to wild-type white and opaque cells (Figure 13B). Consistent with the key role of *WOR1* in directing the white-opaque switch, the expression profile of Δ *wor1* strains was similar to that of wild-type white cells, while strains overexpressing *WOR1* had an expression profile similar to that of wild-type opaque cells (Figure 13B). However, although the global expression patterns were similar, many genes were differentially expressed between Δ *wor1* strains and white cells (Tables S7 and S8,) and between *WOR1* overexpression strains and opaque cells (Tables S9 and S10). Expression of *WOR1* was also compared by RT-PCR and was found to have the following relative transcription levels: *WOR1* overexpresser > wild-type opaque > wild-type white > *wor1* mutant (Figure 13C). *WOR1* expression was ~2-8-fold higher in *WOR1* overexpression strains relative to opaque strains, ~4-10-fold higher in opaque cells relative to white cells, and ~1000-fold higher in white cells relative to *wor1* mutants. The difference in expression levels between white cells and *wor1* mutants is consistent with low-level expression of this gene in the white state. We also note that *WOR1* expression levels were in line with the relative mating efficiencies of these strains (Figure B4).

In *C. albicans*, the white-opaque switch is regulated by Wor1 together with three other factors, Wor2, Czf1, and Efg1, as part of an interacting transcriptional circuit [29]. The *EFG1* gene is missing from the *C. tropicalis* genome although a gene encoding a related APSES transcription factor, *EFH1*, is present [20]. Previous studies failed to observe differential expression of *WOR2*, *CZF1*, or *EFH1* between *C. tropicalis* white and opaque **a** cells [5]. We examined the expression of these genes in the larger white-opaque data set present in **a**/ α cells, and between *wor1* mutant and *WOR1* overexpression **a**/ α strains. *CZF1* and *EFH1* did not show significant expression differences between these profiles, while *WOR2* expression was decreased in opaque cells, the opposite of that expected based on its expression in *C. albicans*. These results are therefore consistent with transcriptional control of the white-opaque switch being divergent between *C. tropicalis* and *C. albicans* (outside of the conserved role of Wor1).

We also identified several other transcription factors to be regulated by the *C. tropicalis* white-opaque switch. Among these factors, *UME6*, a gene known to have a role in regulation of filamentous growth in *C. albicans*, had consistently higher expression in *C. tropicalis* opaque cells. Conversely, upregulated genes in white cells included *RME1* and *NDT802*, genes with homologs involved in regulating meiosis in *S. cerevisiae*, as well as *ADAEC*, a gene that is also white-specific in *C. albicans*. Further studies are now required to determine if any of these transcription factors play an active role in regulating the white-opaque switch in *C. tropicalis*.

3.2.f *Wor1 Regulates Filamentation and Biofilm Formation in C. tropicalis*

In several diverse fungal species, Wor1 homologs do not mediate phenotypic switching but still act as transcriptional regulators of cellular morphogenesis. This is evident in *S. cerevisiae* and *H. capsulatum*, where Wor1 homologs regulate the transition from budding yeast forms to filamentous forms [31,32]. Colony morphologies of *C. tropicalis* strains were compared on several media, and it was found that *WOR1* overexpression strains were markedly more wrinkled than wild-type white or opaque cells when grown on Spider medium (Figure 14A). Spider medium is a low nutrient medium that also induces filamentation in *C. albicans* [34]. Examination of cells from the wrinkled colonies confirmed that Wor1 overexpression strains were significantly more filamentous than other cell types due to a higher percentage of both hyphal and pseudohyphal cells (Figure 14B). In addition, filamentation generally increased as *WOR1* gene expression levels increased. Thus, for **a**/ α cells, filamentation increased from *wor1* mutants (4.4%) to white cells (10.7%) to opaque cells (23.9%) to *WOR1* overexpressing cells (32.9%, Figure 14B). These results indicate that filamentation correlates with *WOR1* expression in *C. tropicalis*, and that Wor1 regulates filamentous growth independent of its regulation of the white-opaque switch.

Filamentous growth is directly associated with biofilm formation in *C. albicans*. Biofilms are surface-associated communities of cells and often involve both yeast and filamentous cells in stable, complex structures that form on biotic and abiotic surfaces [35,36]. Furthermore, *C. albicans* biofilms are responsible for the seeding of serious bloodstream infections and are associated with antifungal

drug resistance [35,36]. We therefore examined whether *C. tropicalis* *wor1* mutant cells, white cells, opaque cells, or cells overexpressing *WOR1* displayed differences in their ability to form biofilms using an adherence to plastic assay. White cells, opaque cells, and *wor1* mutants generally performed poorly in these adherence assays, regardless of *MTL* configuration (Figure 15A). In contrast, cells overexpressing *WOR1* generated robust biofilms on the polystyrene plates, as noted by visual inspection as well as quantification of the biofilm by optical density (Figure 15A). Increased biofilm formation was also demonstrated by an elevated level of staining with crystal violet, which indicates extracellular matrix production (Figure 15B), and by increased XTT reduction, indicating that there were significantly more adherent, viable cells in the *WOR1* overexpressing biofilms (Figure 15C). Many cells within the *WOR1*-overexpressing biofilms were filamentous (22-59%), whereas comparatively few cells were filamentous in white, opaque, or Δ *wor1* cells (0-12%, Figure 15D).

Transcriptional profiling was performed under biofilm culture conditions on *WOR1* overexpression strains and compared to gene expression in wild-type opaque strains. This revealed that 58 genes were differentially expressed (>4-fold) between these strains (Figure 15E). Gene expression changes included *CHT3*, which encodes a chitinase whose expression is repressed in *C. albicans* hyphal cells [37], and which was also repressed in the *C. tropicalis* *WOR1* overexpression strain. Conversely, *JEN2* was upregulated in *C. tropicalis* *WOR1*-mediated biofilms, and encodes a dicarboxylic acid transporter whose expression is also upregulated in *C. albicans* biofilms [38]. In addition, several *C. tropicalis* white-specific genes (e.g.,

ADAEC and *WH11*) were further downregulated in the *WOR1*-overexpressing cells relative to opaque cells (Figure 15E). However, no genes with defined roles in filament regulation were obtained from these profiling studies, although many of the differentially regulated genes currently have no known function.

Together, these results demonstrate that *Wor1* promotes filamentous growth and biofilm formation in *C. tropicalis* and these functions are independent of the white-opaque switch. The role of *Wor1* in this species is therefore analogous to that of several distantly related *Wor1* homologs in the fungal lineage. The increase in filamentous growth is presumably a major reason for the increased adhesion and biofilm formation in the *C. tropicalis* *Wor1*-overexpression strains.

3.2.g Contribution of *MTL* Configuration to *C. tropicalis* Fitness

We next determined if the configuration of the *MTL* locus contributes to overall fitness in *C. tropicalis*. In particular, we investigated the possibility that **a**/ α cells exhibit increased fitness over *MTL* homozygous cells. We first analyzed 150 natural *C. tropicalis* isolates (Table S1) to determine their *MTL* configuration and found that 119 isolates (~80%) were **a**/ α strains, while 23 (~15%) were **a** strains and 8 (~5%) were α strains. A similar analysis performed by Xie *et al.* showed an even higher prevalence of **a**/ α strains (145/150 isolates) in the natural population [8]. Thus, similar to the case in *C. albicans* [17,39], **a**/ α genotypes are the predominant cell types in natural *C. tropicalis* isolates.

We speculated that the predominance of **a**/ α cells could result from increased fitness of these cell types relative to **a** or α cells. Thus, the ability to

switch as an **a**/ α cell could allow cells to retain their optimal fitness yet also be competent to undergo the white-opaque switch. The fitness of *C. tropicalis* **a**, α , and **a**/ α strains was addressed using an *in vivo* model of murine candidiasis.

Competition experiments were performed between **a**/ α and **a** cells, or between **a**/ α and α cells, using strains carrying different auxotrophic markers. Neutropenia was induced prior to infection with *C. tropicalis* cells as this increases the fungal load associated with systemic disease [40]. Mixtures of strains were injected into the tail veins of neutropenic mice and cells recovered from the brain and kidneys 72 hours following infection and characterized for cell type.

Regardless of the combination of auxotrophic markers used, **a**/ α cells consistently colonized host organs at a higher fungal burden than **a** or α cells in competition assays (Figure 16). This difference was statistically significant for fungal colonization of the brain, where approximately 70% of the recovered cells were **a**/ α cell types. These experiments indicate that **a**/ α cells are fitter than **a** or α cells in this *in vivo* model, as they outcompeted *MTL* homozygous cell types during systemic infection. We propose that phenotypic switching in **a**/ α cells allows these cells to have the potential to adopt both white and opaque forms while still maintaining their fitness advantage over **a** or α strains.

3.3 Discussion

In this study, we show that a *Wor1*-regulated phenotypic switch operates in *C. tropicalis* **a**, α , and **a**/ α cell types and is therefore independent of *MTL* control. Even in strains expressing intact **a1** and $\alpha 2$ genes, white cells could stably switch to opaque cells, indicating that *MTL* genes do not block switching. The regulation of the white-opaque switch in *C. tropicalis* therefore deviates from that in *C. albicans*, where the action of **a1**/ $\alpha 2$ prohibits efficient opaque formation in **a**/ α cells [18]. The discovery that *C. tropicalis* **a**/ α cells can undergo stable formation of the opaque state raises new questions as to the role(s) of the switch *in vivo*, as well as the transcriptional regulation of the switch in this species. We note that Xie *et al.* also recently observed a white-opaque-like morphological switch in two *C. tropicalis* **a**/ α strains [8], and although the integrity of **a1** and $\alpha 2$ genes was not reported, it is likely that these strains were also capable of *MTL*-independent white-opaque switching.

3.3.a *C. tropicalis* Mating and the White-Opaque Switch

Despite its apparent independence from *MTL* control, the *C. tropicalis* white-opaque switch still regulates sexual mating in this species. Mating between *C. tropicalis* **a** and α opaque cells occurs approximately 100 times more efficiently than that between white cells. It was not known, however, if mating of white cells required that cells first switch to the opaque state prior to mating, or if white cells were themselves mating competent. Analysis of *C. tropicalis* white-white and opaque-opaque mating products addressed this question, as they revealed that

mating products inherited the phenotypic state of the parental cells (i.e. mating between white cells generated white mating products while those between opaque cells generated opaque mating products). This simple observation established two important facts about phenotypic switching and mating in *C. tropicalis*. First, it demonstrated that the *C. tropicalis* white-opaque switch is independent of *MTL* status, as α/α cells formed by mating would be expected to be in the white state, regardless of the phenotype of the parental cells. Indeed, this is what has been observed in *C. albicans*, where the products of mating are white or, if opaque, have undergone concomitant loss of *MTL* genes [18]. Second, it showed that *C. tropicalis* white cells are themselves mating competent; if they had switched to opaque prior to mating then the products of mating would also have been opaque. Thus, *C. tropicalis* white and opaque cells are both mating competent, although the efficiency of mating is higher in the opaque state than in the white state.

The discovery that *C. tropicalis* white cells are mating competent is interesting given that mating is completely abolished in mutant cells lacking *WOR1*. Thus, although *C. tropicalis* white cells mate inefficiently, their mating frequency is still more than 1000-fold higher than that of *wor1* mutants [5]. This indicates that significant *WOR1* expression occurs in *C. tropicalis* white cells (supported by array and qPCR data), and that this expression is sufficient for basal induction of genes necessary for conjugation. In opaque cells, *Wor1* levels are further increased over white cells (4-10 fold) and higher mating efficiencies are observed in cells with the opaque phenotype. Candidate mating genes include *STE4* and *BAR1*, both of which are elevated in the opaque state and are known to regulate mating in diverse fungal

species [41-43]. Mating efficiency was further increased in Wor1 overexpression strains compared to wild-type opaque cells. These results demonstrate that mating efficiency can be separated from the white-opaque switch *per se* and, at least to a first approximation, *C. tropicalis* mating correlates directly with Wor1 expression levels.

Mating was also observed between *C. tropicalis* **a**/ α cells and either **a** or α partners. The efficiency of **a**/ α cell mating was very low ($\sim 10^{-6}$) regardless of whether white or opaque cells were used, and was approximately 5 orders of magnitude lower than conventional mating between opaque **a** and α cells. In fact, mating of **a**/ α cells was 1000-fold lower than that between white **a** and α cells. Furthermore, the majority of **a**/ α mating products had lost *MTL* genes or had undergone recombination at the *MTL*, and therefore had presumably mated as **a** or α cell types. Aberrant mating of **a**/ α cells has also been described in *C. albicans* cells overexpressing *WOR1* and was also attributed to rare loss of *MTL* genes [25]. Moreover, it is known that *C. albicans* **a**/ α cells undergo occasional mating upon loss of **a1** or $\alpha 2$ genes, as **a1**/ $\alpha 2$ represses the expression of haploid-specific genes necessary for mating [18,21].

3.3.b Profiling of *C. tropicalis* Phenotypic States

Transcriptional profiling of the three different genotypes (**a**, α , and **a**/ α) in *C. tropicalis* revealed that there was significant overlap between white-opaque regulated genes in each cell type. This is to be expected given that Wor1 regulates the switch irrespective of *MTL* configuration. The key role of this transcription

factor in determining the phenotypic state was further illustrated by comparing the profiles of white cells, opaque cells, *wor1* mutants, and *WOR1* overexpression strains. The profiles of white cells and *wor1* mutant cells were similar, as were those of opaque cells and *WOR1* overexpressing cells. In addition, *wor1* mutant strains did not undergo switching to opaque, while *WOR1* overexpression locked cells in the opaque state. Together, these results establish Wor1 as the master regulator of the *C. tropicalis* white-opaque switch in **a**/ α cells, similar to its role in **a** and α cells.

Surprisingly, significant differences in white-opaque regulated genes were noted between *MTL* homozygous and *MTL* heterozygous strains. For example, *STE4* and *BAR1* were expressed at elevated levels in opaque **a** or α cells, but not in opaque **a**/ α cells. This may reflect the fact that the white-opaque switch regulates mating between **a** and α cells, but does not significantly promote mating in **a**/ α cells. In addition, ~400 switch-regulated genes were unique to **a**/ α cells and were not observed in **a** or α cell types. The gene set unique to opaque **a**/ α cells included a significant association with translation, biosynthetic processes, and gene expression control. A direct implication of these transcriptional differences is that behavioral differences (including effects on host interactions and virulence) may occur between white and opaque cells from *MTL* homozygous and *MTL* heterozygous cell types, and additional experiments will now test this possibility.

3.3.c Evolution and Function of Phenotypic Switching in *Candida* Species

The white-opaque switch affects virtually every aspect of *Candida* biology, from mating to interactions with host immune cells to pathogenesis [44-47]. *MTL* regulation of the switch in *C. albicans* ensures that only those cells that are capable of mating undergo the switch to the mating-competent form [18]. The discovery that *C. tropicalis* can form opaque cells regardless of *MTL* status suggests that the switch may have originally evolved to regulate processes other than sexual mating. Furthermore, the majority of natural *C. tropicalis* and *C. albicans* isolates are **a**/ α strains (80-95%), thus while most *C. albicans* strains are unable to switch, it appears that the majority of *C. tropicalis* isolates are competent for switching to the opaque form.

Many of the genes controlled by the white-opaque switch in *C. albicans* are metabolism genes, perhaps reflective of the fact that white and opaque cells colonize different host niches [9,21]. *C. albicans* white cells exhibit greater virulence in models of systemic infection, while opaque cells are more efficient at colonization of the skin and are unstable at 37°C, rapidly switching back to the white form [48,49]. In contrast to *C. albicans*, *C. tropicalis* opaque cells are stable at 37°C [5] and may colonize host niches that are refractory to *C. albicans* opaque cells. In addition, we found that *C. tropicalis* **a**/ α cells exhibited increased fitness relative to **a** or α strains in a neutropenic model of candidiasis. Thus, the ability of **a**/ α cells to switch phenotypes may be beneficial to *C. tropicalis* as it allows cells to propagate with optimal fitness, but still have the potential to form opaque cells and thereby adapt to different host niches.

Alternatively, the white-opaque switch could have evolved to regulate mating in the ancestor to *C. tropicalis* and *C. albicans*, and *MTL* control of the switch subsequently evolved in *C. albicans* as a means of restricting switching to *MTL* homozygous strains. If the major role of the white-opaque switch is to regulate mating, then fine-tuning of the switching mechanism could have been beneficial to help prevent futile switching to the opaque state in **a**/ α cells that are sterile. Certainly, the regulation of mating by the white-opaque switch is stricter in *C. albicans* than in *C. tropicalis*; white cells of *C. tropicalis* undergo appreciable mating frequencies while *C. albicans* white cells do not.

Finally, it is formally possible that *MTL* control of the white-opaque switch was present in the ancestor to *C. albicans* and *C. tropicalis*, but has since been lost in *C. tropicalis*. Experiments will now be required to characterize *C. tropicalis* white and opaque states *in vivo*, and to determine if they exhibit different preferences for host colonization and pathogenesis as has been documented for *C. albicans*.

3.3.d A Dual Role for *Wor1* in Regulating Phenotypic Switching and Filamentation in *C. tropicalis*

An unexpected outcome of our analysis of white and opaque cells was the discovery of a link between *C. tropicalis* *Wor1* and filamentous growth.

Overexpression of *WOR1* in *C. tropicalis* **a**, α , or **a**/ α cells resulted in increased filamentation relative to wild-type white or opaque cells. *C. tropicalis* *Wor1* is therefore a key regulator of filamentation, and this control can be separated from regulation of the white-opaque switch. The role of *Wor1* was most clearly

manifested in biofilm assays, where increased expression of *WOR1* led to enhanced biofilm formation. This is presumably a direct result of the increase in filamentous growth, as filamentation and biofilm formation are inter-related processes in multiple *Candida* species [35,36,50]. Regulation of filamentation by the white-opaque switch has also been noted in *C. albicans*, although here white cells were shown to be more conducive to undergoing filamentation than opaque cells [23,24].

The observation that *C. tropicalis* Wor1 induces filamentation shows interesting parallels with the role of Wor1 homologs in more distantly related ascomycete species. In *S. cerevisiae* and *H. capsulatum*, the Wor1 homologs Mit1 and Ryp1, respectively, are both master regulators of filamentation [31,32]. It has therefore been proposed that the ancestral function of Wor1/Mit1/Ryp1 was to control morphological transitions such as that between yeast form and filamentous form [31]. The discovery that *C. tropicalis* Wor1 promotes filamentation suggests that it has retained the ancestral function of Wor1 in this species.

In contrast to filamentous growth, the white-opaque switch evolved only recently in the ascomycete lineage, probably immediately prior to the divergence of *C. tropicalis* and *C. albicans* [5,8,51]. We therefore propose that *C. tropicalis* Wor1 has retained both the ancestral role of the Wor1 family of transcription factors (regulation of filamentous growth), as well as adopting control over the more recently evolved white-opaque switch. In contrast, overexpression of *C. albicans* Wor1 did not promote biofilm formation under the conditions tested (data not shown) and can even inhibit filamentous growth [8]. Further studies on *C. tropicalis* Wor1 will therefore shed light on its role as a key regulator of both morphological

switches: the recently evolved white-opaque switch, as well as the ancestral program of filamentous growth.

3.6 Figures

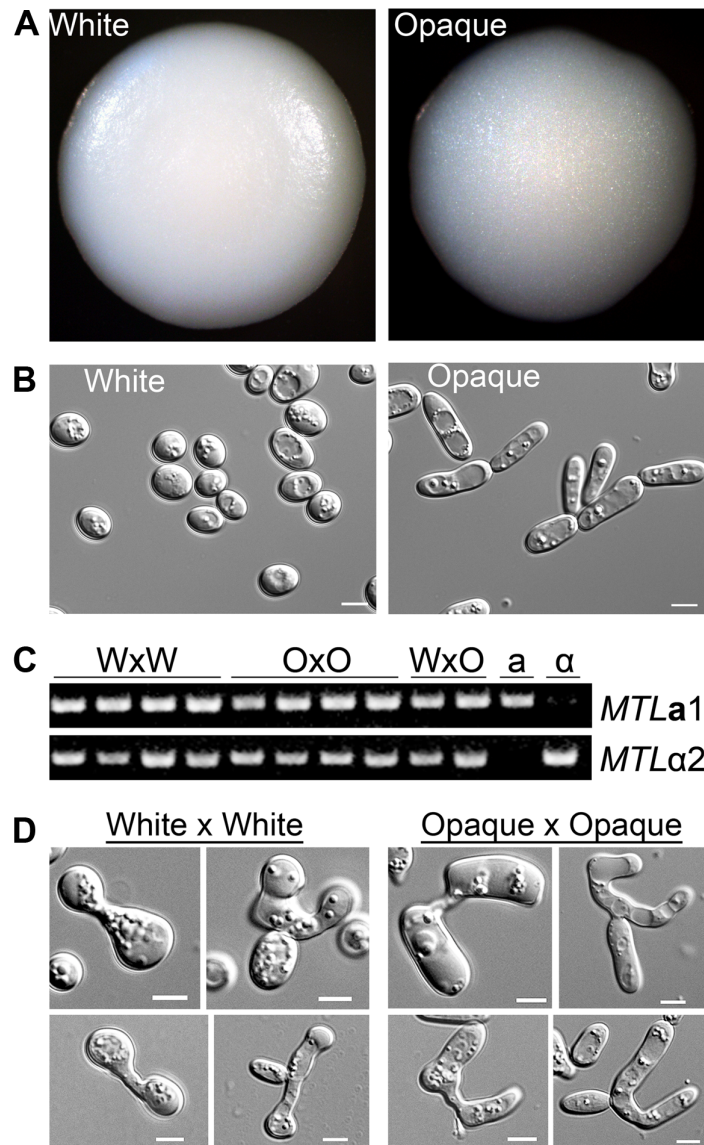


Figure 10. Products of *C. tropicalis* white × white and opaque × opaque mating maintain parental phenotypes. (A) Colony morphology of mating products. Left, product of mating *C. tropicalis* white **a** (CAY3376) with white **α** (CAY3391) cells. Right, product of mating opaque **a** (CAY3378) with opaque **α** (CAY3392) cells. Colonies were grown on SCD medium lacking histidine and arginine for 3 days at room temperature to select for mating products. (B) Cell morphology of cells taken from the corresponding colonies. Scale bars = 5µm. (C) PCR to verify the presence of *MTLα1* and *MTLα2* genes in white × white (WxW) mating products, opaque × opaque (OxO) mating products, white × opaque (WxO) mating products, and in the parental **a** and **α** strains. (D) Left, pictures of zygotes analyzed from white × white mating experiments and, right, pictures of zygotes analyzed from opaque × opaque mating experiments. Scale bars = 5µm.

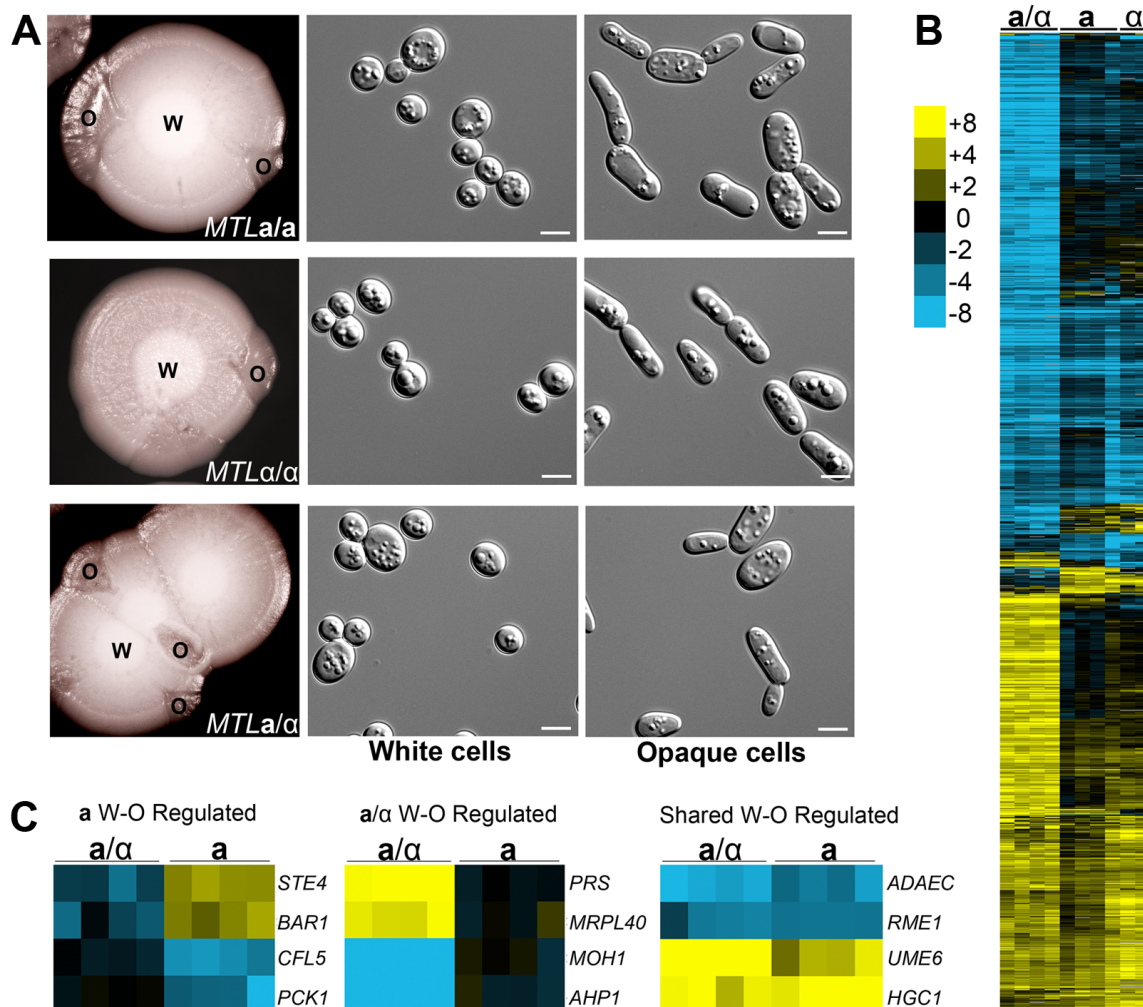


Figure 11. *C. tropicalis* a/α cells undergo a white-opaque phenotypic switch related to that in a and α cells. (A) White (W) colonies sectoring to opaque (O) in a (CAY1503), α (CAY1505), and a/α (CAY1511) strains and the corresponding cell morphologies observed from these colonies. Cells were grown in SCD at 37°C for 5 days and plated to Lee's + N-acetylglucosamine at room temperature for 10 days. Scale bars = 5 μ m. (B) White-opaque gene expression profiles of a/α , a , and α cells. cDNA prepared from white and opaque states of CAY1511 (a/α), CAY1504 (a), and CAY1505 (α) in independent experiments was hybridized against a universal reference. Opaque cell gene expression was divided by that in white cells and filtered for those genes with a fold-change greater than 4 and clustered by Average Linkage Clustering. Genes with elevated expression in white cells are shown in blue and genes upregulated in opaque cells are shown in yellow. (C) Examples of genes whose expression was white-opaque regulated in a cells (left), a/α cells (middle), or in both a and a/α cells (right).

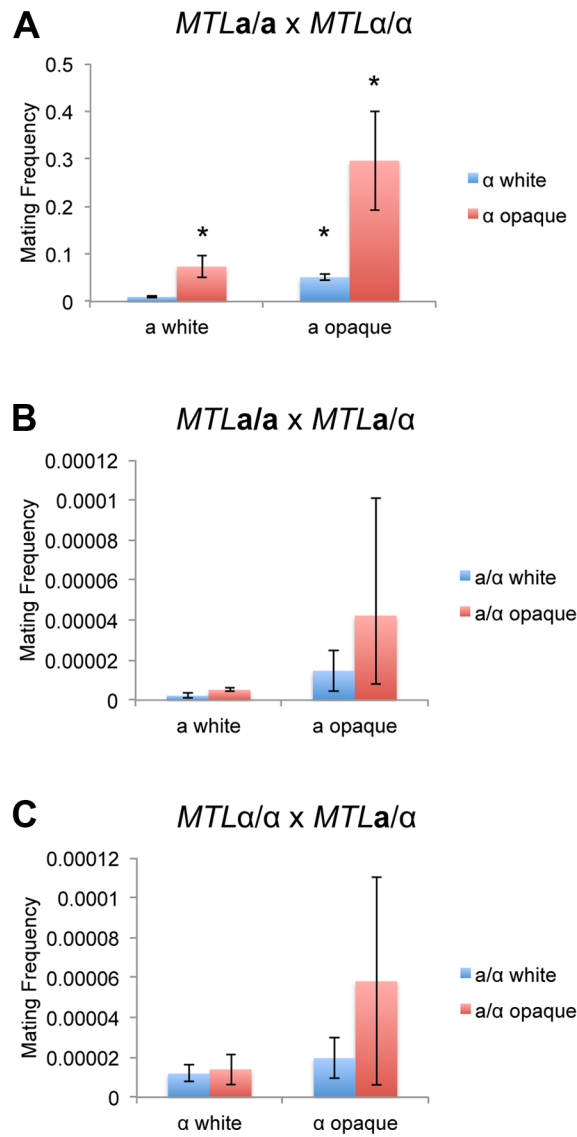


Figure 12. The white-opaque switch regulates *C. tropicalis* mating in a, α , and a/ α cells. Mating frequency of (A) a $\times$ α (CAY1503 $\times$ CAY1505), (B) a $\times$ a/ α (CAY1503 $\times$ CAY1511), and (C) α $\times$ a/ α (CAY1505 $\times$ CAY1513) white and opaque cells. Mating experiments were performed on Spider medium for 3 days at room temperature and plated to selective media to quantify mating frequency (see Materials and Methods). Error bars indicate SEM for 3 independent experiments. * = significantly different from white $\times$ white crosses, $p < 0.01$.

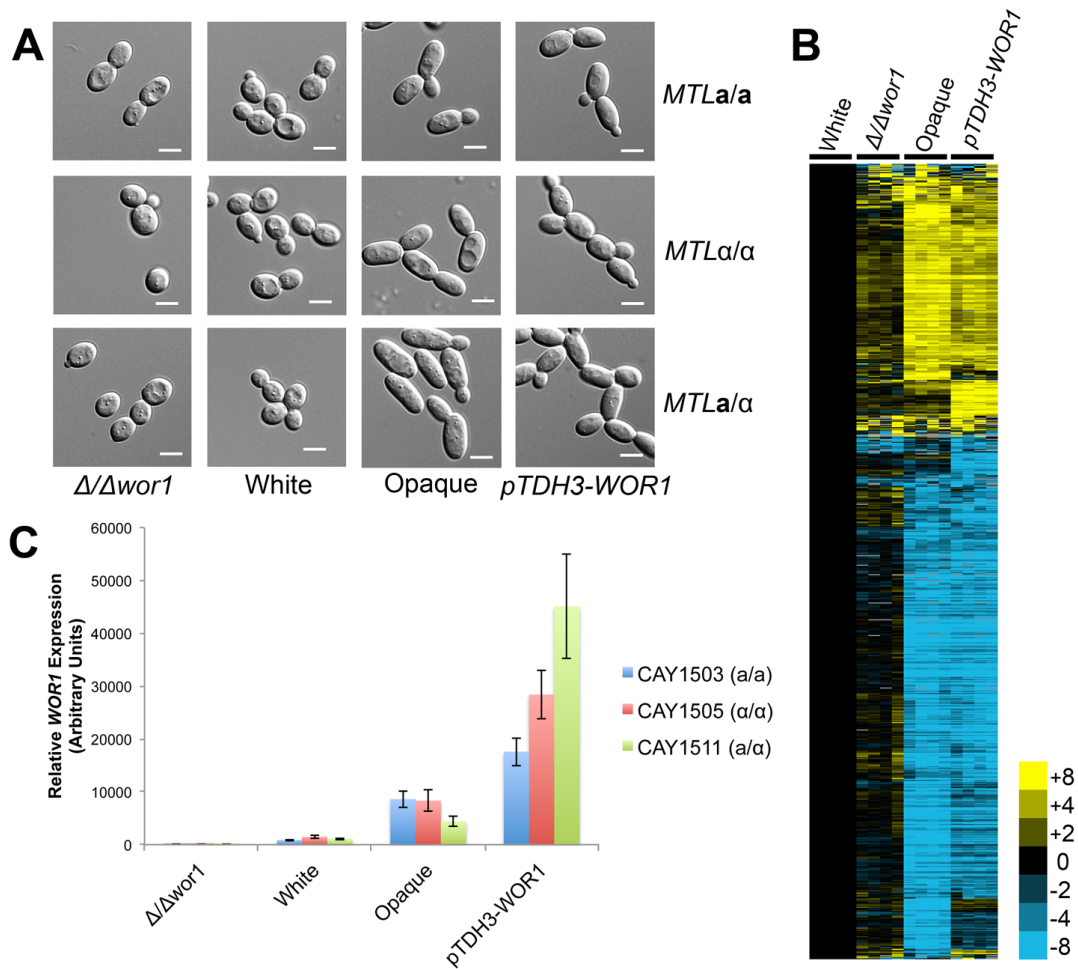


Figure 13. *WOR1* is the master regulator of the white-opaque switch in *C. tropicalis*. (A) Cell morphologies of $\Delta wor1$, white, opaque, and *pTDH3-WOR1* (*WOR1*-overexpressing) strains. Cells were grown in Spider medium at room temperature to 0.8-1.0 OD₆₀₀. Scale bars = 5 μ m. (B) Gene expression in *a/a* white cells (CAY1511), $\Delta wor1$ cells (CAY4043), opaque cells (CAY4048), and *pTDH3-WOR1* cells (CAY4045), relative to white cells (CAY1511). Expression profiles for each state were divided by white expression values and filtered for those genes with an expression change greater than 4-fold in 4 or more experiments. (C) *WOR1* expression in $\Delta wor1$, white, opaque, and *pTDH3-WOR1* strains derived from *a*, α , and *a/a* cells. Expression levels measured by qRT-PCR. Error bars indicate SEM for replicate experiments from 3 different biological replicates.

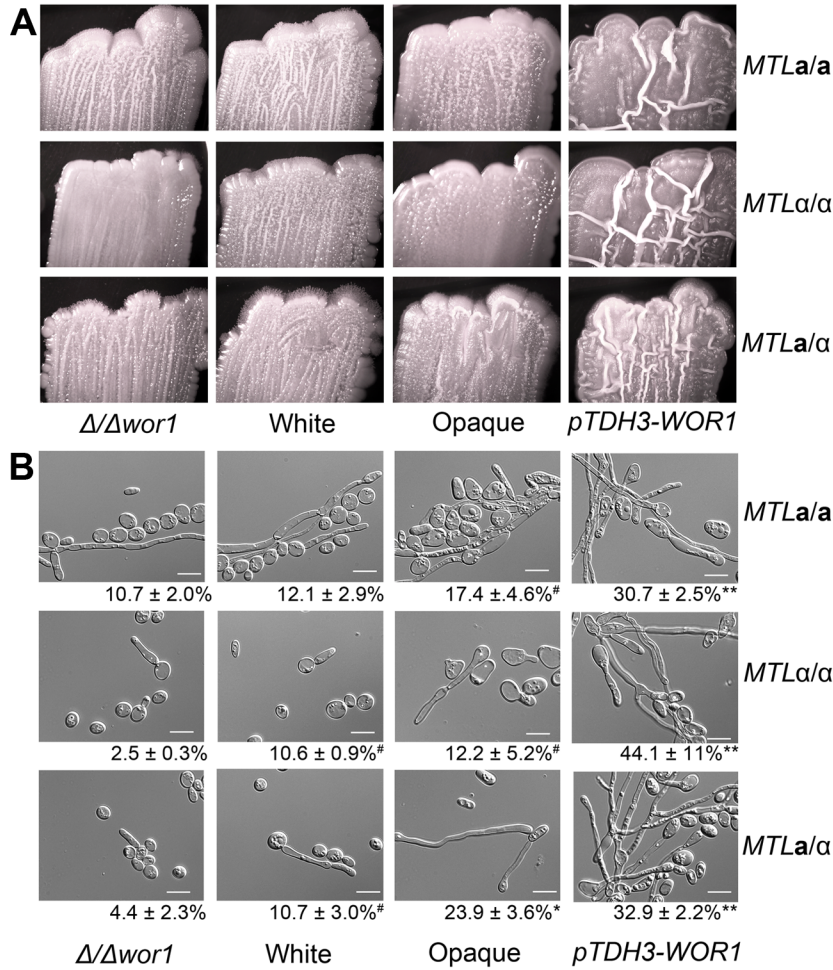


Figure 14. *C. tropicalis* *WOR1* expression regulates filamentous growth. (A) Wild-type white, wild-type opaque, *Dwor1* mutant, and *WOR1*-overexpression (*pTDH3-WOR1*) strains were grown on Spider medium at 37°C for 7 days and photographed. (B) Cells from corresponding patches in (A). The percentage of filamentous cells is shown below each image. Scale bar = 10μm. ** = significantly different from opaque, white, and $\Delta wor1$ strains, $p < 0.001$. * = significantly different from white and $\Delta wor1$, $p < 0.001$. # = significantly different from $\Delta wor1$, $p < 0.05$.

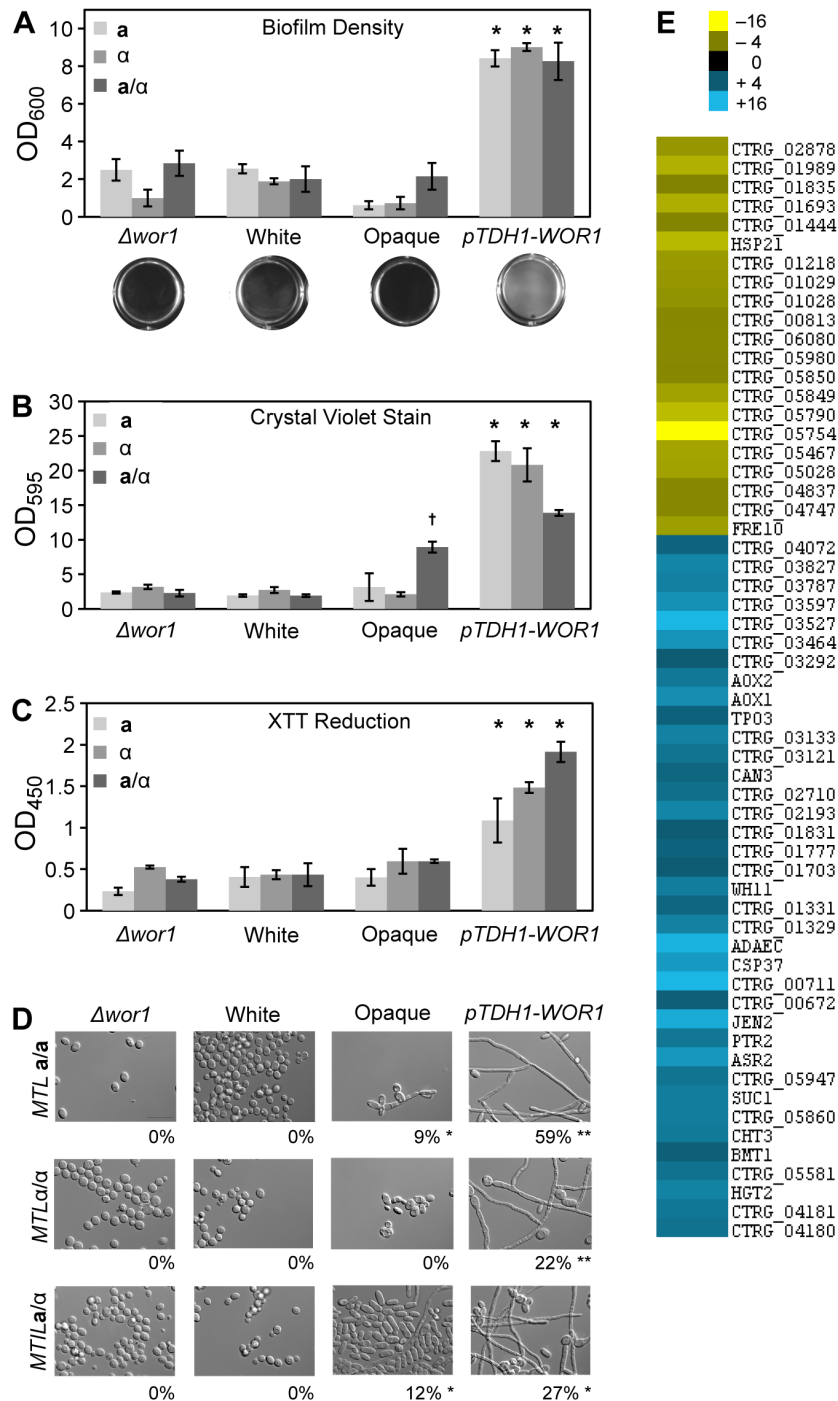


Figure 15. Biofilm formation is induced by *WOR1* overexpression in *C. tropicalis*. (A) Quantification of cells adhering to a 12-well polystyrene dish following a 48-hour incubation in Lee's medium. Representative images of wells appear below the corresponding strains. Data from three experimental replicates, error bars indicate SD. * = $p < 0.001$ when compared to *Δwor1*, white, and opaque strains. (B, C) Quantification of biofilm production by colorimetric assays. (B) Beta-glucan content was determined by crystal violet staining. (C) Cellular viability was

measured by formazan formation upon XTT reduction. Each biofilm experiment includes three or more replicates, error bars indicate S.D. * = $p < 0.001$ when compared to $\Delta wor1$, white, and opaque strains. † = $p < 0.001$ when compared to $\Delta wor1$, white, and $pTDH3-WOR1$ strains. (D) Morphology of adherent cells taken from (A). Percentage of filamentous cells indicated below image. Scale bars = $20\mu\text{m}$. Statistical significance was determined using Mann-Whitney pair-wise tests. ** = significantly different from opaque, white, and $\Delta wor1$ strains, $p < 0.001$. * = significantly different from white and $\Delta wor1$ strains, $p < 0.001$. (E) Genes induced in $MTLa/a$ $WOR1$ overexpressing cells under biofilm conditions. Heat map shows relative expression changes between $pTDH3-WOR1$ (CAY3853) and opaque (CAY3378) strains in the adherence to plastic biofilm assay. Data set was filtered for genes with a fold-change greater than 4 and clustered by Average Linkage Clustering. Data shows average of two independent biological replicates. Arrowheads highlight *C. albicans* homologs that are biofilm regulated.

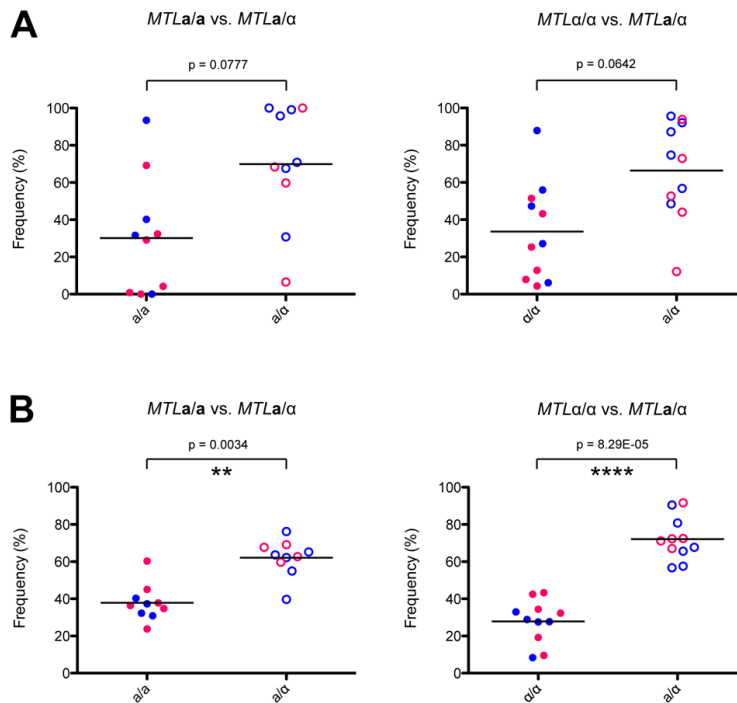


Figure 16. Heterozygosity at the *MTL* provides a fitness advantage *in vivo*. *MTL* homozygous and heterozygous *C. tropicalis* cells were mixed 1:1 and injected systemically via the mouse tail vein. After 72 hours, *C. tropicalis* cells were recovered from the kidney (A) and the brain (B), and strain genotypes identified by plating to auxotrophic media. In both organs, a/α cells (CAY1511 or CAY1513) showed increased fitness relative to a (CAY1502 or CAY1503) or α (CAY1505 or CAY1509) cells, and this difference was statistically significant in the brain. ** = $p < 0.01$, **** = $p < 0.0001$. Blue circles indicate strains were His-, red circles indicate strains were Arg-.

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Chapter 4: Transcriptional Regulation of a White-Intermediate- Opaque Tristable Switch in *Candida tropicalis*

Supplemental material that accompanies this paper is located in **Appendix C**.

AMP and NW performed strain construction and phenotypic switching assays on deletion mutants. AMP performed phenotypic analysis of *TDH3* and *MAL2* experiments. CAC contributed to RNA-Seq and analysis.

Allison M. Porman¹, Na Wang¹, Christina A Cuomo², and Richard J. Bennett^{1*}

¹Department of Microbiology and Immunology, Brown University, Providence, RI, 02912, USA. ²Broad Institute of MIT and Harvard, 7 Cambridge Center, Cambridge, MA 02142.

*Correspondence: Richard_Bennett@brown.edu

4.1 Abstract

Switching between 'white' and 'opaque' phenotypic forms plays a key role in the lifestyle of the human fungal pathogen, *Candida albicans*. This switch occurs epigenetically and is regulated by an interlocking network of six transcription factors, of which Wor1 is the master regulator of the opaque state. *C. albicans* white and opaque forms exhibit many different attributes, particularly the fact that only opaque cells are mating competent. Previous work showed that the related *Candida* species, *C. tropicalis*, also undergoes a Wor1-regulated white-opaque switch. Here, we establish that a third stable phenotypic state, the intermediate state, exists in multiple clinical isolates of *C. tropicalis* and is distinct from conventional white and opaque states. Cells in the intermediate state show a cell and colony phenotype, switching frequency, and gene expression profile that are intermediary between white and opaque cells. Thus, in place of a bistable white-opaque switch there is a tristable white-intermediate-opaque switch in this species. We also address the transcriptional regulation of the phenotypic switch in *C. tropicalis* and show that Wor1 and Wor3 are conserved inducers of the opaque state while Czf1 expression induces formation of the intermediate state. However, the Wor2 transcription factor that promotes opaque formation in *C. albicans* drives induction of the white state in *C. tropicalis*. We also identify novel regulators of phenotypic switching including the *C. tropicalis* transcription factors Ume6, Ndt802, and CTRG_03621. Overall, this work characterizes a stable third phenotypic state in *C. tropicalis* and identifies regulatory factors involved in controlling the switch, providing

mechanistic and evolutionary insights into white-opaque phenotypic switching in *Candida* species.

Author Summary

Epigenetic transitions can drive rapid and heritable changes in cell identity without any change in the primary sequence of the genome. These transitions are critical to the biology of *Candida* species that are the most common human fungal pathogens. The white-opaque switch is a bistable switch between two alternative heritable states that regulates multiple aspects of *C. albicans* biology, including sexual competency. Here, we demonstrate that the related species, *C. tropicalis*, undergoes a tristable switch between white, opaque, and a novel 'intermediate' form. The intermediate state is a stable state that appears truly intermediary to that of white and opaque forms based on phenotypic analysis and gene expression profiling. We also compare the transcription regulation of the phenotypic switch in *C. albicans* and *C. tropicalis*. We demonstrate that some factors have a conserved function (e.g. Wor1 and Wor3), while other factors have diverged (e.g. Wor2 promotes the opaque state in *C. albicans* but the white state in *C. tropicalis*). Together, our experiments provide the first mechanistic insights into a tristable switch in *C. tropicalis*, and reveal striking regulatory differences with the analogous switching mechanism in *C. albicans*.

4.2 Introduction

Phenotypic switches are widespread in biology and play important roles in enabling adaptation to environmental cues. In many microorganisms, epigenetic transitions are used to promote phenotypic variation within mainly clonal, genetically identical populations. One such phenotypic transition is the “white-opaque” switch, which is conserved between the human fungal pathogens *Candida albicans*, *Candida dubliniensis*, and *Candida tropicalis* [1-3]. In this system, cells can reversibly switch between two phenotypic states: the white state, in which cells are round and smooth and form dome-shaped colonies, and the opaque state, where cells are elongated, have an uneven surface texture, and form flatter, darker colonies [1, 4, 5]. White and opaque forms are stably inherited for many generations, with stochastic interconversion between the two states [6]. However, environmental conditions also impact switching frequencies; high temperatures induce opaque-to-white switching *en masse* in *C. albicans*, while N-acetylglucosamine has been shown to induce opaque formation in both *C. albicans* and *C. tropicalis* [6-8]. The white-opaque switch regulates the differential expression of ~1300 genes in *C. albicans* and affects metabolic preferences, immune cell interactions, filamentous growth, and niche specificity [9-12]. Furthermore, one of the best-studied features of the white-opaque switch is its ability to regulate sexual mating, as in *C. albicans*, *C. dubliniensis* and *C. tropicalis* opaque cells mate efficiently while white cells do not [2, 3, 13, 14].

In *C. albicans*, a complex interacting transcriptional network controls the white-opaque transition. At least 6 transcription factors form interlocking feedback

loops to ensure an “on-off” epigenetic switch [15]. Wor1 is the master regulator of the opaque phenotype; in white cells expression of *WOR1* is off while induction of *WOR1* induces a stable switch to the opaque state due, at least in part, to positive feedback of Wor1 protein on its own promoter [4, 16, 17]. Efg1 is an essential regulator of the white state in *C. albicans*, as deletion of this factor led to the inability to exhibit a normal white phenotype [18]. Efg1 binds to its own promoter as well as the 5 other factors in the white-opaque network, and acts to antagonize expression of *WOR1* [15, 19]. The other key transcriptional regulators, Wor2, Wor3, Czf1, and Ahr1, also contribute to the white-opaque circuit via positive and negative feedback loops [15, 19-21]. In addition to this transcriptional circuit, the *C. albicans* white-opaque switch is regulated by histone-modifying enzymes [22, 23] and by Mediator complex [24], indicating a complex interplay between histone epigenetic marks and transcriptional regulation.

In *C. tropicalis*, the role of Wor1 as the master regulator of the opaque state has been conserved [3, 8, 25]. However, several other features of the white-opaque switch are divergent between *C. albicans* and *C. tropicalis*. For example, *C. albicans* opaque cells are unstable at 37°C and rapidly switch back to the white state whereas *C. tropicalis* opaque cells are stable at this temperature [3]. Wor1 also regulates additional phenotypes in *C. tropicalis* other than the white-opaque switch, including filamentation and biofilm formation [25]. Moreover, while most white-opaque regulatory factors are conserved in *C. tropicalis*, the *EFG1* gene, an important morphogenetic regulator of the white state and filamentation in *C. albicans*, is apparently absent from *C. tropicalis* [26, 27]. Finally, analysis of cell morphologies

led to the hypothesis that *C. tropicalis* cells could exist in an intermediate state between classical white and opaque forms [8]. Many of these distinct features of white-opaque switching are expected to be the result of divergent transcriptional regulation between the two species.

The evolution of transcriptional networks is now recognized to play a major role in the generation of novel phenotypes. A number of studies have identified *cis*-regulatory mutations with corresponding consequences for morphology, physiology, and behavior [28, 29]. Based on studies in yeasts, large-scale rewiring of transcriptional circuits appears to be common during evolution [30]. For example, rearrangement of transcriptional regulatory components of metabolism have occurred among the ascomycetes [31]. Common changes in regulatory networks include genes moving into and out of regulons through changes in *cis*-regulatory sequences, transcription factor substitution, and formation of new combinatorial interactions [30]. Since the white-opaque switch is conserved in several closely related but divergent species, it provides an excellent system for studying the evolution of the regulatory network controlling this process.

In this work, we identify and characterize a stable phenotype in *C. tropicalis* that is intermediate between that of conventional white and opaque states in terms of colony and cell morphology, switching behavior, and gene expression. We therefore propose that *C. tropicalis* undergoes a white-intermediate-opaque phenotypic switch between three stable states. Analyses of mating phenotypes reveals that intermediate cells mate at frequencies similar to that of opaque cells. We identify genes that regulate phenotypic switching in *C. tropicalis* and find that,

while the function of some genes are conserved with orthologs in *C. albicans*, including *WOR1* and *WOR3*, others such as *WOR2* have altered functions in regulating the switch. Furthermore, regulatory factors including *NDT802* and *UME6* have been discovered as novel regulators of the *C. tropicalis* white-opaque transcriptional circuit. Surprisingly, we also show that ectopic induction of *WOR1* is not sufficient to induce stable opaque formation in *C. tropicalis*. This is in contrast to the situation in *C. albicans* where ectopic *WOR1* expression induces stable expression of endogenous *Wor1* and heritable activation of the opaque circuit. Together, these experiments provide the first insights into the regulation of *C. tropicalis* white-opaque switching, and contrast its regulation with that in *C. albicans*.

4.3 Materials and Methods

Media. Media was prepared as previously described [32, 33]. Yeast extract peptone dextrose (YPD) plates or Spider plates containing 200 µg/ml nourseothricin (NAT) were used for selection of strains that were resistant to nourseothricin (SAT^R strains) [34]. For induction of genes under the *MAL2* promoter, Spider medium was made with mannitol excluded and supplemented with 2% maltose.

Strain/Plasmid Construction. All strains and plasmids used in this study are listed in Supplementary Tables C11 and C12, respectively. Transformations were performed as previously described for *C. tropicalis* [3]. Nutritional gene deletions were constructed using the *SAT1* flipper strategy [34]. Plasmids to delete *HIS1* and *ARG4* were made as described [3, 25]. *AHR1* was also deleted using the *SAT1* flipper strategy: the 5' UTR was cloned using *Apal* and *XhoI* and 3' UTR using *SacI* and *SacII*. The deletion cassette was liberated for transformation using *Apal*/*SacI*. Deletion of transcription factors was performed using a previously described method [35]. 5' and 3' regions flanking the ORF were PCR amplified (Table C13). *HIS1* and *ARG4* auxotrophic markers were PCR amplified from plasmids pSN52 and pSN64, respectively. Fusion PCR was then performed to fuse 5' and 3' flanks to nutritional markers. This PCR product was transformed into auxotrophic strains lacking *HIS1* and *ARG4*. PCR was used to confirm correct genomic integration, and this process was repeated to delete the second copy of the ORF. PCR was performed to confirm deletion of the target gene.

To overexpress transcription factors, target genes and the *TDH3* promoter were amplified by PCR and the two fragments fused by PCR amplification. The fusion PCR products cloned into pSFS2a [34] using restriction enzymes *ApaI* and *XhoI*. The plasmids were linearized in the *TDH3* promoter by cutting with *SmaI* or *PacI* and transformed into *C. tropicalis*. Correct genomic integration was confirmed by PCR. To express transcription factors under the *MAL2* promoter, plasmid pRB324 was constructed by cloning the *C. tropicalis MAL2* promoter into pSFS2a using *KpnI* and *ApaI*. PCR was used to amplify transcription factors that were then cloned into this plasmid using *ApaI/XhoI* or *ApaI/BamHI* as noted in oligonucleotide table (Table C13). Plasmids were linearized in the *MAL2* promoter using a partial digest with *PvuI*. Correct integration at the *C. tropicalis MAL2* promoter was confirmed by PCR.

Mating assays. Quantitative mating analyses were performed as previously described [3].

Microscopy. Digital images of colonies were collected using a Zeiss Stemi 2000-C microscope equipped with an Infinity 2 digital camera and Infinity Analyzer software (Lumenera Corporation, Ottawa, Canada). Differential interference contrast (DIC) of cells were captured using a Zeiss Inverted Microscope (Axio Observer. Z1) fitted with an AxioCam HR. Images were processed with AxioVision Rel. 4.8 (Zeiss, Germany).

RNA extraction and RNA-Seq. White (CAY3051), intermediate (CAY3393), and opaque (CAY3053) cells derived from strain CAY1505 (*MTL α / α*) were grown at 25°C in liquid Spider medium to 1.6-1.8 OD₆₀₀. Two biological replicates were prepared for each strain. RNA was isolated using the Ribopure-Yeast Kit (Ambion). RNA was treated with Turbo DNaseI (Ambion). RNA quality was measured on an Agilent 2100 Bioanalyzer at the Brown University Genomics Core Facility, and RNA with RIN scores ≥ 7 used for RNA-Seq.

PolyA RNA was isolated and used to construct strand-specific libraries using the dUTP second strand marking method [36, 37] as previously described [38]. Samples were pooled and sequenced on the Illumina HiSeq to generate 101 base reads.

To measure gene expression, reads were aligned to transcripts from the *C. tropicalis* MYA-3404 genome. RNA-Seq reads were then mapped to the transcripts with Bowtie (version 0.12.9) [39], and placement of multiply mapped reads was estimated using RSEM (version 1.1.18) [40]. Differentially expressed genes were identified using EdgeR (version 3.4.2) [40].

Isolation of single cells for phenotypic stability. Cells from a white, intermediate, or opaque colony were spread onto a plate of Spider medium. Single cells were picked using a micromanipulator and individually plated onto Spider media over a grid so that the resulting colonies could be analyzed for phenotype. For intermediate cells, a distribution of sizes from round to elongated were picked. Cells were allowed to

grow at 30°C for 7 days and phenotypes were analyzed by colony and cellular morphology.

Phenotypic switching assays on C. tropicalis mutant strains. Strains were grown overnight at 30°C in Spider medium. Cells were diluted in water and plated onto Spider medium at a concentration of ~100 colonies per plate. Plates were incubated for 2 days at 30°C and for 8 additional days at room temperature and colonies examined for sectors.

Phenotypic switching assays for strains expressing genes under the control of the TDH3 promoter. White (CAY4599) and opaque (CAY4600) cells were grown at 30°C for 5 hours in YPD liquid. Cells were checked for phenotypic purity and inoculated into YPD liquid and grown at 25°C overnight. These cells were transformed with plasmids containing target transcription factors under the *TDH3* promoter, as well as a control plasmid containing *TDH3*, as previously described [3]. Cells were plated to Spider medium containing 200 µg/mL nourseothricin and incubated at 30°C for 7 days. Phenotypes were analyzed and replica patched to Spider plates for PCR testing. Correct integration of constructs was confirmed by PCR.

Phenotypic switching assays for strains expressing genes under the control of the MAL2 promoter. Strains in the white or opaque state were inoculated from Spider plates into liquid Spider and grown overnight at 30°C. Cells were diluted in water and plated to Spider medium or Spider +Maltose medium to produce ~100 colonies/plate. Plates were incubated at 30°C for 7 days and phenotypes analyzed.

Phenotypic stability of ectopically induced states. Strains containing *pMAL2* driven target genes were grown on Spider +Maltose plates to induce expression of transcription factors. Colonies were inoculated into Spider +Maltose liquid medium and grown at 30°C overnight. Cells were diluted in water and plated to Spider +Maltose or regular Spider media at a concentration of ~100 colonies/plate. Plates were incubated at 30°C for 7 days and phenotypes analyzed.

4.4 Results

4.4.a A stable, third phenotypic state in *C. tropicalis*

During culturing of *MTL* homozygous derivatives of *C. tropicalis* strain ATCC3419 ST-120, we observed that, rather than a bistable switch between white and opaque states, colonies generated multiple phenotypes, including a state that appeared intermediate between that of conventional white and opaque cells (Figure 17 and Figure C1). This phenotype had a distinct colony morphology that appeared wrinkled with dark edges. In general, it appeared darker than white colonies but lighter than opaque colonies, and the edges often exhibited darker stripes (Figure C1). Cells from intermediate colonies contained a range of morphologies: most cells were egg shaped and had a morphology between that of white and opaque cells, but there were also round cells that resembled white cells and elongated cells that resembled opaque cells (Figure 17B-D). Thus, the colony and cellular morphologies associated with the intermediate state are distinct from, but in between that, of white and opaque phenotypes. There were also sub-variants of white and opaque phenotypes, as colonies formed could be smooth or wrinkled, depending on the strain (Figure C1). Although we only discuss *MTL* homozygotes in this study, the *MTLa*/ α parental strain was also observed to form the intermediate state (data not shown).

Switching assays were performed on each of the three phenotypic states in *C. tropicalis*. While switching between white and opaque states was observed, this was less frequent than switching between white and intermediate states, or between intermediate and opaque states (Figure 17E, F). For example, in the *MTLa/a*

background white-to-intermediate switching was 7% and intermediate-to-opaque switching was 3.9%, while direct white-to-opaque switching was only 0.12%. The same was true for switching in the opposite direction, where opaque-to-intermediate and intermediate-to-white switching occurred more frequently than opaque-to-white switching. These results are consistent with cellular and colony morphologies, and suggest that the third phenotypic state represents a true intermediate state between that of white and opaque forms.

To assess the inheritance of the intermediate state, a number of single cells were selected from an intermediate colony and allowed to grow up to form new colonies. Significantly, all of the single cells isolated from intermediate colonies re-formed new intermediate colonies, regardless of whether the initial cells were more spherical or more elongated (Table 1). This result establishes that cells from intermediate colonies truly represented the intermediate state, and that this state is stably inherited for multiple generations. White and opaque cells were also stably inherited when single cells were analyzed, with some sectoring to the intermediate state observed in the colonies once grown up (Table 1).

To test if the intermediate phenotype is a common feature of different *C. tropicalis* strains, we examined 5 other clinical isolates that we had previously observed to undergo white-opaque switching [25] for their ability to form the intermediate state. Four of these isolates (ATCC20663.084, b31083/6/04, WC04-200748, and SCS501148) also formed an intermediate state (data not shown), while one isolate (AM2005/0093) exhibited only a bistable switch between conventional white and opaque forms (Figure 17G-I) [25].

4.4.b *C. tropicalis* cells in the intermediate phenotype undergo efficient mating

The white-opaque switch is a stringent regulator of mating in both *C. tropicalis* and *C. albicans*, as only opaque cells undergo efficient mating [13, 25]. We therefore tested the ability of *C. tropicalis* MTL homozygous cells in the intermediate state to undergo sexual mating. Cells in the intermediate state were able to undergo efficient mating at a level similar to that of opaque cells. Thus, mating crosses containing intermediate or opaque cells mated at a significantly higher frequency than mating crosses that contained white cells ($p < 0.001$) (Figure 18A). The intermediate and opaque states therefore represent mating-competent states of *C. tropicalis*, while cells in the white state mate only infrequently.

4.4.c Global gene expression in white, intermediate, and opaque cells

Genome wide RNA sequencing (RNA-seq) was performed to investigate the gene expression profiles of *C. tropicalis* white, intermediate, and opaque cells. A detailed analysis of differentially expressed genes in each state is presented in Tables C1-6. A comparison of expression profiles reveals that cells in the intermediate state more closely resemble the overall expression pattern of cells in the opaque state than cells in the white state (Figure 18B). In fact, using a cut-off of 3-fold and $p < 1e-10$, there were only 33 differentially-expressed genes between the opaque and intermediate states. In contrast, there were 456 genes differentially expressed between white and intermediate states. Furthermore, there were 129 genes specifically upregulated in white cells, 4 genes specifically upregulated in opaque cells, while intermediate cells did not have any state-specific genes

compared to white and opaque cells. These results indicate cells in the intermediate state express a combination of white- and opaque-specific genes, consistent with it being an intermediate phenotype. However, the expression profile of intermediate cells are biased towards genes preferentially expressed in the opaque state rather than the white state.

Significantly enriched GO-Term processes in white cells included oxidation-reduction, amino acid catabolism, and other catabolic and metabolic processes ($p < 0.01$, Table C7). Opaque-specific genes included *MDR1* and *TPO2*, genes involved in drug transport ($p = 0.003$), and *OP4*, which is also an opaque-specific gene in *C. albicans* (Table C8). White-repressed genes included many involved in ribosome biogenesis and gene expression, as well as nitrogen compound metabolism ($p < 1e-60$, Table C9). Opaque-repressed genes were enriched for genes involved in carbon utilization, including *PCK1* and *SFC1* ($p = 0.012$, Table C10). One gene, the yeast-specific cell wall protein *YWP1*, was repressed in intermediate cells.

4.4.d Transcriptional regulation of the *C. tropicalis* white-opaque switch

To compare regulation of the white-opaque switch between species, we examined the function of the core transcriptional regulators of the *C. albicans* switch in *C. tropicalis*. Transcription factors Wor1, Wor2, Wor3, Czf1, Ahr1, and Efg1 all regulate the white-opaque circuit in *C. albicans*, and orthologs of these factors are present in *C. tropicalis* with the exception of Efg1, which is absent from the sequenced genome [26]. We therefore included a related APSES transcription factor, *EFH1*, in our analysis of *C. tropicalis* switching. In *C. albicans*, overexpression

of *EFH1* was shown to induce opaque-to-white switching, although this switching was dependent on *EFG1* [41].

Analysis of RNA-seq data was first performed to determine the relative expression levels of these transcription factors between the three phenotypic states. *C. tropicalis* *WOR1* is upregulated approximately 2-fold in intermediate cells and 5-fold in opaque cells relative to white cells, while *WOR3* expression increased 2-2.5-fold in these cells types relative to white cells. Conversely, *CZF1* expression was ~2-fold upregulated in white cells relative to intermediate or opaque cells. *EFH1* expression appeared to be up in white cells, but expression of this gene was very low in all three phenotypes. *WOR2* and *AHR1* were not differentially expressed between white, intermediate, and opaque states. Relative expression data for these genes is shown in Figure 18C.

We previously showed that deletion of *WOR1* locks *C. tropicalis* cells in the white state while *WOR1* overexpression induces opaque formation, confirming its role as a conserved master regulator of the opaque state [25]. Here, we generated deletion mutants for *WOR2*, *WOR3*, *CZF1*, and *EFH1*, in *MTLa/a C. tropicalis* cells. We were able to generate a deletion mutant for *AHR1* in the AM2005/0093 strain background, and this strain had no obvious switching defects, although this was not quantified. In the strains that were quantified, white-to-opaque switching in the mutants was not significantly different from that of wild type (Figure C2A). In the case of the $\Delta wor2/\Delta wor2$ mutant, this strain exhibited a distinct intermediate colony morphology that was wrinkled compared to wild type, and cells appeared intermediate or opaque (Figure 19). This contrasts with *C. albicans*, where

Δwor2/Δwor2 mutants are locked in the white state and unable to switch to opaque [19].

4.4.e Identification of novel regulators of phenotypic switching in *C. tropicalis*

To expand our search for transcriptional regulators of the *C. tropicalis* white-opaque switch, we identified factors differentially expressed between these two phenotypic states using previous expression profiling data [3]. White-enriched genes encoding transcription factors included orthologs of *C. albicans* genes *NDT802*, *RME1*, *FGR27*, *ASK10*, *ZCF18*, and *ZCF22*, while opaque-enriched factors were *UME6*, *TAN1*, and *CTRG_03621*. *NDT802* is an ortholog of the *S. cerevisiae* meiosis-specific transcription factor *NDT80*. In *C. albicans* and *C. tropicalis*, there are two *NDT80* paralogs, *NDT801* (*orf19.211*) and *NDT802* (*orf19.513*). *C. albicans* *NDT801* has been shown to regulate cell separation, filamentation, biofilm formation, and virulence, while *NDT802* has no function reported [42-44]. *RME1* is a meiotic regulator in *S. cerevisiae*, and although its function is unknown in *C. albicans* it is also a white-specific gene in this species [45]. *FGR27* encodes a zinc finger transcription factor that regulates *C. albicans* filamentation and adherence to silicone substrates [46, 47]. *ASK10* is involved with *SKN7*-dependent transcription in *S. cerevisiae* and is a component of the RNA polymerase II holoenzyme [48]. *UME6* encodes a zinc finger transcription factor that has roles in hyphal extension and virulence in *C. albicans* [49] and hyphal formation in *C. tropicalis* [50]. *TAN1* is involved in tRNA modification in *S. cerevisiae* [51]. *ZCF18*, *ZCF22* and *CTRG_03621* are predicted to encode zinc finger transcription factors. The ortholog of

CTRG_03621 in *C. albicans* is orf19.4972, and is also upregulated in the opaque state [10, 45], although deletion of orf19.4972 did not show a major affect on switching [19].

To determine if these genes function in regulating the white-opaque switch in *C. tropicalis*, deletion mutants were attempted for the 9 transcription factors listed above. We were successful in obtaining double deletion mutants for 7 of these genes, although repeated attempts at creating mutants for *ZCF22* and *CTRG_03621* were unsuccessful, possibly indicating that these genes are essential. A deletion mutant for *NDT802* was only constructed in the AM2005/0093 background and not in the background analyzed here for switching, although this strain had no obvious phenotype. For the remaining target genes, we performed three replicates of quantitative switching assays on cells starting in the intermediate phenotype [25] using two independent deletion mutants for each candidate gene. None of these mutants had a significant affect on switching to white or opaque, although the *ume6/ume6* mutant exhibited decreased filamentation relative to wild type controls (Figure C2 and Figure 19). Thus, although each of these transcription factors is differentially expressed between white and opaque forms, none of them influenced switching when deleted. We therefore pursued the phenotypes associated with overexpression of these transcription factors, as outlined below.

4.4.f Analysis of constitutive overexpression of potential white-opaque regulators in *C. tropicalis*

To examine the effect of constitutive overexpression of target transcription factors in *C. tropicalis*, we placed these factors under control of the constitutive *TDH3* promoter. This promoter has been extensively used in *C. albicans* to drive high expression of target genes {Delgado, 2003 #1472}{Chauvel, 2012 #1473}. To facilitate analysis of switching events, we used strain AM2005/0093 that typically exhibits a ‘cleaner’ bistable switch between white and opaque forms, with little evidence for a stable intermediate form. We transformed both white and opaque versions of AM2005/0093 with plasmids containing target transcription factors under the control of the *TDH3* promoter, plated transformants on selective media, and analyzed phenotypes after 7 days of growth at 30°C.

Consistent with previous studies [25], constitutive expression of *WOR1* resulted in increased phenotypic switching in white colonies. On transformation plates, *pTDH3-WOR1* expression led to formation of intermediate and opaque colonies that showed a range of filamentous phenotypes from smooth colonies consisting of yeast cells to wrinkled colonies that exhibited aerial hyphae and contained 100% filamentous cells (Figure 20C). Constitutive expression of *WOR3*, *CZF1*, and *CTRG_03621* also generated distinct phenotypes in white cells compared to the control strain (Figure 20). White cells expressing *pTDH3-WOR3* generated a mixture of colonies, some with a wrinkled volcano-like appearance whose cells appeared intermediate, and flat, dark, wrinkled colonies containing cells that looked opaque (Figure 20D). Expression of *CZF1* gave rise to smoother, flatter colonies, and

cells in these colonies maintained the round, white phenotype. Occasionally, slightly darker colonies also formed from which cells looked egg-shaped or intermediate (Figure 20E). Overexpression of *CTRG_03621* gave rise to colonies that were slightly darker and more wrinkled than the control, and contained cells with a mixture of shapes that resembled the intermediate phenotype (Figure 20F).

The opaque phenotype was less stable during transformation and showed a high rate of switching back to the white form even in the control strain (Figure 20G, H). Constitutive expression of *WOR1* stabilized the opaque state and caused increased invasive growth into the agar and filamentation (Figure 20I). Constitutive expression of *WOR3* also stabilized the opaque state at the level of cell morphology, but generated shiny colonies that were distinct from normal opaque colonies (Figure 20J). *CTRG_03621* expression was another stabilizer of the opaque phenotype, and forced expression generated colonies that were more wrinkled than those of wild-type opaque colonies (Figure 20K). Interestingly, overexpression of *NDT802* generated opaque colonies with multiple white sectors, giving the colonies a striped look and contributing to the presence of both round and elongated cells (Figure 20L). Together, these experiments identified several regulators of white-intermediate and white-opaque switching, but were unable to identify factors that significantly induce opaque-to-white switching due to instability of the opaque state. We therefore developed a regulatable promoter for controlled expression of *C. tropicalis* transcription factors as described below.

4.4.g Regulatable expression of *C. tropicalis* transcription factors by the *MAL2* promoter

To gain further insight into the phenotypes induced by the expression of specific transcription factors, we employed the regulatable *C. tropicalis MAL2* promoter. The *MAL2* promoter has been successfully used in *C. albicans* for regulated gene expression, as this promoter is induced in the presence of the sugar maltose [52]. We created strains that expressed target transcription factors under the *C. tropicalis MAL2* promoter, with two independent strains generated for each factor. For switching assays, experiments were performed on Spider medium, which represents uninduced conditions, or on Spider+Maltose to induce expression of the relevant gene. This analysis was performed in the AM2005/0093 strain background of *C. tropicalis*. Unfortunately, generating the plasmid for induction of *CTRG_03621* proved to be difficult, so this strain has not yet been tested for switching.

We found that for *C. tropicalis* white cells expressing *pMAL2-WOR1*, a phenotypic change was induced on Spider medium containing maltose, while no phenotypic change was observed on regular Spider medium. Thus, all cells plated on Spider medium lacking maltose maintained their white phenotype, while overexpression of *WOR1* in the presence of maltose led to the formation of intermediate-looking colonies with darker sectors (Figure 21A, B). Interestingly, induction of *WOR1* was insufficient to drive complete switching from white to opaque in white cells; even the darker sectors contained egg-shaped cells that resembled intermediate cells rather than the more elongated true opaque cells (Figure 21B). Overall, 98% of colonies switched to the intermediate state upon

WOR1 overexpression, with few colonies containing conventional opaque cells (Figure 21A). This contrasts with the situation in *C. albicans*, where ectopic expression of *WOR1* is sufficient to induce 100% of cells switching to opaque [4, 16, 17]. It is possible that the level of *WOR1* expression under the *C. tropicalis* *MAL2* promoter is insufficient to promote the full switch from white to opaque, or that other parts of the opaque transcriptional circuit are not activated upon ectopic *WOR1* expression.

When *WOR1* expression was induced in opaque cells this also led to a change in phenotype. Growth of *pMAL2-WOR1*-expressing opaque cells on Spider-Maltose generated normal looking, smooth opaque colonies, whereas growth on Spider+Maltose resulted in highly wrinkled, invasive colonies (Figure 21C, D). Analysis of cells from these wrinkled colonies revealed a large number of highly filamentous cells. This resembles what was also seen with overexpression of *WOR1* in opaque parental cells using the *pTDH3* promoter (Figure 20I). We therefore note that induction of *WOR1* expression generates different phenotypes when performed in white or opaque cells, indicating that that phenotypic output is dependent on the initial state of the parental cell.

For strains expressing *pMAL2-WOR3* and *pMAL2-CZF1*, *C. tropicalis* phenotypes were consistent with those generated by *TDH3* overexpression. *WOR3* induction in white cells led to a high percentage (98%) of wrinkled colonies containing cells that appeared intermediate (Figure 21A, B), while it generated shiny opaque sectors when induced in opaque cells (Figure 21C, D). *CZF1* expression led to increased formation of intermediate colonies and sectoring in

white cells (28% of cells showed switching, Figure 21A, B), yet no phenotypic differences were observed when induced in opaque cells (Figure 21C).

Interestingly, induction of *WOR2* expression led to the increased formation of white sectors in opaque colonies (10%), indicating a role for *WOR2* in induction of switching to the white state (Figure 21C, D), but had no effect on the phenotype of white cells (Figure 21A). *NDT802* expression induced frequent switching in opaque cells, with 96% of cells showing opaque-to-white or opaque-to-intermediate switching (Figure 21C, D), similar to what was observed in the *TDH3* overexpression assay (Figure 20G). Interestingly, *NDT802* expression also caused induction of the intermediate state in white cells, although this was observed at low frequency (8%, Figure 21A, B). This suggests that induction of *NDT802* may destabilize both white and opaque states. *UME6*, a gene that was not tested in the *TDH3* overexpression experiments, showed induction of filamentation in both white and opaque cells (Figure 21B, D), as well as opaque-to-white sectoring, consistent with a role in promoting formation of the white state (Figure 21C, D).

4.4.h Inheritance of ectopically induced phenotypic states

In *C. albicans*, ectopic expression of *WOR1* in a wildtype strain leads to stable formation of the opaque state even after turning off ectopic expression, due to activation of endogenous *WOR1* expression and initiation of a positive feedback loop on its own promoter [4, 16, 17]. Additional experiments showed that ectopic expression of *C. albicans* *CZF1* drives switching to opaque but does not affect heritability of the opaque state once turned off. Conversely, *C. albicans* *WOR2*

expression is necessary for the stability of the opaque state but not for switching from white to opaque [19].

To determine if the phenotypes induced by expression of *C. tropicalis* transcription factors were heritable, we examined the effect of shutting off ectopic gene expression by transferring cells from maltose-containing media to media without maltose. We examined the white-inducers *WOR2*, *NDT802*, and *UME6*, as well as opaque/intermediate inducers *WOR1*, *WOR3*, *CZF1*, and *CTRG_03621*. Strikingly, all intermediate- and opaque-like phenotypes induced by *WOR1* in white cells were unstable, with 100% of cells returning to the white state on Spider-Maltose media (Figure 22A, B). Similar results were observed with *WOR3* and *CTRG_03621*, as although ectopic expression of these genes induced high levels of white-to-intermediate switching, shutting off ectopic expression on media lacking maltose led to cells returning to the white state (Figure 22A, B).

On the other hand, the transcription factors that induced switching to the white state, particularly *WOR2*, *NDT802*, and *UME6*, often showed partial maintenance of the white phenotype, although there was some reversion to the opaque state without continuous expression of these transcription factors (Figure 22). For example, in the case of *UME6*, while induction of ectopic *UME6* in opaque cells resulted in switching to intermediate in 60% of colonies (Figure 21D), shutting off ectopic expression in these colonies caused 35% of colonies to remain intermediate and 65% of colonies to switch back to opaque (Figure 22C, D).

4.5 Discussion

The white-opaque switch represents a model system for understanding epigenetic switching. Much of our understanding of the switch has come from studies in *C. albicans*, which have revealed that the switch is regulated by a transcriptional network involving multiple interacting feedback loops between at least six transcription factors [15]. In this paper, we examined the regulation of white-opaque switching in *C. tropicalis*, and found both similarities and surprising differences with the regulation of the switch in *C. albicans*.

First, our work establishes that a stable intermediate phenotype exists in *C. tropicalis*, as initially proposed by Xie *et al.* [8]. These authors noted a cell type that showed physical characteristics of both white and opaque *C. tropicalis* cells, but did not address other aspects of the stability of this cell type or its switching behavior. We demonstrate that the intermediate form represents a true stable state that is intermediary to both white and opaque states for multiple traits including cellular and colony morphology, gene expression profile, and switching frequency. Thus, while white-to-opaque switching and opaque-to-white switching was relatively rare, switching both to and from the intermediate phenotypic state occurred at a higher frequency. In addition, although there was a distribution of cellular morphologies associated with the intermediate state, all of these cells were deemed to be truly intermediate by selection of single cells and analysis of the resulting colonies. The intermediate state is not, therefore, simply a mixture of cells from white and opaque states. We consequently propose that a tristable switch exists in

C. tropicalis, in which cells can exist in (at least) three stable states represented by white, intermediate, and opaque.

Expression profiling of white, intermediate, and opaque cells revealed that gene expression in the intermediate state was closer, overall, to that of the opaque state than the white state. Consistent with this observation, intermediate cells mated with a similar efficiency to opaque cells, and significantly higher than that of white cells. Furthermore, while expression of many white-specific and opaque-specific genes were identified, there were relatively few genes expressed specifically only in the intermediate state. These results are further evidence of this state representing a true intermediary form between that of white and opaque.

Recent studies in *C. albicans* have similarly revealed the existence of phenotypic states that differ from conventional white and opaque forms. For example, Pande *et al.* demonstrated that Wor1 regulates the formation of a GUT phenotype in *C. albicans* α/α cells [53]. The GUT phenotype was induced in *C. albicans* cells passaged in the mammalian gastrointestinal tract and, although consisting of elongated cells and dependent on Wor1 overexpression, exhibited a distinct transcriptional profile to that of similar-looking opaque cells. In addition, Tao *et al.* recently showed that a subset of clinical isolates of *C. albicans* undergo tristable switching between white, opaque, and grey phenotypes [54]. These three phenotypes each showed distinct transcriptional profiles and niche adaptations, with grey cells showing the most growth in a cutaneous infection model [54]. We emphasize that neither the grey phenotype nor the GUT phenotype appears to represent an intermediary form between white and opaque states. For example,

cells in the grey and GUT phenotypes expressed many state-specific genes that distinguished them from white and opaque cells, whereas *C. tropicalis* intermediate cells exhibited a profile between that of white and opaque cells. Furthermore, at least in the case of the grey phenotype, *C. albicans* white cells can stochastically switch to this state in the absence of the Wor1 transcription factor [54]. In contrast, the intermediate form in *C. tropicalis* was dependent on Wor1, as *Dwor1/Dwor1* cells appeared locked in the white phenotypic state.

Regulation of the *C. albicans* white-opaque bistable switch involves an interlocking regulatory circuit with reinforcing positive and negative feedback loops. *C. albicans* Wor1 positively regulates its own expression, and this positive feedback loop is thought to be key for stable inheritance of the opaque state [4, 16, 17]. Wor1 acts in concert with at least 5 other transcription factors, Wor2, Wor3, Czf1, Ahr1, and Efg1 to regulate white-opaque switching. In fact, Wor1 binds to the promoters of each of these transcription factors, and this highly integrated network provides resistance to perturbations to the circuit [15, 19]. The white-opaque transcriptional circuit shows striking topological similarities to other regulatory circuits, including those controlling *C. albicans* biofilm formation [43], and pseudohyphal growth in *S. cerevisiae* [55, 56], indicating that related transcriptional networks regulate many important developmental transitions.

In comparing the regulation of the white-opaque circuits in *C. albicans* and *C. tropicalis* there are clear similarities as well as notable differences. In particular, the role of Wor1 as the master regulator of the opaque state has been conserved, as this gene is essential for the opaque state [4, 16, 25]. However, the *EFG1* gene is absent

from the *C. tropicalis* genome, which is surprising given that Efg1 is a key controller of the white state in *C. albicans* [15]. We have ruled out the possibility that the related APSES transcription factor, Efh1, has assumed the role of Efg1 in regulating *C. tropicalis* switching, as deletion or overexpression of *EFH1* had no apparent effect on switching. Overexpression of *AHR1* also did not have a significant effect on *C. tropicalis* switching, unlike in *C. albicans* [20]. The inability of *AHR1* to effect switching is perhaps not surprising; Ahr1 has been shown to direct non-canonical binding of Mcm1 [57], and *C. tropicalis* lacks these non-canonical Mcm1 binding motifs [58]. Of the other 3 transcriptional regulators in the *C. albicans* white-opaque circuit, Wor3 function appears to be the most similar in *C. tropicalis*, as overexpression of Wor3 resulted in efficient white-to-opaque (and white-to-intermediate) switching. Overexpression of *C. tropicalis* *CZF1* also increased white-to-intermediate switching, but deletion of this gene did not block switching as in *C. albicans* [19]. Surprisingly, overexpression of *C. tropicalis* *WOR2* actually resulted in increased switching to the white state, whereas in *C. albicans* *WOR2* promotes the stability of the opaque state [19]. *WOR2* expression was also elevated in *C. albicans* opaque cells relative to white cells, whereas its expression did not differ between *C. tropicalis* white and opaque cells. It therefore appears while *WOR2* regulates the white-opaque switch in both *Candida* species, its precise role has been reversed in terms of which phenotypic state it is promoting.

Another striking difference between *C. albicans* and *C. tropicalis* circuits was that transient induction of *WOR1* did not lead to stable formation of the opaque state in *C. tropicalis*. Thus, ectopic expression of *WOR1* induced formation of opaque

or intermediate forms in white cells, but after repression of ectopic expression cells reverted to the white state. This contrasts sharply with *C. albicans*, where a pulse of ectopic *WOR1* expression is sufficient for stable expression of the opaque state due to activation of endogenous *WOR1* expression [4]. There are several possibilities for this difference, including the fact that *C. tropicalis* *WOR1* may not self-regulate its own promoter. However, we have observed putative *WOR1* binding sites upstream of the *WOR1* gene in *C. tropicalis* [25] and *WOR1* has a conserved DNA binding domain [59], making this hypothesis unlikely. Alternatively, it seems likely that induction of endogenous *C. tropicalis* *Wor1* is not sufficient for establishment of the opaque state, and that other parts of the circuit must also be activated for heritable opaque formation. Interestingly, a recent study showed the ectopic *WOR1* expression also fails to activate stable opaque formation in *C. albicans* cells lacking the Med3 component of Mediator, even though endogenous *WOR1* expression is activated [24]. Mediator is large protein complex that functions to link DNA-bound activators with the core transcriptional machinery, and also regulates epigenetic chromatin modifications [60]. It is therefore possible that chromatin changes required for stable establishment of the opaque state do not occur in *C. tropicalis* cells ectopically expressing *Wor1*. Additional experiments will be required to define what factors stabilize the opaque state in *C. tropicalis* or, alternatively, what mechanisms destabilize the white state upon switching to opaque.

We also think it is notable that different phenotypes were induced by the same transcription factor when overexpressed in white cells versus opaque cells. This was most evident for overexpression of *WOR1* and *WOR3* genes. For example,

ectopic *WOR1* expression in white cells induced formation of smooth intermediate or smooth opaque colonies, whereas ectopic expression in opaque cells resulted in highly invasive and filamentous opaque colonies. In the case of *WOR3*, overexpression of this gene in white cells resulted in highly wrinkled colonies containing intermediate and filamentous cells, whereas smooth opaque colonies were produced upon overexpression in opaque cells. Our results establish that the transcriptional circuits produced by overexpression of these genes in white and opaque cells are distinct. In particular, they provide further evidence that ectopic *WOR1* expression results in different phenotypic states when induced in white or opaque cells. Thus, ectopic *WOR1* (or *WOR3*) expression in white cells does not result in the same state as ectopic *WOR1* (or *WOR3*) expression in opaque cells.

Our work also identifies novel regulators of the white-opaque switch. *NDT802* and *UME6* genes were selected for investigation in *C. tropicalis* due to differential expression between white and opaque cells. Induction of *NDT802* or *UME6* induced instability in the opaque state and either opaque-to-white or opaque-to-intermediate switching. In *C. albicans*, orthologs of these genes are key regulators of filamentation and biofilm formation [43, 61, 62], and *UME6* also regulates these processes in *C. tropicalis* [50]. These results therefore extend previous observations [12, 19, 25, 44, 63] that there is a close relationship between the transcriptional regulation of the white-opaque switch and filamentation in *Candida* species. This overlap includes *EFG1* and *CZF1* in *C. albicans* and *WOR1* in *C. tropicalis*, which regulate both phenotypic switching and filamentation [19, 25, 63]. Indeed, *Wor1* orthologs are known to regulate filamentous growth in other fungal

species [56, 64]. It is therefore expected that future studies will reveal further crosstalk between the circuits regulating white-opaque switching and filamentous growth.

Together, the work presented here establishes the existence of a tristable switching system in *C. tropicalis* and demonstrates differences in regulation with the related switching mechanism in *C. albicans*. Recent work has shown that phenotypic switching in *C. tropicalis* is associated with changes in virulence and antifungal susceptibility [65]. Whether or not these phenotypic transitions are related to the white-opaque switching system, they further demonstrate the relevance of heritable changes on interactions with the host. The discovery of a white-intermediate-opaque tristable switch provides a new phenomenon to study in the context of human disease. Additionally, the fact that the white-opaque switch appears to be a relatively recent adaptation, thought to have arisen just prior to the divergence of *C. albicans* and *C. tropicalis*, provides an interesting context for studying evolution of the switch. The differences in white-opaque switching between *C. albicans* and *C. tropicalis* are numerous, including phenotypic stability (bistability versus tristability), gene expression profiles, sensitivity to environmental cues, and transcriptional regulation. Further studies will continue to illuminate how the white-opaque switch has evolved and its role(s) in these two human fungal pathogens.

4.6 Figures and Tables

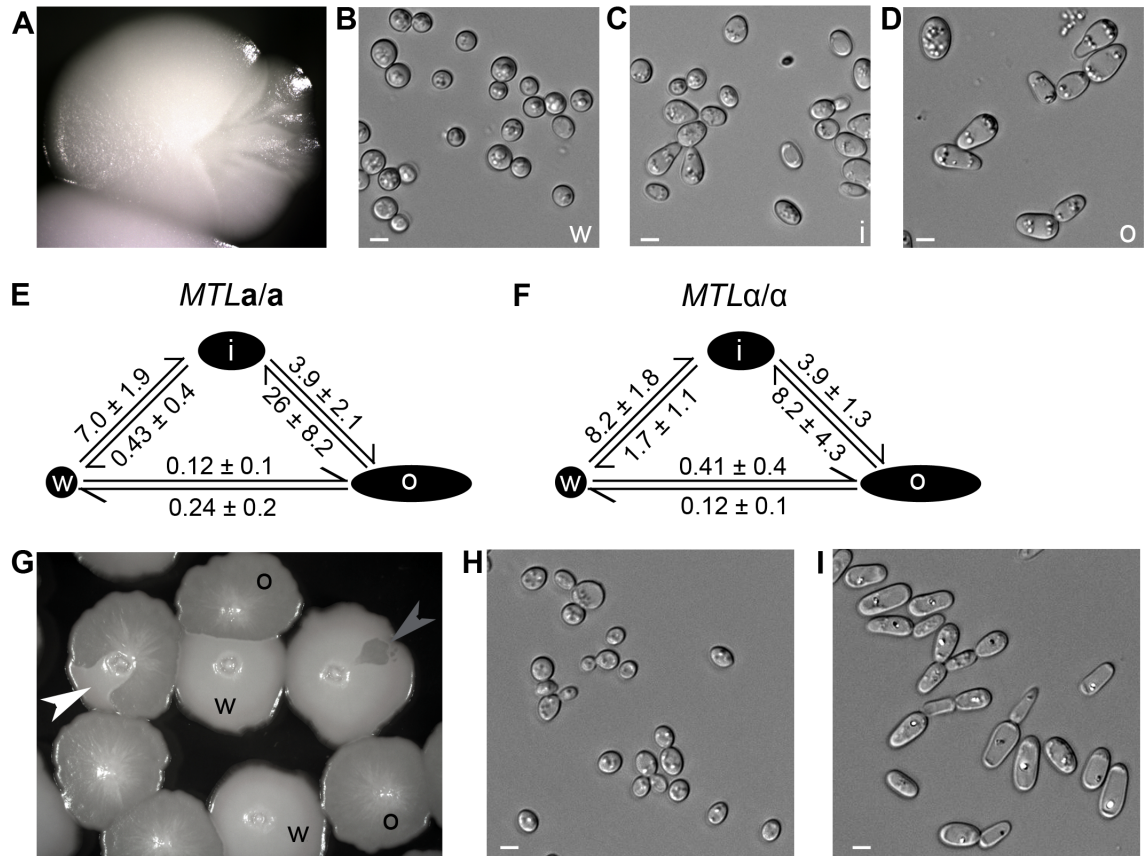


Figure 17. A white-intermediate-opaque switch in *C. tropicalis*. (A) White colony of *MTLα/a* strain CAY1504 sectoring to intermediate (light gray arrowhead) and then to opaque (dark gray arrowhead). Colony was grown on Spider plates at room temperature for 10 days. (B-D) Representative cellular morphologies of (B) white cells (CAY3051), (C) intermediate cells (CAY3393), and (D) opaque cells (CAY3053) from *MTLα/α* strain CAY1505. Cells were taken from corresponding colonies shown in Figure C1B. Scale bars = 5μm. (E-F) Switching frequencies between the three phenotypic states. (E) *MTLα/a* and (F) *MTLα/α* strains were grown on Spider media at room temperature for 10 days and switching frequencies determined (see Materials and Methods). (G) Colony morphology of white (w) (CAY4599) and opaque (o) (CAY4600) states of strain CAY2597. Gray arrowhead indicates an opaque sector in a white colony; white arrowhead indicates a white sector in a white colony. (H) Cell morphology of the white phenotype of CAY2597 (CAY4599). (I) Cell morphology of the opaque phenotype of CAY2597 (CAY4600). Scale bar = 5μm.

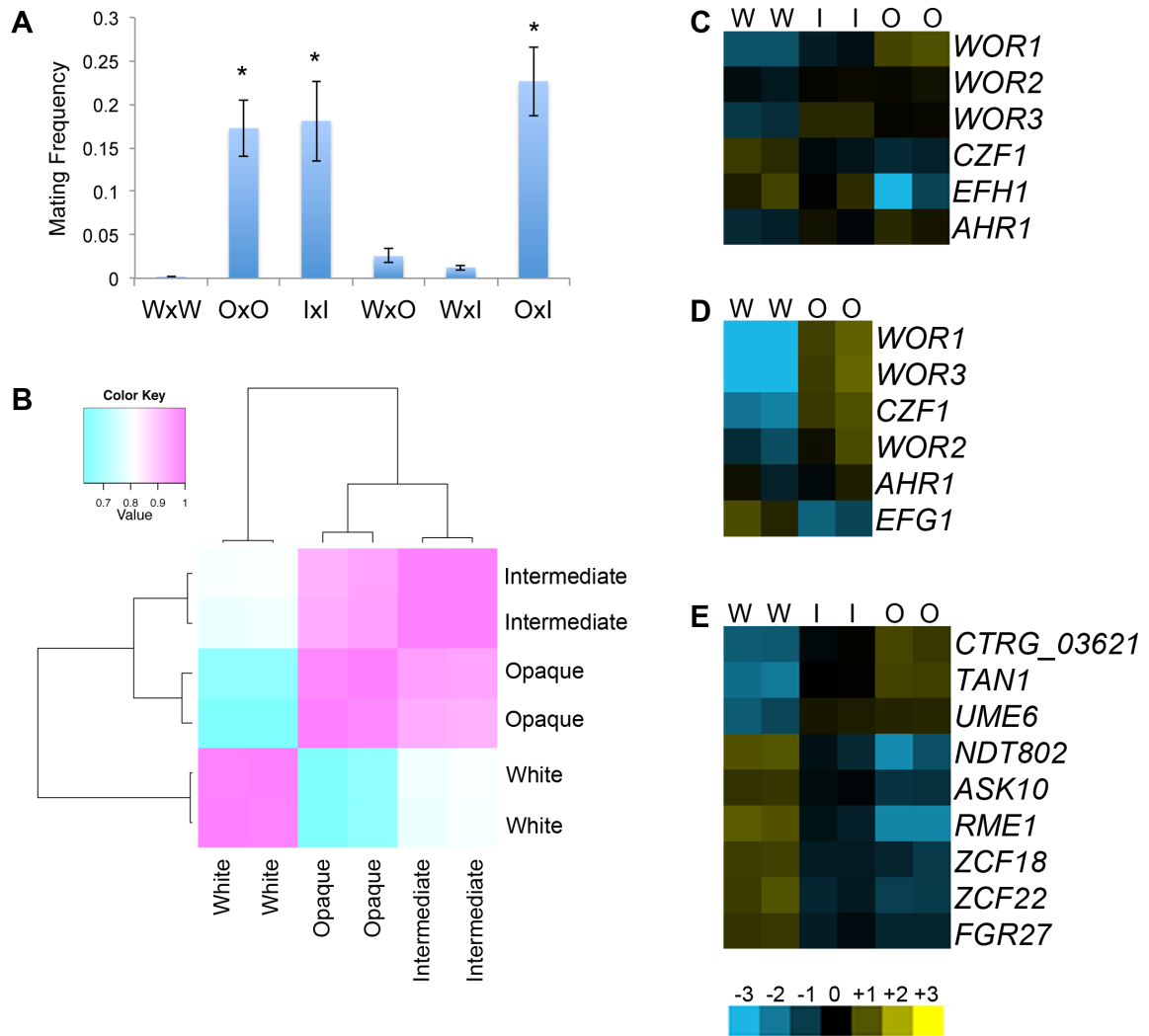


Figure 18. Analysis of the intermediate state of *C. tropicalis*. **(A)** Mating frequencies of white (W), opaque (O), and intermediate (I) phenotypes. *MTLa* strains were arg⁻ and include CAY3371 or CAY3384 (W), CAY3373 or CAY3386 (O), and CAY3381 or CAY3389 (I). *MTL α* strains were his⁻ and include CAY3051 or CAY3391 (W), CAY3393 or CAY3394 (I), and CAY3053 or CAY3392 (O). Matings were performed for 1 day at room temperature on Spider media. Error bars show standard error, with each data point containing at least 9 replicates. All starred data points are significantly higher than all unstarred points ($p < 0.001$, two-tailed T-test). **(B)** Global pairwise comparison of expression profiles of two replicates of RNA-Seq analysis for white, intermediate, and opaque phenotypes. Pink indicates similar gene expression, while blue indicates divergent gene expression. **(C)** Comparative expression levels of *C. albicans* white-opaque regulators in *C. tropicalis* white, intermediate, and opaque states. **(D)** Expression of *C. albicans* white and opaque regulators for comparison (*C. albicans* data from [9]). **(E)** Expression levels of several transcription factors that differ between *C. tropicalis* white, intermediate, and opaque states. Expression data is shown as log₂ values.

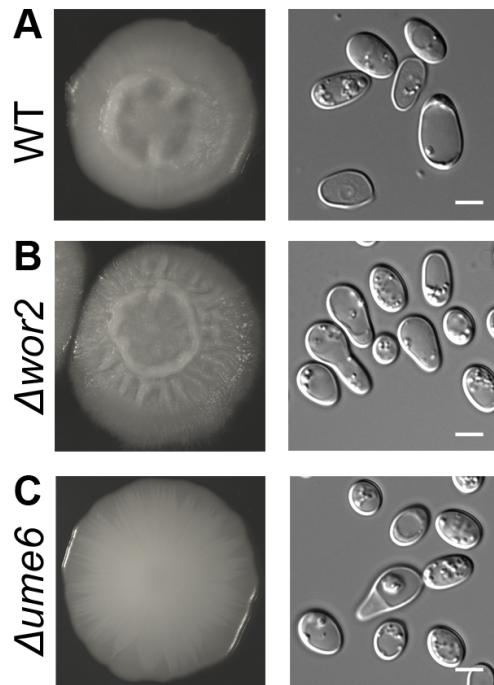


Figure 19. *C. tropicalis* *wor2* and *ume6* mutants display distinct phenotypes.

Strains lacking *WOR2* or *UME6* have distinct phenotypes. Strains were grown on Spider medium at 30°C for 2 days followed by growth at room temperature for 8 days. Representative colonies and cells in the intermediate phenotype from **(A)** WT, **(B)** $\Delta/wor2$, and **(C)** $\Delta/ume6$ strains.

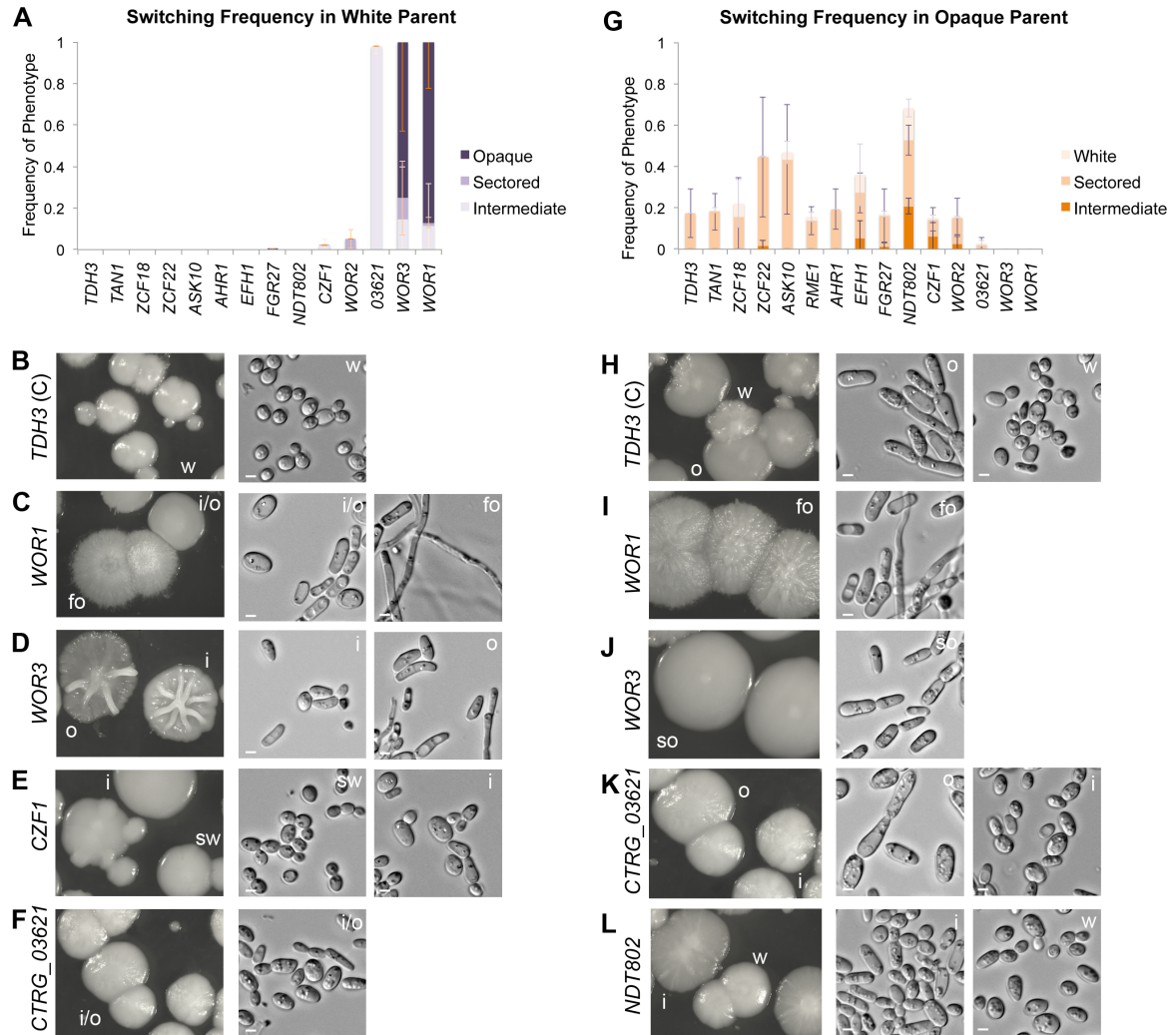


Figure 20. Analysis of constitutive expression of *C. tropicalis* transcription factors on the white-opaque switch. *C. tropicalis* transcription factors were constitutively expressed under the *TDH3* promoter to determine potential affects on phenotypic switching. **(A)** Frequency of phenotypic switching in white cells upon transformation with transcription factors under *pTDH3* control. **(B-F)** Colony morphology (left) and cell morphology (right) from white cells transformed with the indicated transcription factor and grown at 30°C for 7 days. (B) *TDH3* control, (C) *WOR1*, (D) *WOR3*, (E) *CZF1*, and (F) *CTRG_03621*. **(G)** Frequency of phenotypic switching in opaque cells upon transformation with the indicated *TDH3* constructs. **(H-L)** Colony morphology (left) and cell morphology (right) from opaque cells transformed with the indicated transcription factor and grown at 30°C for 7 days. (H) *TDH3* control, (I) *WOR1*, (J) *WOR3*, (K) *CTRG_03621*, and (L) *NDT802*. Scale bars = 5 μ m. Phenotypes are indicated as follows: white (w), intermediate (i), opaque (o), smooth white (sw), shiny opaque (so), filamentous white (fw), filamentous opaque (fo).

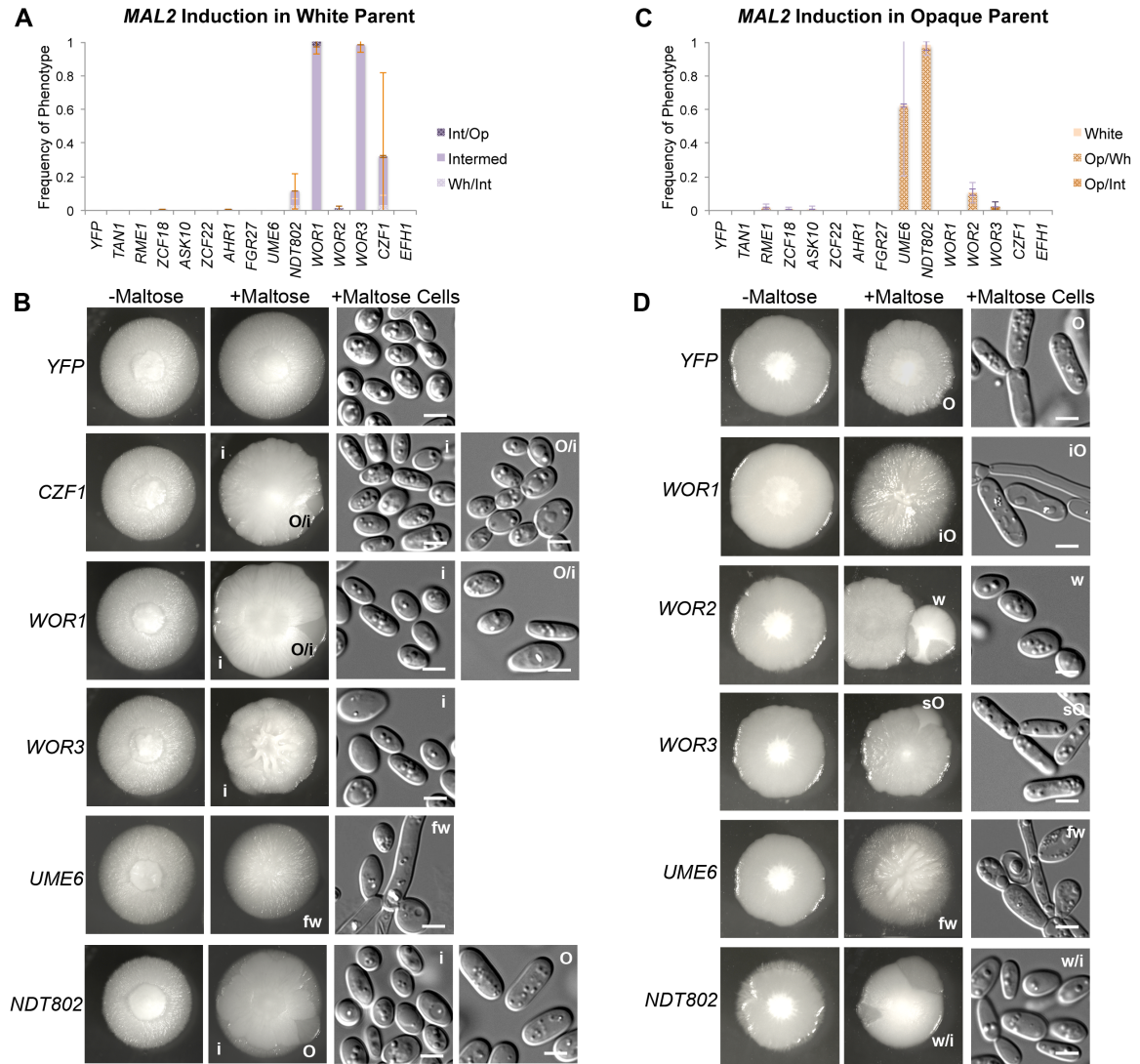


Figure 21. Analysis of phenotypes in *C. tropicalis* cells expressing transcription factors under the control of the regulatable *MAL2* promoter. (A) Frequency of switching in white cells upon transcription factor induction (growth on Spider+Maltose). **(B)** Left, colonies on non-inducing medium (-Maltose) and inducing medium (+Maltose) after growth at 30°C for 7 days. Colony phenotypes are indicated by “i” (intermediate), “O” (opaque) or “O/i” (opaque/intermediate). Right, cells from Spider +Maltose plates, from phenotypes noted as above. Genes whose induction led to alternative phenotypes in white cells are shown. **(C)** Frequency of switching in opaque cells upon transcription factor induction (growth on Spider+Maltose). **(D)** Left, colonies on non-inducing medium (-Maltose) and inducing medium (+Maltose) after growth at 30°C for 7 days. Phenotypes on plates are noted as “O” (opaque), “iO” (invasive opaque), sO (shiny opaque), “w” (white), and fw (filamentous white). Right, cells from Spider+Maltose plates, with phenotypes noted as above. Genes whose induction led to alternative phenotypes in opaque cells are shown.

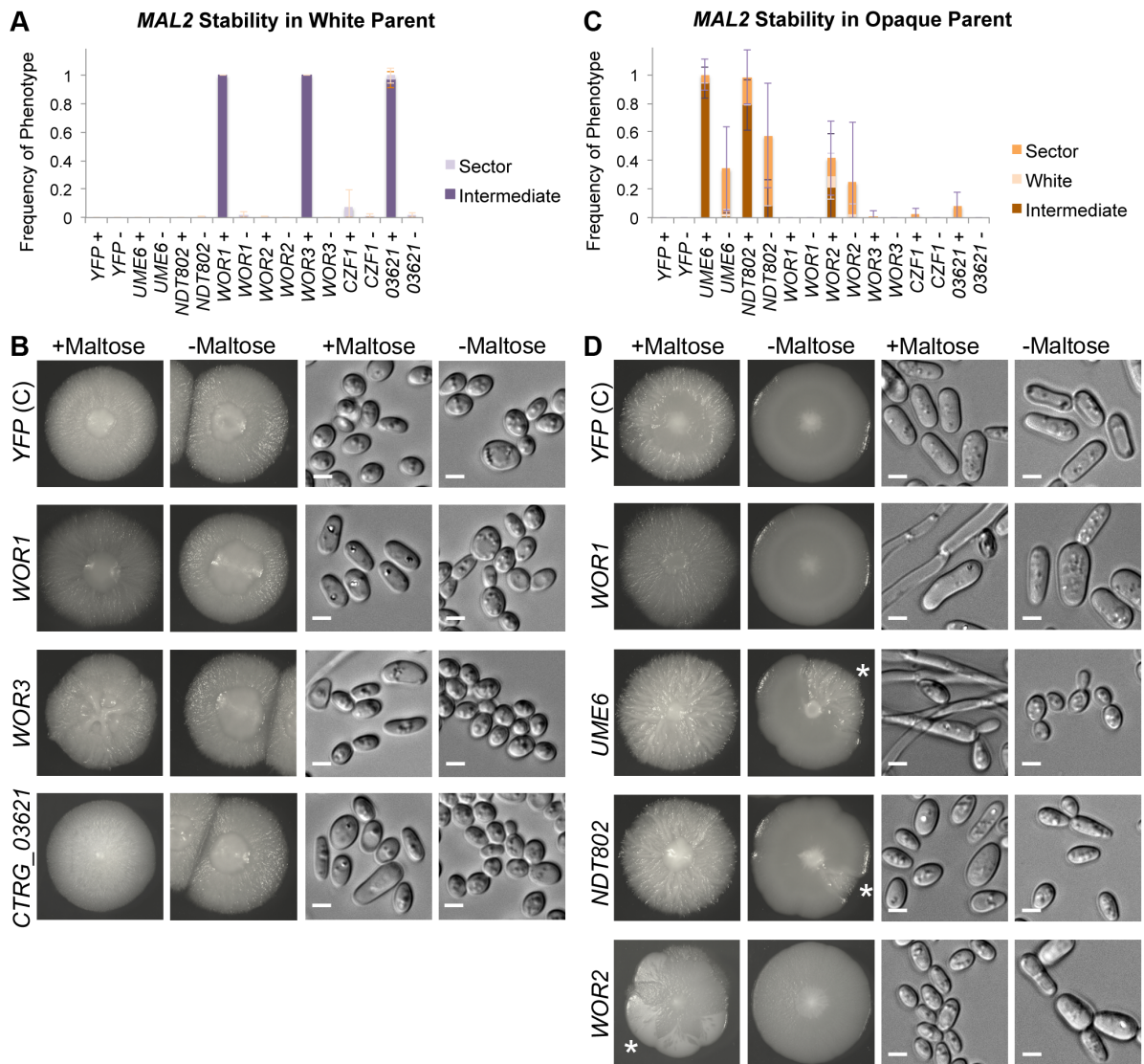


Figure 22. Analysis of the heritability of induced phenotypic states. Phenotypic states of *C. tropicalis* cells expressing genes under the control of the regulatable *MAL2* promoter. Cells were grown under inducing conditions (+Maltose) and then gene expression turned off (-Maltose). **(A)** Analysis of phenotypic states in white parental cells. Cells were transferred from Spider+Maltose to Spider-Maltose. **(B)** Left, colonies on Spider+Maltose plates or Spider-Maltose plates. Right, cells from Spider+Maltose plates or Spider-Maltose plates. **(C)** Analysis of phenotypic states in opaque parental cells. Cells were transferred from Spider+Maltose to Spider-Maltose. **(D)** Left, colonies on Spider+Maltose plates or Spider-Maltose plates. Right, cells from Spider+Maltose plates or Spider-Maltose plates. Cells taken from starred sectors where noted in colony images.

Table 1. Analysis of single cells isolated from different phenotypic states.

Single cells were picked using a micromanipulator and allowed to develop into colonies on Spider plates for 7 days at 30°C. A range of cell shapes were picked to account for variable phenotypes from each state. % Stability indicates percent of colonies that maintained phenotype from the original colony. Results show that cells picked from each phenotype grew up to give similar colony morphologies. Sectors indicates the percentage of colonies that contained minority sectors to alternative phenotypes as noted in parentheses.

Strain / Phenotype	# Cells	Colony Phenotype	% Sectors
CAY3384 a/a White	25	100% White	8% (intermediate)
CAY3386 a/a Opaque	21	100% Opaque	0%
CAY3389 a/a Intermediate	18	100% Intermediate	0%
CAY3391 α/α White	20	100% White	5% (intermediate)
CAY3392 α/α Opaque	20	100% Opaque	0%
CAY 3393 α/α Intermediate	18	100% Intermediate	6% (opaque)

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Chapter 5. Conclusion

5.1 Summary and general discussion

This thesis presents an examination of white-opaque phenotypic switching in *C. tropicalis*, a human fungal pathogen. Importantly, it identifies for the first time an extant mating cycle in a species of yeast that was previously believed to be asexual. The identification of the ability of the white-opaque switch to regulate sexual mating in *C. tropicalis* revealed that another pathogenic species of yeast outside of *C. albicans* and *C. dubliniensis* has conserved this trait, providing further evidence of unusual mating cycles being common in fungal pathogens. The discovery of the white-opaque switch regulating mating in *C. tropicalis* opened new avenues for studying the evolution of phenotypic switching and its control of mating.

I have identified several characteristics of the *C. tropicalis* white-opaque switch that are not conserved with *C. albicans*. This includes the temperature sensitivity of the opaque state to 37°C and absorption of the dye phloxine B by opaque colonies. Additionally, the white-opaque switch in *C. tropicalis* does not appear to be regulated by the *MTL* as it is in *C. albicans*. Regulation by *WOR1* is shared by both species but there are also important differences; overexpression of *WOR1* induces opaque formation in both species, but filamentation and biofilm formation are also induced in *C. tropicalis* but not in *C. albicans*. There are other fundamental differences in regulation, including transcription factors that regulate the switch in only one species (e.g., Ahr1) and the roles that they play in regulation. One of the most surprising results of this work was the identification of a third

stable phenotypic state in *C. tropicalis*. The identification of this tristable switch provides a foundation for the study of how this epigenetic switch is regulated. As examples of tristable switching are rare in biology, mechanistic analysis of tristability in *C. tropicalis* has the potential to be highly informative for how reversible transitions between three stable states can occur.

5.2 Future Directions: White-opaque switching and the environment

The novelty of this project has opened a new field of study for phenotypic switching in *C. tropicalis*. As this work is the first to identify mating in this species, various aspects of the mating process are now being examined. Work in the lab has examined the ability of *C. tropicalis* to form sexual biofilms, or biofilms that require a sexual interaction between **a** and α cells. These studies have revealed that opaque cells of *C. tropicalis* are able to form biofilms when they are mixed with cells of the opposite mating type and a mating response is allowed to occur [1]. This is a process that has not been observed in *C. albicans*, again emphasizing the differences in white-opaque switching between these two species. Other aspects of the parasexual process, including the completion of a parasexual cycle via chromosome loss, have also been assessed. The study by Seervai *et al.* demonstrated that *C. tropicalis* tetraploids can be induced to return to the diploid state under certain environmental stress conditions, and that this occurs by a process of chromosome loss similar to that observed in *C. albicans* [2]. Future studies may examine additional aspects of the parasexual cycle in *C. tropicalis* and how this might compare with *C. albicans*. For example, ongoing projects in the lab address the

potential for generating variation by the parasexual cycle in *C. albicans*, and future studies may examine this process in *C. tropicalis*. Moreover, the identification of haploids in *C. albicans* [3] has also provoked a search for haploids in *C. tropicalis* in our laboratory. The identification of haploids in the *C. tropicalis* parasexual cycle would provide a useful tool for the field and enable further comparisons with *C. albicans*. The work presented here only scratches the surface of the study of the mating cycle in *C. tropicalis*, and provides a foundation for many future studies.

How environmental cues affect the white-opaque switch in *C. tropicalis* is still an open question. During my time in the lab, I did several screens of various conditions known to induce white-opaque switching in *C. albicans*, including oxidative stress, carbon dioxide, anaerobic conditions, N-acetylglucosamine, and others, but was unable to identify any condition that induced stable switching at an appreciable level. The inability to identify conditions to reproducibly induce switching in either direction was a major hindrance, as getting cells in the proper phenotypic state required for experimental analysis proved difficult. These preliminary studies on the environmental influences of switching in *C. tropicalis* will be combined with additional studies in the lab that aim to compare conditions that induce switching between *C. albicans* and *C. tropicalis* to emphasize the difference in environmental regulation of white-opaque switching between these two species.

Recent studies in the lab have identified nicotinamide, a histone deacetylase inhibitor, as a condition that induces stable white-opaque switching in *C. tropicalis*. This is also the case in *C. albicans*, where the induction of white-to-opaque switching is due to epigenetic modifications, presumably including those at the *WOR1*

promoter [4]. Future work may examine the mechanism of action of nicotinamide in *C. tropicalis*, including effects on *WOR1* expression. Examining other compounds that effect histone modifications or other components of the epigenetic machinery will also be informative. To identify environmental conditions that induce switching in *C. tropicalis*, Biolog® phenotype microarray plates may be employed [5]. These plates contain multiple different conditions, including alternative carbon sources, nitrogen sources, compounds, and peptides, providing a diverse platform to screen growth of strains. Ongoing work in the lab applies these plates to examine growth of switching in *C. albicans* white and opaque cells, and may be used for *C. tropicalis* in the future.

Studies focused on how the white-opaque switch affects interactions with the host in *C. tropicalis* are also of interest. In *C. albicans*, the white-opaque switch alters interactions with host immune cells, and similar studies are now being performed with *C. tropicalis* white and opaque cells. Future work should also include cells in the intermediate state, as these may also interact with phagocytic cells differently from white or opaque. Other studies have shown that *WOR1* and *EFG1* regulate virulence and gut commensalism in *C. albicans* [6, 7]. With Ben Cowan, an undergraduate in the lab, we have examined the effect of *WOR1* overexpression on gut commensalism in *C. tropicalis*. This work has revealed that opaque cells and cells overexpressing *WOR1* are generally less fit to proliferate in the gut than cells in the white state. These results contrast with those from *C. albicans*, where overexpression of *WOR1* promotes colonization of the mouse gut [7]. Based on the differences observed thus far, I predict that additional studies on the white-opaque

regulators in *C. tropicalis* and their ability to effect virulence and commensalism will reveal further differences from *C. albicans*, illustrating the divergence of the white-opaque switch and its role in these species.

5.3 Future Directions: Regulation of white-opaque switching

A major focus of this thesis was to examine the regulation of the white-opaque switch in *C. tropicalis*. Much of this work was informed by previous studies done in *C. albicans*, a species that now has a very well defined white-opaque regulatory network. Due to the narrow conservation of the white-opaque switch among a few species, the comparison of regulatory function has the potential to be highly informative of when and how the white-opaque switch evolved and what transcriptional regulators are essential for this process, such as *WOR1*.

It was surprising that the genome sequence of *C. tropicalis* lacked *EFG1*, a highly conserved morphogenesis regulator in multiple fungal species [8-10]. However, in collaboration with Eugenio Mancera Ramos of Alexander Johnson's laboratory at UCSF, we have identified an *EFG1* gene in *C. tropicalis* through the application of PCR against a conserved region of *EFG1* along with RNA-Seq (unpublished data). Current studies in the lab are focused on creating deletion mutants and inducible expression strains for the newly identified *EFG1* gene. Preliminary results have demonstrated that *EFG1* has a role in maintenance of the white and intermediate phenotypes in *MTL* homozygous cells of *C. tropicalis*, but not in *a/a* cells. This is interesting considering the ability of *a/a* cells to undergo switching in *C. tropicalis*; there may be another level of regulation that we have not

yet identified in α/α strains. Eugenio has also examined the role of *EFG1* in filamentation and biofilms of *C. tropicalis*, where preliminary results also suggest a role for this conserved gene. Further studies on *EFG1* in *C. tropicalis*, such as its role in virulence and commensalism, are of interest to the lab.

The function of *WOR1* in the regulation of white-opaque switching in *C. tropicalis* remains unclear. While this work shows that overexpression of *WOR1* induces opaque cell, hyphae, and biofilm formation, it also illustrates that these induced phenotypes are unstable upon repression of exogenous *WOR1* expression. As discussed in Chapter 4, this is very different from the case in *C. albicans*, where *WOR1* induction induces stable opaque cell formation. Does *WOR1* activate its own promoter in *C. tropicalis*? To determine this, studies are planned that will examine induction of the endogenous *WOR1* gene upon exogenous *Wor1* expression. In *C. albicans*, this was shown by Northern blot, as transcripts generated from exogenous and endogenous *WOR1* were different lengths, and the longer endogenous transcript of *WOR1* was induced by exogenous *WOR1*. As an alternative to this approach, we are designing an RT-PCR based approach to detect induction of the native *WOR1* promoter. To do this, we can take advantage of the fact that *WOR1* expression generated from the *MAL2* promoter will have a distinct 5' UTR from that of endogenous *WOR1*. We can examine this in the context of *MAL2*-induced *Wor1* expression as well as constitutively highly expressed *TDH3*-induced *Wor1*. Results of these studies will be informative on how *WOR1* regulates its own expression in *C. tropicalis*, and could provide insights into the instability of the induced opaque state.

How *WOR1* is acting in wild-type opaque cells versus the unstable, induced intermediate/opaque phenotype is another major question. We are currently performing ChIP-Seq on myc-tagged *WOR1* strains in parallel with RNA-Seq in wild-type white and opaque strains. Furthermore, ChIP-Seq is being performed in cells in which *WOR1-myc* expression has been induced under the *MAL2* promoter. This work will illustrate the different targets that *WOR1* has as well as its effects on these targets in wild type and artificially induced cells, shedding light on the mechanism by which *WOR1* functions to maintain opaque cells in a wild-type situation, or induce the intermediate state (in white cells) or hyphae (in opaque cells). This data will also be used to establish whether *WOR1* is able to bind and induce expression of its own promoter. It is also possible that the induction of *WOR1* is unable to induce stable opaque cells due to an epigenetic block at the *WOR1* promoter. Current and future studies in the lab will examine epigenetic histone modifications in white and opaque cells, as well as in the unstable induced opaque-like state. This could provide information on why upon shutting off expression of exogenous *WOR1* cells rapidly switch back to the white state.

While the work presented here identifies regulatory factors that induce phenotypic changes in *C. tropicalis*, it remains unclear which genes are regulated by these factors and how these factors interact to create a white-opaque regulatory network. This question is of interest, as it would illustrate the evolutionary rewiring between *C. albicans* and *C. tropicalis*. How transcription factor networks are rewired, especially in the context of phenotypic variation, is a major question in biology, and work on the *C. tropicalis* white-opaque regulatory network would be

highly informative when compared with *C. albicans*. Additionally, analysis of the regulation of switching in *C. tropicalis* would provide information on how intermediate formation is regulated. The intermediate phenotype is unique in that it seems to be a stable state between white and opaque, creating a tristable switch in *C. tropicalis*. How can a stable intermediate state exist based on the regulatory network we observe? Studies in the lab have attempted to model this question; however, these studies would be supported by experiments examining the interaction of regulatory factors and structure of the regulatory network in *C. tropicalis*.

The work presented in Chapter 4 provides a foundation for epistasis studies of white-opaque regulators in *C. tropicalis*. The regulatory factors *WOR1*, *WOR3*, *CTRG_03621*, *WOR2*, *NDT802*, and *UME6* (in addition to the new finding of *EFG1*) can now be examined epistatically to determine specific interactions between factors. For example, induction of *WOR1* in a wild-type strain does not generate stable opaque formation, as these cells return to white upon shutting off exogenous expression. However, identifying a situation where *WOR1* induction does promote stable opaque formation, for example in a *WOR2* or *NDT802* mutant, would identify which factor is important for maintenance of the white state and why *WOR1* induction of opaque cells is unstable in wild-type white cells. Along with epistasis studies of these regulatory factors, ChIP-Seq would also be helpful in identification of targets of these transcription factors, as well as their interactions at specific promoters. In *C. albicans*, the white-opaque regulatory network is highly interwoven and every regulatory factor interacts with every other factor in the

network, especially at promoters. This is characteristic of several state-specific regulatory networks, including mammalian stem cells and cancer. It is likely that *C. tropicalis* also has a highly interconnected network regulating phenotypic switching, and future experiments will examine this possibility.

5.4 Future directions: Features of white-opaque regulation

While studying the white-opaque switch, an interesting feature was noted among white-opaque regulatory factors: many of these factors contained glutamine (Q)-rich domains or poly-Q stretches. While this can be a general feature of transcriptional activators [11, 12], it is also characteristic of aggregation-prone or prion-forming proteins [13]. Prions are proteins that can adopt two stable conformations: a soluble conformation, often considered the functional variant, or an alternative conformation that promotes conversion of new protein to this alternative state, leading to aggregation of itself. While prion formation can be a pathological process leading to disease, there are examples of prion aggregates that play important biological functions, including in environmental responses [14]. Prions are another mechanism of epigenetic inheritance in yeast, where they have been shown to be regulators of phenotype. Prions can be a source of phenotypic diversity, which may be beneficial to the organism to survive a fluctuating, stressful environment [15].

Properties of prions and the amino acid sequences that are conducive for prion formation are of great interest, and many groups have designed algorithms to predict prion domains based on experimental evidence. One such algorithm is the

protein aggregation prediction algorithm (PAPA) developed by Toombs *et al.* and specifically designed to predict the prion propensity of Q/N-rich proteins [16]. This algorithm generates a prion propensity score based on the amino acid sequence of a protein, where a score greater than 0.05 indicates a high probability for prion propensity. If we enter the amino acid scores of the white-opaque regulators in *C. albicans*, we find that several of these proteins have a high prion propensity score (Table 2). To test the hypothesis that *C. albicans* white-opaque regulators may be functioning as prions, I have developed a strategy to use *S. cerevisiae* as a model for testing the prion-forming ability of these proteins. The methods we plan to use are based on a screen performed by Alberti *et al.* to identify prionogenic proteins in *S. cerevisiae* [17]. There are several biochemical assays we plan to employ, including aggregation of GFP-tagged proteins, SDD-AGE analysis of aggregates, and ability to generate read-through when fused to the *S. cerevisiae* SUP35 C-terminus. These studies are currently the project of an undergraduate in the lab, Natasha Nelson.

There is also evidence that poly-Q domains stabilize protein-protein interactions, and that expansion of these domains can lead to altered interactions and protein aggregation [18]. Working with *CTRG_03621* in *C. tropicalis*, I noticed through PCR and sequencing that this gene contained a stretch of 14 glutamine residues, which was expanded from the previously reported genome sequence of 8 glutamine residues. It would be interesting to examine the effect of expanding or contracting poly-Q stretches, as this may be a source of variation and evolution within this regulatory network. In general, examination of the role of these Q-rich stretches would provide evidence of whether these play a role in protein-protein

interaction, regulatory complex formation, or other forms of aggregation that may cause prion-enabled inheritance of the white or opaque state.

Other epigenetic properties of the white-opaque switch are also of interest to the lab. There is a wealth of evidence suggesting that epigenetic histone modifications play a major role in the regulation of white-opaque switching in *C. albicans*, and we are also finding evidence of this in *C. tropicalis*, as suggested by the nicotinamide studies described in Section 5.2. Future studies in the Bennett lab will examine epigenetic histone modifications in white and opaque states. Other work in mammalian cells has shown that the highest levels of active chromatin marks are present at the promoters of master regulators of cell identity [19, 20], and we are interested in testing if this is also the case white and opaque cells of *Candida* species.

5.5 Conclusion

How fungal pathogens are able to survive and thrive within the human host remains a major question for the field. It is clear that phenotypic transitions play crucial roles in regulating survival within the host, as well as the transition between commensalism and virulence. This work identifies a white-opaque switch in *C. tropicalis*, demonstrating the conservation of this switch among three related *Candida* species. The study of how the white-opaque switch contributes to the fitness of *C. tropicalis* will provide additional information as to the role of this phenotypic switch in different species.

How phenotypic transitions are regulated and how regulatory networks evolve are also important questions. This work demonstrates that physically similar

phenotypic switches can have divergent regulation between related *Candida* species. The identification of a tristable switch begs the question, how are the three heritable states regulated? The differences in regulation of the switch between *C. albicans* and *C. tropicalis* provide an excellent system for studying the evolution and divergence of regulatory networks. Other studies in yeast demonstrate that there can be multiple regulatory paths to the same output [21], and this work further demonstrates this point – divergent regulation can lead to a similar phenotypic output.

Evolution has been happening for a long time; we may never know when the very first biological organism burst into existence. However, evolution doesn't necessarily mean improvement. Change occurs for the better or worse, and mutations can be beneficial or detrimental. However, in the context of a different environment, that same mutation may have the opposite effect. Fungi have presumably been co-evolving with humans for thousands of years and have adapted to survive on mucosal surfaces of the human host. New drug treatments have altered the host; immunosuppressive drugs have expanded the niche of opportunistic fungal pathogens, which are now able to penetrate mucosal barriers and grow systemically, causing deadly infections. In the end, evolution acts by selection on fitness, and selective pressures are subject to change. As humans, we have the ability to transform the environment, so it is up to us to figure out how best to improve the world around us and be mindful of how our actions alter selective pressures.

5.6 Tables

Table 2. Prion propensity scores of white-opaque regulators generated by PAPA. Only scores for regulators with positive values are shown. Significant prion scores are highlighted in red.

Gene	<i>C. albicans</i> PAPA score	<i>C. tropicalis</i> PAPA score
<i>WOR1</i>	0.09	0.10
<i>WOR2</i>	0.03	0.07
<i>WOR3</i>	0.09	0.02
<i>EFG1</i>	0.12	0.05

5.7 References

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Appendix A: Supplementary Information for “A phenotypic switch regulates sexual mating in *Candida tropicalis*”

A.1 Supplemental Methods

Media. Media was prepared as described previously (1, 2). Yeast extract peptone dextrose (YPD) plates containing 200 µg/ml nourseothricin (NAT) was used for selection of strains that were resistant to nourseothricin (*SAT^R* strains)(3).

Strain/Plasmid Construction. All strains are listed in Supplementary Table 1. *C. tropicalis* strains were derived from ATCC 34139 ST-120 (*MTLa/α*). *MTLa/a* (CAY677) and *MTLa/α* (CAY679) derivatives were obtained by growth on sorbose medium as described previously for *C. albicans* (4), and were generously provided by Brian Tuch and Alexander Johnson (UCSF). Gene deletions were constructed using the *SAT1* flipper strategy as described previously (3). Strains were transformed with 1-4 µg of DNA using a modified electroporation protocol (3, 5). Cells were grown to OD₆₀₀≥2, resuspended in 0.1 M lithium acetate, 7.5 mM Tris-HCl, pH 8.0, 1 mM EDTA, 10 mM DTT (dithiothreitol) and incubated at room temperature for 1 hour. Cells were washed twice with water and once in 1 M sorbitol. The sorbitol wash was decanted and cells were resuspended in the residual liquid. Approximately 40-50 µl of cells were used per transformation. Cells and DNA were electroporated at 1.8 kV using a BioRad MicroPulser Electroporator and immediately resuspended in 1 ml cold 1 M sorbitol. Cells were subsequently resuspended in 1 ml YPD and allowed to recover for 4 hours at 30°C before plating

on selective media (YPD + 200 µg/ml NAT). To delete *WOR1*, *HIS1*, *ARG4*, *STE2*, and *STE3* genes using the *SAT1*-flipper method, oligonucleotides were used to amplify approximately 900 bp of the 5' and 3' homologous flanks of each gene. The resulting PCR products were digested with restriction enzymes noted in Supplementary Table 2, and cloned into the plasmid pSFS2a (3). The resulting plasmids were digested with restriction enzymes as noted in Supplementary Table 3 to liberate cassettes containing the 5' and 3' gene flanks as well as the *SAT1* selectable marker, and used for transformation into strains CAY677 and CAY679. Correct genomic integration of transformant colonies was confirmed by PCR of the 5' and 3' disruption junctions (for oligos see Table A2). The *SAT1* marker was recycled by growing transformants on maltose media at room temperature or 30°C, and subsequently replica patching to both YPD and YPD+NAT plates. The transformation process was repeated to delete the remaining copy of the gene, and loss of the open reading frame (ORF) was confirmed by PCR. To complement, *Dste2/Dste2* and *Dste3/Dste3* mutants with a copy of the original gene, oligonucleotides were used to amplify the promoter region and ORF. The resulting PCRs were digested with restriction enzymes as noted in Table A2, and cloned into pSFS2a (3). The resulting plasmids were digested with restriction enzymes (see Table A3) to either liberate gene cassettes or to linearize the plasmid within the promoter region. Correct genomic integration was confirmed by PCR. To fluorescently label the nuclei, histone H2B chimeras fused to either GFP or mCherry were constructed. PCR with oligonucleotides 44/45, containing sequences homologous to the *HTB* gene (*C. tropicalis* H2B homolog), were used to amplify GFP or mCherry, together with the *SAT1* gene, from plasmids

pADH76 (GFP) and pADH77 (mCherry). The resulting PCR product was used to transform strains CAY2059/2060 and CAY2061/2062 to obtain strains CAY2136/2261 and CAY2134/2135, respectively.

Scanning electron microscopy. *C. albicans* cells were grown on synthetic complete dextrose (SCD) medium and *C. tropicalis* grown on Spider medium at 25°C. Cells were resuspended in water, attached to poly-L-lysine coated-coverslips, fixed in 2.5% (w/v) glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.4, and washed with 0.1 M sodium cacodylate buffer. Cells were then postfixed with 4% aqueous osmium tetroxide in water and washed in 0.1 M sodium cacodylate buffer. Following fixation, cells were dehydrated gradually using a gradient ethanol series (37% to 100%) and subsequently critical point dried in 100% ethanol. The samples were then coated with 20 nm of gold palladium (60:40) in an Emitech K550 sputter coater. Cells were viewed with a Hitachi S-2700 scanning electronic microscope and images were acquired with Quartz PCI software.

Phenotypic Switching assays. White phase cells were inoculated into liquid Spider medium and incubated overnight at room temperature. Cultures were checked for the purity of white cell forms by microscopy (>99%), diluted in H₂O, and plated onto solid Spider medium at a concentration of ~100 colonies per plate. Colonies were scored for opaque sectors after growth at room temperature for 7 days.

Staining of Cell Nuclei. Tetraploid cells from mating progeny were fixed in 70% ethanol for 10 min, washed twice in water, and resuspended in 0.25 µg/ml DAPI (4',6'-diamidino-2-phenylindole). After incubation for 10 min, cells were washed in water and resuspended in 50% glycerol. Samples were analyzed on a Zeiss Axio-Observer.

Microarray design. An 8 x 15 K custom microarray manufactured by Agilent Technologies (Wilmington, DE) was used in these studies. Nucleotide sequence data for *C. tropicalis* transcripts were obtained from the Broad Institute website at <http://www.broadinstitute.org> (6). Sequencing of *C. tropicalis* was performed at Stanford and the Biotechnology Research Institute of the National Research Council Canada with the support of the NIDR and the Burroughs Wellcome Fund. Two tiled probe sets were selected for each of the 6,258 annotated transcripts of the *C. tropicalis* genome, resulting in a DNA microarray containing 12,558 probe sets, including Agilent hybridization controls. Information about coding sequences and proteins were obtained from CandidaDB available at <http://genodb.pasteur.fr.CandidaDB> (7).

Preparation of cDNA for microarray experiments. 3 ml liquid cultures of white or opaque cells of strain CAY1504 were grown overnight in liquid Spider medium at room temperature and used to inoculate a 25 ml liquid culture at OD₆₀₀ = 0.2. When the OD₆₀₀ of the culture was approximately 1.2, 10 ml aliquots were harvested and frozen in a liquid nitrogen bath and stored at -80°C. Total RNA was obtained from

the cells using the Ribopure™-Yeast Kit (Ambion) and treated with Turbo DNase (Ambion). cDNA was generated from 10 µg of total RNA by first annealing the RNA with oligo(dT)₁₉V (8.3 µg) and pdN9 (6.3 µg). cDNA was then synthesized using Superscript III reverse transcriptase (Invitrogen, 334 Units/reaction) in reaction mixtures containing 0.5 mM DTT, 0.5 mM (total) deoxynucleoside triphosphates (aminoallyl-dUTP and deoxynucleoside triphosphates [3:2]). After cDNA synthesis for 3 h at 42°C, the RNA was hydrolyzed with 0.3 M sodium hydroxide and 0.03 M EDTA. The reaction was neutralized with 0.3 M HCl to pH 7.0. A Zymo kit with 25 µg capacity columns (Zymogen) was used to clean and recover the cDNA. cDNA was aliquoted to achieve 2 µg per sample, dried in a speed-vac, resuspended in 9 µl ddH₂O, and stored at -80°C.

Coupling of cDNA and hybridization to microarrays. The cDNA was coupled with fluorescent dyes by addition of 1 µl fresh 1M sodium bicarbonate, pH 9.0, and incubated with either 1.25 µl Cy3 or Cy5 dye (Amersham Biosciences) for 1 h at room temperature in the dark. Unincorporated dye was removed using a 25 µg capacity Zymo column (Zymo Research). cDNA samples were analyzed on a NanoDrop 2000C spectrophotometer (Thermo Scientific) using the “microarray” setting to quantitate total yield and dye-coupling efficiency. 300 ng of the appropriate Cy3-cDNA and Cy5-cDNA samples were then mixed in a total volume of 25 µl ddH₂O and heated for 3 min at 95°C before adding 25 µl 2X GEx hybridization buffer (HI-RPM, Agilent). The samples were applied to the microarray slide and

were hybridized for ~17 hours at 65°C rotating at 10 rpm. The slides were washed and dried with commercial Agilent buffers, and analyzed as described below.

Analysis of microarrays. Arrays were scanned on a GenePix 4000 scanner (Axon Instruments, Foster City, CA), and data were quantified using GENEPIX PRO version 3.0 and normalized using Goulphar (<http://transcriptome.ens.fr/goulphar/>). Statistical analysis was performed using SAM (<http://www-stat.stanford.edu/~tibs/SAM/>) (8), and statistically significant genes were further analyzed. Pairwise average linkage clustering analysis was performed using the program CLUSTER and visualized using TREEVIEW (9). The Candida genome database (<http://www.candidagenome.org>)(10), the Candida DB GenoList Browser (<http://genolist.pasteur.fr/CandidaDB/>)(7), and the *S. cerevisiae* Comprehensive Yeast Genome Database (<http://mips.gsf.de/proj/yeast/CYGD/db/index.html>) were used to facilitate further analysis.

A.2 Supplemental Data

A.2.a Phloxine B staining of *C. tropicalis* and *C. albicans* white and opaque cells

Phloxine staining has been used as a standard method for facile identification of opaque cells of *C. albicans* and *C. dubliniensis*, as this dye is preferentially taken up by opaque cells, but not white cells, of the species (11, 12). To determine if *C. tropicalis* opaque cells are also preferentially stained by phloxine B, white and opaque cells were mixed and plated onto Lee's medium containing 5 µg/ml phloxine B, and subsequently incubated for 4 days at room temperature. As shown in Figure A1, *C. albicans* white and opaque colonies are clearly differentially stained by phloxine B (opaque colonies stain red, while white colonies remain white). In contrast, *C. tropicalis* white and opaque colonies did not show a marked difference in staining with phloxine B. This was not due to instability of the *C. tropicalis* states, as recovery of colonies from the Lee's medium and re-plating on Spider medium confirmed that both white and opaque colonies were present on the Lee's plates.

We also note that the white-opaque switch in *C. albicans* has been shown to be highly sensitive to multiple environmental stimuli. Thus, increased switching to opaque is observed in the presence of N-acetylglucosamine, carbon dioxide, low oxygen tensions, and chemical stressors such as hydrogen peroxide, methyl methanesulfonate and hydroxyurea (13-17). Surprisingly, however, none of the conditions that induce elevated rates of switching in *C. albicans* have been found to influence the white-opaque switch in *C. tropicalis* to a significant degree. These findings further illustrate the differences in the switch between the two species, including the fact that *C. tropicalis* opaque cells are stable at body temperature.

*A.2.b Mixtures of C. tropicalis white **a** and α cells do not show an efficient mating response*

Co-incubation of *C. tropicalis* opaque **a** and α cells leads to a rapid and efficient induction of mating projections (shmoos) and the formation of zygotes (see Figure 3). In contrast, similar mixtures of *C. tropicalis* white **a** and α cells do not show the induction of mating shmoos or zygotes, and continue to display the typical round phenotype of white cells (Figure A2). Occasionally, rare mating zygotes were observed in mixes of white cells consistent with the low level of mating observed in quantitative crosses.

A.2.c Images of C. tropicalis and C. albicans white cells, opaque cells, and mating zygotes

The phenotypes of white and opaque cells from *C. tropicalis* and *C. albicans* are further compared in Figure A4. In both species, white cells appear round and have a smooth appearance in scanning electron microscope images, while opaque cells are elongated and have an uneven or pimpled cell surface. These images are consistent with previous differences noted between *C. albicans* white and opaque cells (18). We also show zygotes from mating experiments between opaque **a** and α cells from both *C. tropicalis* and *C. albicans*. In general, mating zygotes appeared similar in both species. Mating projections from the two partners fused to form a single zygotic cell, and this cell then begun to divide via budding from the conjugation bridge formed between the two parental cells.

A.2.d Annotation and summary of significant genes from microarrays

Tables S4 and S5 represent *C. tropicalis* genes that are significantly upregulated in white and opaque forms, respectively, using the statistical analysis package SAM (<http://www.stat.stanford.edu/~tibs/SAM/>)(8). *C. albicans* orthologs to *C. tropicalis* genes were determined using the Broad Institute Fungal Orthogroups Repository (http://www.broadinstitute.org/cgi-bin/regev/orthogroups/show_orthogroup.cgi?orf=orf19.6925).

Overall, there are 250 white-specific genes and 146 opaque-specific genes in *C. tropicalis*. Of that number 218 and 134, respectively, have homologs in *C. albicans*. 62 white-specific and 25 opaque-specific genes are shared between *C. tropicalis* and *C. albicans* based on previous analysis of *C. albicans* white and opaque genes (see Figure 4)(19-23). Statistical analysis of the overlap of white- and opaque-specific genes between species indicates that this overlap is significant ($p = 2.05 \text{ E-}13$ for white genes and $p = 1.579 \text{ E-}5$ for opaque genes, using a Pearson's chi-squared test).

Common white-specific genes include *ADAEC*, a gene of unknown function, *RME1*, a zinc finger transcription factor that represses meiosis in *S. cerevisiae*, and *MSC1*, a conserved gene that is involved in meiotic recombination events between sister chromatids in *S. cerevisiae*. Notable opaque-specific genes include *WOR1*, the master regulator of white-opaque switching in *C. albicans*, *UME6*, a transcriptional regulator of meiotic genes in *S. cerevisiae*, and *STE4*, a gene involved in the mating signaling pathway in multiple fungal species. In addition, 31 genes that are white-specific in *C. tropicalis* are opaque-specific in *C. albicans*, and 15 genes that are

opaque-specific in *C. tropicalis* are white-specific in *C. albicans*. Overall, this data indicates that, while there are physical similarities between the white and opaque forms of *C. tropicalis* and *C. albicans*, there are also fundamental differences between the transcriptional programs in the two species.

We further analyzed the *C. tropicalis* white-opaque gene sets by determining which gene ontology (GO) terms are represented (Figure A5). Gene classes that are overrepresented in both white and opaque sets include transmembrane transport and several metabolic pathways. In particular, white genes are overrepresented by classes of genes involved with carbohydrate and amino acid metabolism, whereas opaque genes are overrepresented by genes involved with small molecule metabolism, lipid metabolism, and amino acid metabolism. However, only genes involved in organic substrate transport were enriched in *C. tropicalis* white cells ($p=0.09391$) and those involved with cellular component biogenesis were significantly enriched in *C. tropicalis* opaque cells ($p=0.04405$). Previous analysis of the white-opaque gene sets in *C. albicans* suggested that white cells were specialized for a fermentative metabolism whereas opaque cells were specialized for an oxidative metabolism (19). However, the white-opaque switch in *C. tropicalis* did not differentially regulate many of the genes associated with these two pathways in *C. albicans*. For example, *HXK1*, *PFK2*, *CDC19*, *HXT3*, *HXT4*, and *HXT5* are all white-specific genes in *C. albicans* that are involved with glycolysis (19), but none of these genes were regulated by the white-opaque switch in *C. tropicalis*. Conversely, *FAA2*, *POX1*, *ECL1*, *FOX2*, *FOX3*, *ADH3*, and *ALD6* are involved in fatty acid oxidation and are opaque-specific *C. albicans*, but not are phase-specific in *C. tropicalis*. In general, it

therefore appears that metabolic genes are a common regulatory target of the white-opaque switch in *Candida* species, but that the precise pathways involved have been reprogrammed between species.

A.3 Supplemental Figures

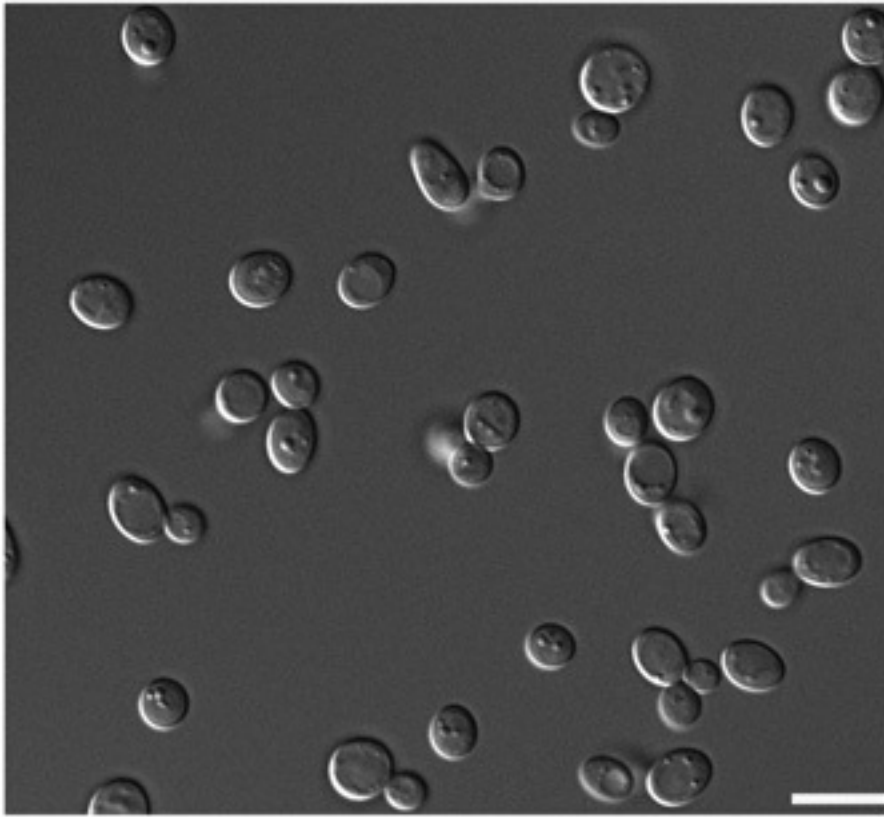


Figure A1. Mixtures of *C. tropicalis* white a and α cells do not undergo efficient mating and zygote formation. Cells from strains CAY2134 and CAY2136 containing white a and α cells, respectively, were mixed and incubated on Spider medium for 24 h, and analyzed by DIC (differential interference contrast) microscopy. Unlike opaque cells (see Figure 4 and Figure A3), the majority of cells showed no change in morphology or evidence of a mating response. Scale bar, 10 μ m.

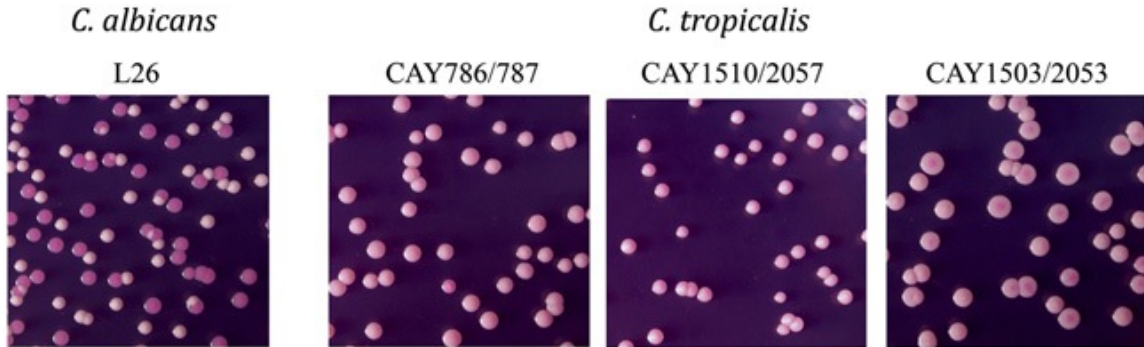


Figure A2. *C. tropicalis* white and opaque forms are not differentially stained with phloxine B. To compare the white and opaque forms of *C. tropicalis* with those previously described in *C. albicans*, an approximately 50:50 mixture of white and opaque cells were deposited on Lee's medium containing 5 μ g/ml phloxine B. This dye preferentially stains the opaque form of *C. albicans* (left panel) but does not preferentially stain the opaque form of *C. tropicalis* (right panels). Strains used: *C. albicans* L26 white/opaque (**a/a**), *C. tropicalis* CAY786/787 (α/α), CAY1510/2057 (α/α), and CAY1503/2053 (**a/a**).

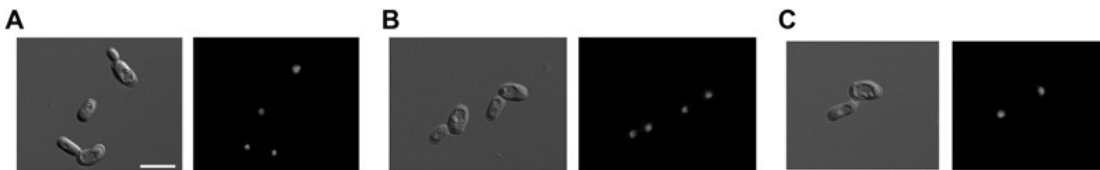


Figure A3. The products of *C. tropicalis* mating are mononuclear, tetraploid cells. Staining of tetraploid mating products with DAPI (4',6'-diamidino-2-phenylindole) revealed that they contain single nuclei. Three different tetraploid products (analyzed by flow cytometry) were stained with DAPI and shown to be mononuclear. Left panel, DIC image. Right panel, DAPI channel. Scale bar, 10 μ m.

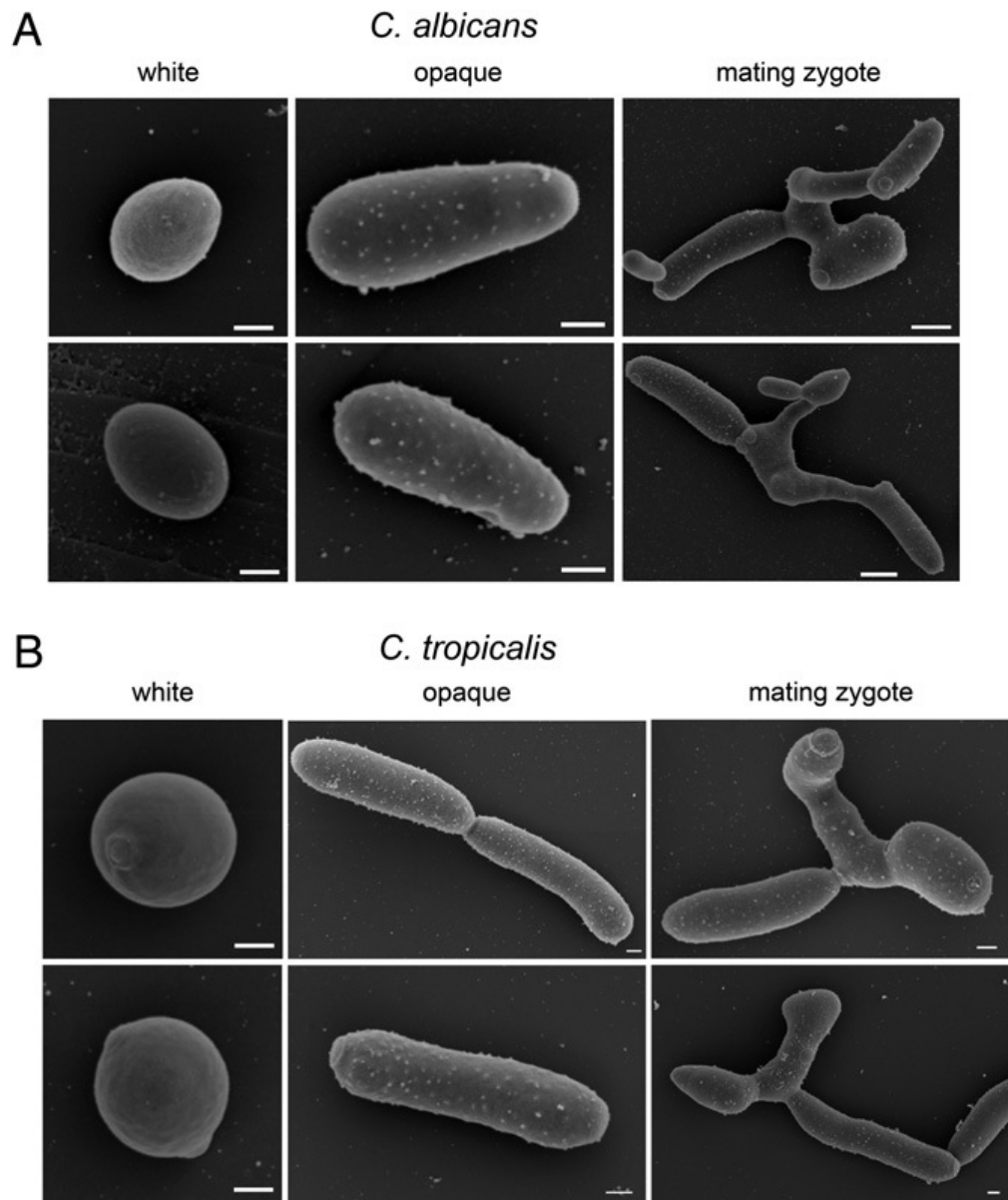


Figure A4. Scanning electron microscope images of white cells, opaque cells, and mating zygotes from *C. albicans* and *C. tropicalis*. **(A)** White and opaque cells from *C. albicans* strain WO-1 (an *a/a* strain), and mating zygotes from a cross between opaque WO-1 cells and opaque P37005 (*a/a*) cells (top panel) or RBY731 cells (an α/α derivative of SC5314; lower panel). Scale bar for white and opaque cells, 1 μm . Scale bar for zygotes, 3 μm . **(B)** White and opaque cells from *C. tropicalis* strain CAY784/785 (an *a/a* strain), and mating zygotes from a cross between CAY784 and CAY786 (an α/α strain). Scale bar, 1 μm . Mating of *C. albicans* and *C. tropicalis* opaque strains was performed by co-incubation on Spider medium for 1 day.

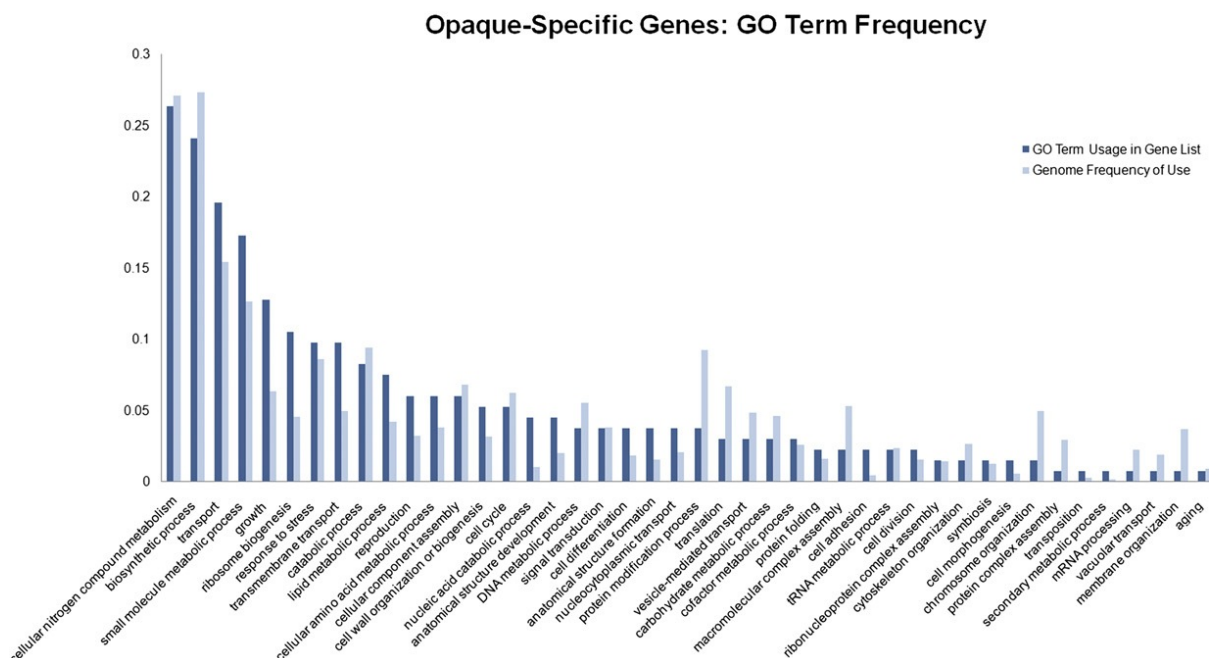
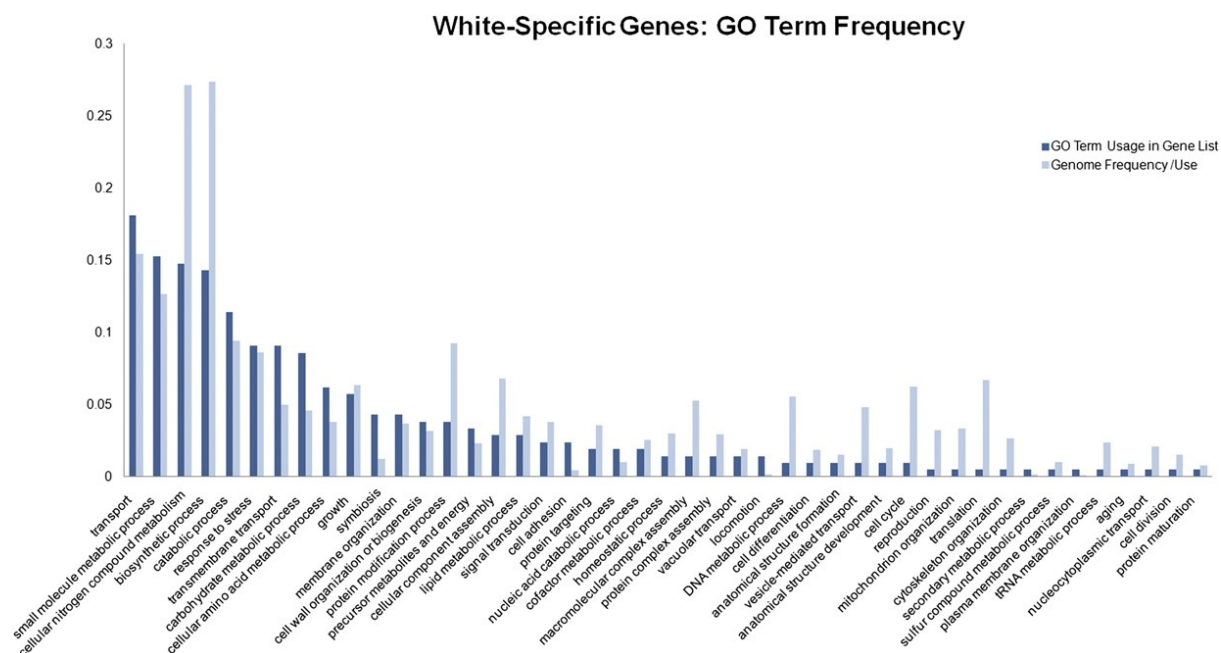


Figure A5. Analysis of GO term frequency of *C. tropicalis* white and opaque specific genes. GO Term Mapper (<http://go.princeton.edu/cgi-bin/GOTermMapper>) was used to map *C. albicans* homologs of *C. tropicalis* white and opaque specific genes to their biological process from the Candida Genome Database (<http://candidagenome.org>).

A.4 Supplemental Tables

Table A1. List of *C. tropicalis* strains used in this study

<i>White Strain</i>	<i>Opaque Strain</i>	<i>Genotype</i>	<i>MTL</i>
CAY675		ATCC 34139 ST-120	a/α
CAY677/785	CAY784	Sorbose selected ATCC 34139	a/a
CAY679/787	CAY786	Sorbose selected ATCC 34139	α/α
CAY1117/1118		<i>wor1/wor1::SAT1</i>	a/a
CAY1502		<i>his1/his1::SAT1</i>	a/a
CAY1503/1504	CAY2053/2275	<i>arg4/arg4::SAT1</i>	a/a
CAY1505/06	CAY2054/2055	<i>his1/his1::SAT1</i>	α/α
CAY1509/1510	CAY2056/2057	<i>arg4/arg4::SAT1</i>	α/α
CAY2058		<i>his1/his1</i>	a/a
CAY2059/2060		<i>arg4/arg4</i>	a/a
CAY2061/2062		<i>his1/his1</i>	α/α
CAY2063		<i>arg4/arg4</i>	α/α
CAY2200		<i>his1/his1 ste2/ste2</i>	a/a
CAY2201/2245		<i>his1/his1 ste3/ste3</i>	α/α
CAY2202		<i>arg4/arg4 ste2/ste2</i>	a/a
CAY2203/2243		<i>arg4/arg4 ste3/ste3</i>	α/α
CAY2247		<i>his1/his1 ste2/ste2/STE2::SAT1</i>	a/a
CAY2246		<i>arg4/arg4 ste2/ste2/STE2::SAT1</i>	a/a
CAY2380		<i>his1/his1 ste3/ste3/STE3::SAT1</i>	α/α
CAY2378/2379		<i>arg4/arg4 ste3/ste3/STE3::SAT1</i>	α/α
CAY2136/2251	CAY2257/2260	<i>arg4/arg4 HTB/HTB-mCherry::SAT1</i>	a/a
CAY2134/2135	CAY2258/2259	<i>his1/his1 HTB/HTB-GFP::SAT1</i>	α/α
CAY2205/2206/ 2248		<i>arg4/arg4 wor1/wor1::SAT1</i>	a/a
CAY2342/2439		<i>his1/his1 wor1/wor1::SAT1</i>	α/α
CAY2347/2348/ 2441		<i>arg4/arg4 wor1/wor1/WOR1::SAT1</i>	a/a
CAY2440/2805		<i>his1/his1 wor1/wor1/WOR1::SAT1</i>	α/α

Table A2. Oligonucleotides used in this study. Underlined sequences denote restriction sites.

<i>Oligo</i>	<i>Name</i>	<i>Sequence</i>
1	<i>WOR1</i> (ApaI) - 900	ggagcggggcccCTACTACATTAGTTGTTCCCTG
2	<i>WOR1</i> (XhoI) 0	gccggcctcgagATAATCTAGACGCCGACATG
3	<i>WOR1</i> (SacII) +2100	ggagcgcgcggGGTAACTACAGGTGACGAATC
4	<i>WOR1</i> (SacI) +3000	ggccgcgagctcTTACCCACAAGTGTGTCTAG
5	<i>WOR1</i> 5' check - 950	GAATCTAAGGCAACAAACGATTTTC
6	<i>WOR1</i> 3' check +3100	GGTGATTAAATCACATTCTCGG
7	<i>WOR1</i> 5' ORF +200	GACTGATGGAATTTCTTGGTC
8	<i>WOR1</i> 3' ORF +550	GCAGCTGTCCATAAACATGG
9	<i>HIS1</i> (ApaI) -900	ggagcggggcccACCTCAAGAGATGAATGACA
10	<i>HIS1</i> (XhoI) 0	gccggcctcgagACTCTAACTATGATTGTATTC
11	<i>HIS1</i> (SacII) +900	ggagcgcgcggAGTTCATCTGTATAAAGTACA
12	<i>HIS1</i> (SacI) +1800	ggccgcgagctcTCCACACATTCATAAATGTTCA
13	<i>HIS1</i> 5' check - 950	CCAAGGACAACACTCGTGCAGT
14	<i>HIS1</i> 3' check +1900	CAAATTGATCACTTCTCTAGTCGA
15	<i>HIS1</i> 5' ORF +200	AGGGTAATTGTGATTTGGGTA
16	<i>HIS1</i> 3' ORF +600	CTTGGAAGAAACCAAGTGAG
17	<i>ARG4</i> (ApaI) -900	ggagcggggcccTGAATGTGTTGGTGGTGATG
18	<i>ARG4</i> (XhoI) 0	gccggcctcgagGACATTGTTGTTAGAATAAGTT
19	<i>ARG4</i> (NotI) +1400	ggagcggcgccgcACATTCATTCTCCTTCCCTCTC
20	<i>ARG4</i> (SacII) +2300	ggccgcccgcggGAATATTGATTGTTGATTGTTG
21	<i>ARG4</i> 5' check - 950	GACGTTGTTTACCCTTGGTATC
22	<i>ARG4</i> 3' check +2400	TTGTGGAATGTAATTTGCACAC
23	<i>ARG4</i> 5' ORF +200	TCAGGATTAGAAGAAATTCGTG
24	<i>ARG4</i> 3' ORF +600	TGGAATTTGATTAACTCTGGT
25	<i>STE2</i> (ApaI) -950	ggcgccgggcccGTCTAGAGTTGCTCTTCTCT
26	<i>STE2</i> (XhoI) 0	gccggcctcgagCACCAGAAGACTGAATAGTATTG
27	<i>STE2</i> (SacII)	ggagcgcgcggCTGAAGTCACTTACAAGAAGG

	+1200	
28	<i>STE2</i> (SacI) +2100	ggccgcgagctcGGGAATGGCACAATTGGAGTC
29	<i>STE2</i> 5' check - 970	CGAGCATTAGTGTAAGTAGTC
30	<i>STE2</i> 3' check +2150	CAATAGGACCAACTGGGAAATG
31	<i>STE2</i> 5' ORF +150	GCTTAGTCATGGGTGTGATG
32	<i>STE2</i> 3' ORF +650	GACATAAGATTGACACTAACGG
33	<i>STE3</i> (ApaI) -900	ggcgccgggcccGATCTTGAAACACGAACAAGC
34	<i>STE3</i> (XhoI) 0	gccggcctcgagCCAGCAGCTGCAGCAATCAT
35	<i>STE3</i> (SacII) +1200	ggagcgccgaggCGCCTAGCGAACATTTCTCA
36	<i>STE3</i> (SacI) +2100	ggccgcgagctcgTCGAGATCAAGAATTTGGG
37	<i>STE3</i> 5' check - 970	CGTTGATGATGAAGAAGCTC
38	<i>STE3</i> 3' check +2150	CATTTGGATATTTGTACCGGAG
39	<i>STE3</i> 5' ORF +100	GCATGGCATATTAAGTGTGC
40	<i>STE3</i> 3' ORF +650	CAGAAGTAAAGAGACAATGGC
42	<i>STE2</i> (XhoI) +1500	gccggcctcgagAACTGGATCTGCAACCATGG
43	<i>STE3</i> (XhoI) +1500	gccggcctcgagGCAATTGGCAGTGAAAGAAATC
44	<i>HTB-FP</i> forward	GTTAGATTGATCTTGCCAGGTGAATTGGCTAAACATGC CGTTTCTGAAGGTACTAGAGCTGTTACCAAATACTCTT CTGCTTCAAACcggatccccgggtaattaacgg
45	<i>HTB-FP</i> reverse	ATGGATTAGCATATTATAAATTAATAATTATGCAAAGT TGGGTACAAGTAATGAAATTGGTTCTCTTCTGTTTGAA ATGGTTATggcggccgctctagaactagtggatc
46	<i>HTB</i> check	GATTGTTTCTCTCTCCTCTC
47	prBT_74 (a2)	GATTTGGTATGAAAAGAGGAACCTAAC
48	prBT_75 (a2)	CTACTAATTTTGAAACCATTTGGAGTCT
49	prBT_70 (alpha2)	TAAACATTAAAGCATAGAGGACAAAGAA
50	prBT_71 (alpha2)	AACTTCAAATGCAAAATGTAAAACATAC
51	<i>ACT1</i> 5Q	GGTGATGGTGTACCCACGTT
52	<i>ACT1</i> 3Q	TGTAACCACGTTTCAGACAAGATCTTC

53	<i>ADAEC</i> 5Q	ATCAATGTATTCCAACCAAAACCA
54	<i>ADAEC</i> 3Q	TGAAACCAAGTACACCACCTCTTC
55	<i>WOR1</i> 5Q	CCGTCTAATGTTATACCTGCATCAA
56	<i>WOR1</i> 3Q	TTCGTCGTACTTATGGTAATTGTTTTCT
57	<i>FUS1</i> 5Q	CCTTCATCAACCACAACCTAGAAGAATT
58	<i>FUS1</i> 3Q	TCCCAAAGCAATGAAAAAATAGC
59	<i>WOR1</i> (Xho I) +2500	gccggcctcgagGTCATATACCCAACACCGAAC

Table A3. Plasmids used in this study.

<i>Plasmid</i>	<i>Name</i>	<i>Oligos used</i>	<i>Enzymes used for transformation</i>
pRB29	<i>WOR1</i> deletion plasmid	1/2, 3/4	<i>Apa</i> I/ <i>Sac</i> I
pRB55	<i>HIS1</i> deletion plasmid	9/10, 11/12	<i>Apa</i> I/ <i>Sac</i> I
pRB59	<i>ARG4</i> deletion plasmid	17/18, 19/20	<i>Apa</i> I/ <i>Sac</i> II
pRB94	<i>STE2</i> deletion plasmid	25/26, 27/28	<i>Apa</i> I/ <i>Sac</i> I
pRB95	<i>STE3</i> deletion plasmid	33/34, 35/36	<i>Apa</i> I/ <i>Sac</i> I
pRB101	<i>STE2</i> complementation plasmid	25/42, 27/28	<i>Sma</i> I
pRB102	<i>STE3</i> complementation plasmid	33/43, 35/36	<i>Afl</i> II
pRB103	<i>WOR1</i> complementation plasmid	1/59, 3/4	<i>Apa</i> I/ <i>Sac</i> I

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Appendix B: Supplementary Information for “*MTL*-Independent Phenotypic Switching in *Candida tropicalis* and a Dual Role for *Wor1* in Regulating Switching and Filamentation”

B.1 Supplemental Results

*B.1.a GO Term analysis of **a/a** and **a/α** white- and opaque-specific genes*

GO term analysis on white- and opaque-specific genes was used to determine if gene sets indicated any specialized functions for white and opaque **a/a** and **a/α** cells. While both shared and **a/a** opaque-specific genes are enriched for genes involved in RNA processing and ribosome biogenesis, the **a/α** opaque-specific gene set also contains genes involved in translation, biosynthetic processes, mitochondrial organization, and protein metabolic processes. **a/a** white-specific genes were enriched for genes involved in metal ion transport, but shared and **a/α** white-specific genes contained no significantly enriched GO terms (see Figure B3).

*B.1.b **a1/α2** binding sites within the *WOR1* promoter*

We analyzed the *WOR1* promoter for **a1/α2** binding sites by searching for the conserved consensus sequence [1,2] within 5 kb upstream of the *WOR1* gene sequence. We found 3 potential binding sites for **a1/α2** at positions -2589 (TGTTTTTATTTTAAAGCAG), -2214 (TTCTGTAATTTATAATACT), and -1902 (TGCTAAAAATCCACAT) upstream of the ATG. At present, we cannot confirm that these are bona fide **a1/α2** sites. However, the presence of putative **a1/α2** binding

sequences suggests that the ability to undergo the white-opaque switch in *C. tropicalis* **a**/ α cells is not due to the loss of **a**1/ α 2 binding sites within the *WOR1* promoter.

*B.1.c Generation of an MTL marker to track MTL status during **a**/ α mating*

To track potential *MTL* loss during mating of **a**/ α cells, a *SAT1* marker was inserted immediately adjacent to the *MTLa* locus in *C. tropicalis*. As shown in Figure B5A, an **a**/ α strain containing the *SAT1* marker upstream of the *MTL α* locus (Arg- strain) was mated with an unmarked α / α strain (His- strain). Mating products were selected on Arg- His- medium and tested by PCR for the presence of the marker (Figure B5B and C). Out of 52 mating products tested from 4 independent experiments, none still contained the marker next to the *MTL α* locus (a subset of products are shown in Figure B5C). Interestingly, 4 out of the 52 products (7.7%) had undergone recombination so that the *SAT1* marker was now adjacent to the *MTLa* locus. This result indicates that recombination events immediately adjacent to the *MTL* can take place at an appreciable frequency in *C. tropicalis*. However, the majority of mating products (48/52) had lost the *SAT1* marker, presumably either by loss of the chromosome containing the *MTL α* locus or by recombination outside of the *SAT1* marker region.

*B.1.d Regulation of mating in C. tropicalis by **a**2 and α 1 transcription factors*

In *C. albicans*, mating of **a** cells is dependent on the **a**2 transcription factor, while mating of α cells is dependent on the α 1 transcription factor [3]. The roles of

a2 and $\alpha 1$ in *C. tropicalis* mating were therefore examined, and **a2** was found to be essential for **a**-cell mating, while $\alpha 1$ was essential for α -cell mating (Table B11), similar to their roles in *C. albicans* [3]. Mating of *C. tropicalis* **a**/ α cells was also dependent on **a1** and $\alpha 2$ transcription factors, as **a**/ α cell mating with **a** cells required **a2**, while **a**/ α cell mating with α cells required $\alpha 1$ (Table B11). It is therefore apparent that *C. tropicalis* **a**/ α cells behave like **a** cells when mating with α cells, and like α cells when mating with **a** cells. We presume that loss or recombination at the *MTL* locus occurs prior to **a**/ α cell mating, subsequently allowing conventional mating with **a** or α partners to occur.

B.1.e Comparison of white- and opaque-specific genes in C. tropicalis and C. albicans

Genes regulated by the white-opaque switch in **a**/ α *C. tropicalis* cells were compared to those regulated by the switch in *C. albicans* **a/a** cells [3]. This approach is similar to a previous analysis between *C. tropicalis* and *C. albicans* white and opaque **a/a** cells [4]. Interestingly, of the 180 *C. tropicalis* **a**/ α opaque-specific genes with homologs in *C. albicans*, only 5 (2.8%) were also opaque-specific in *C. albicans*, while 4 (2.2%) were white-specific in *C. albicans* (Table B6). Similarly, of the 281 *C. tropicalis* **a**/ α white-specific genes with homologs, 23 (8.2%) were also white-specific while 20 (7.1%) were opaque-specific in *C. albicans* (Table B5). These results indicate that most of the genes regulated by the white-opaque switch in *C. tropicalis* **a**/ α cell types are not shared with those that are switch-regulated in *C. albicans*. Furthermore, *C. tropicalis* genes regulated by the white-opaque switch in **a**/ α cells have significantly less overlap with switch-regulated genes in *C. albicans*

(6% for both white and opaque genes) than do those in *C. tropicalis* **a/a** cells (25% for white- and opaque-specific genes). Thus, while the white-opaque switch in *C. tropicalis* **a/a** cells regulates an increased number of genes relative to **a/a** cells, this gene set is more divergent to those regulated by the switch in *C. albicans*. Taken together, these results demonstrate that the white-opaque switch regulates distinct sets of genes between different cell types and between different *Candida* species.

B.1.f Comparative analysis of Δ/Δ wor1 mutant cells with white cells, and pTDH3-WOR1 expressing cells with opaque cells

To compare Δ/Δ wor1 mutant cells and white cells, and pTDH3-WOR1 expressing cells and opaque cells, SAM analyses were performed on the corresponding microarray data (Tables S7-S10). Interestingly, we found that genes upregulated in pTDH3-WOR1 strains relative to opaque strains were enriched for “genes involved in biofilm formation by a single species on an inanimate substrate” based on their function in *C. albicans*. These included *BCR1*, a master regulator of biofilm formation, *CRZ2*, a transcription factor that is involved in biofilms, and *CSR1*, a zinc-finger protein involved in regulation of biofilm matrix (see Table B9). It is therefore possible that some of these genes contribute to the increased biofilm formation observed in *C. tropicalis* WOR1 overexpression strains (see Figure 6). There were also several genes that were down regulated in the *C. tropicalis* wor1 mutant strain versus the white strain, including *STB5*, which has sequence-specific DNA binding transcription factor activity and could potentially play a role in regulation of the switch by Wor1 (see Table B7).

B.2 Supplemental Figures

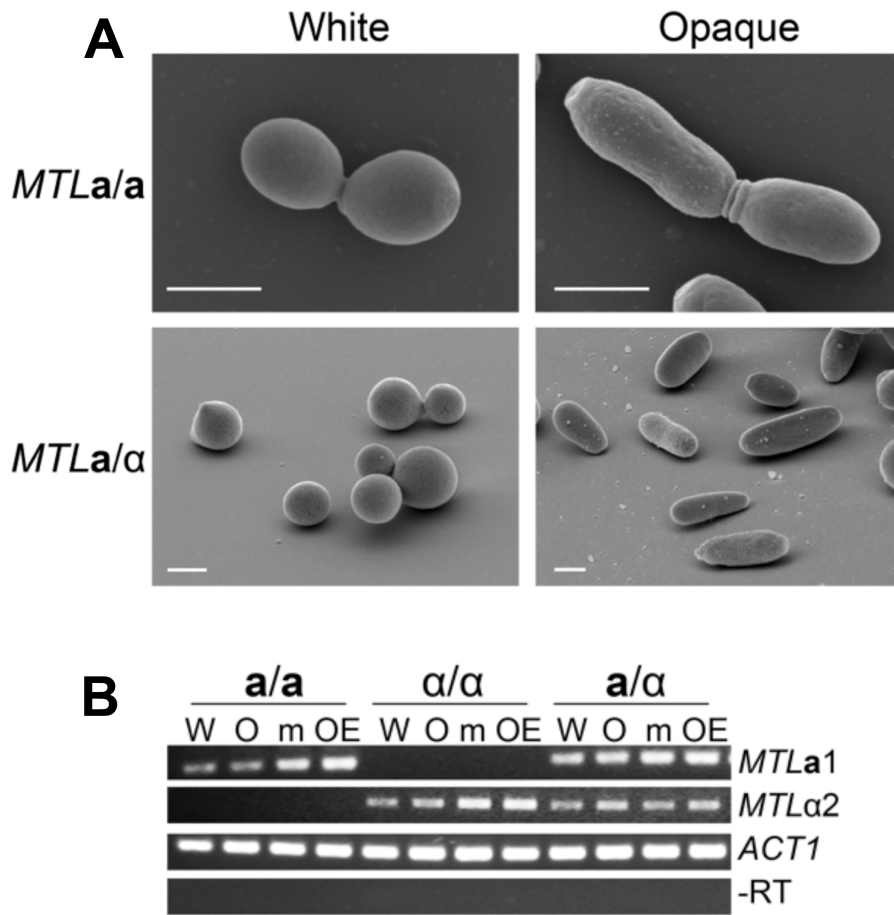


Figure B1. Scanning Electron Micrographs of *MTLa/a* (CAY1504) and *MTLa/α* (CAY1513) white and opaque cells and RT-PCR analysis of $\alpha 1$ and $\alpha 2$ expression. (A) White cells were round and exhibited a smooth cell surface, while opaque cells were elongated and typically had a pimpled or uneven surface. Scale bars = 5 μ m. (B) RNA was extracted from white (W), opaque (O), Δ/Δ *wor1* mutant (m), and *WOR1* overexpressing (OE) cells and examined by RT-PCR for expression of *MTLa1* and *MTLa2* genes. *ACT1* is a positive control performed with primers against *ACT1* and -RT is RT-PCR performed in the absence of reverse transcriptase with primers against *ACT1*.

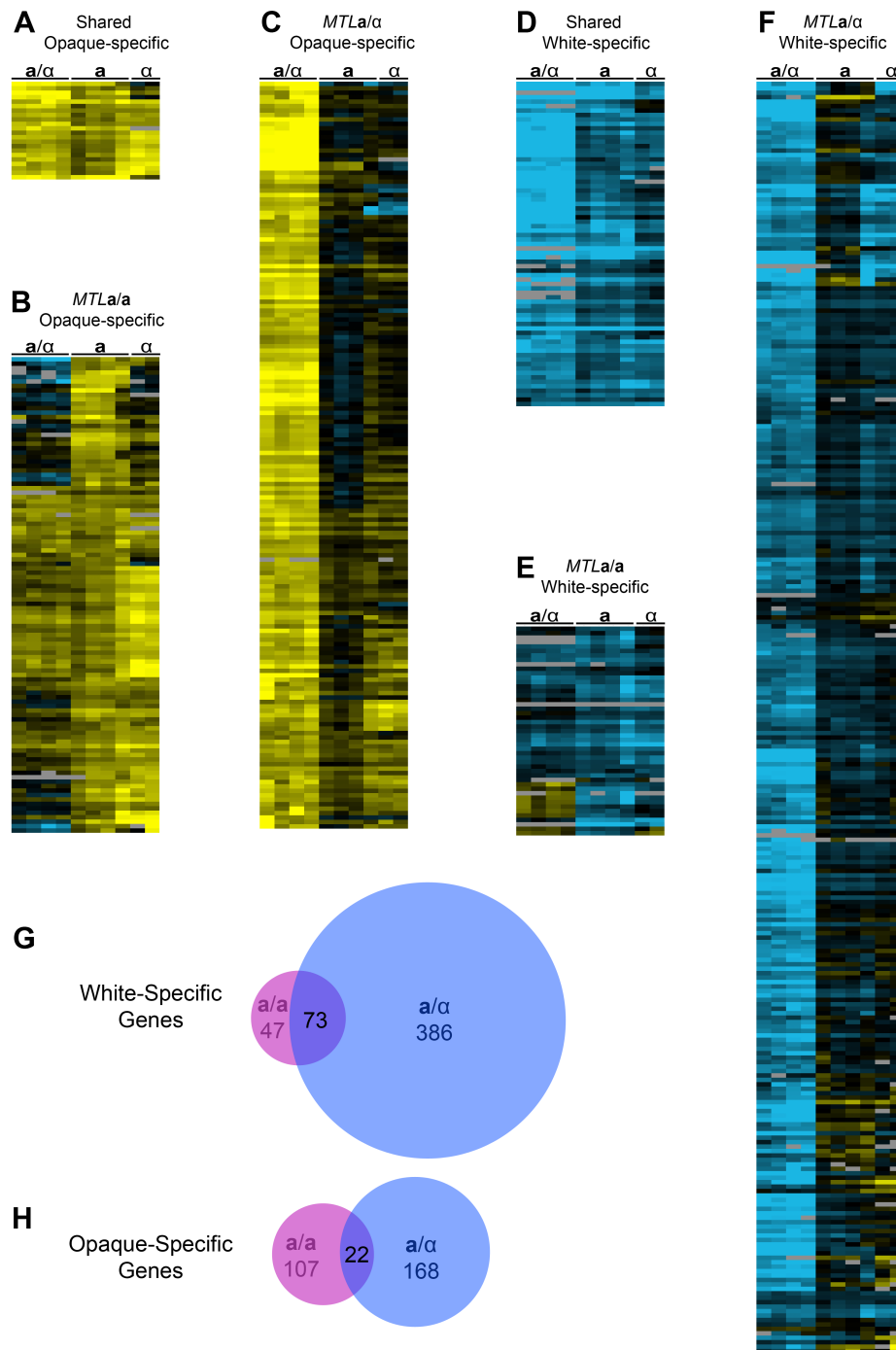


Figure B2. Transcriptional profiles of genes regulated by the white-opaque switch in *C. tropicalis* *a*, α , and *a/α* cell types. Panels A-F show genes that passed SAM analysis that were significantly different between white and opaque *a* or *a/α* cells. cDNA prepared from white and opaque states of CAY1511 (*a/α*), CAY1504 (*a*), and CAY1505 (α) in independent experiments was hybridized against a universal reference. Opaque cell gene expression data sets were divided by white cell

expression data sets. Some genes are regulated by the white-opaque switch in all three cell types (A, D, shared opaque- and white-specific genes). However, many white-opaque regulated genes are unique to *MTL* homozygous cells (**a** cells in panels B and E) or to *MTL* heterozygous cells (**a**/ α cells in panels C and F). Numbers of unique and shared genes between **a** and **a**/ α white and opaque states are illustrated in panels G and H. Note: a subset of genes that did not pass SAM analysis still appear to be similarly regulated by the switch in each cell type (e.g. middle of panel B and bottom of panel C).

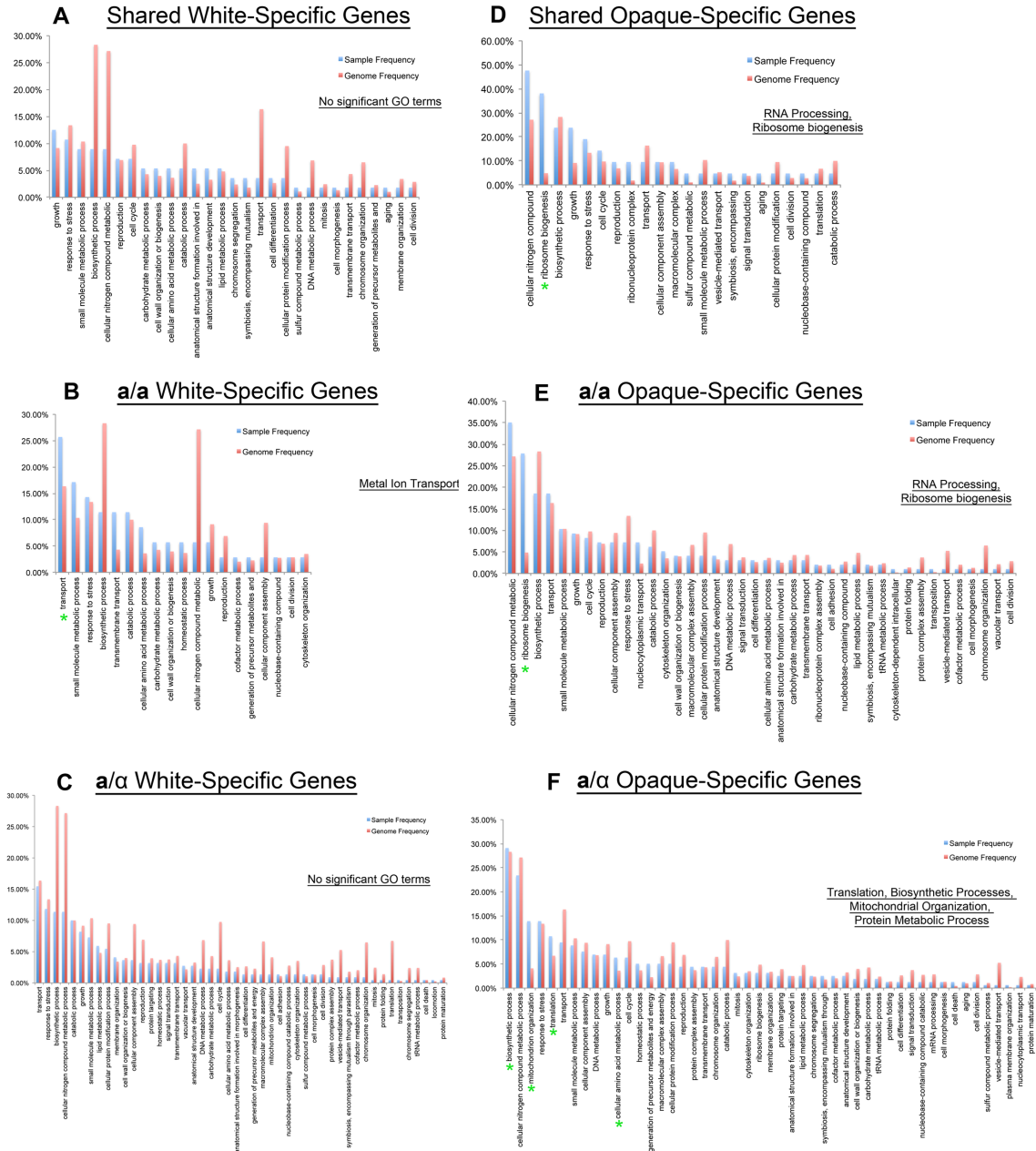


Figure B3. GO Term analysis of genes regulated by the white-opaque switch in *C. tropicalis* *a* and *a/α* cell types. Graphs represent GO Terms for genes that passed SAM analysis that were significantly different between white and opaque *a* or *a/α* cells. GO Term frequencies for genes regulated by the white-opaque switch in *a* and *a/α* cell types are shown in A and D for shared opaque- and white-specific genes, respectively. GO Term frequencies for white-opaque regulated genes are unique to *MTL* homozygous cells (*a* cells in panels B and E) or to *MTL* heterozygous cells (*a/α* cells in panels C and F) are also presented. * = statistically significant number of genes represented in the GO Term category.

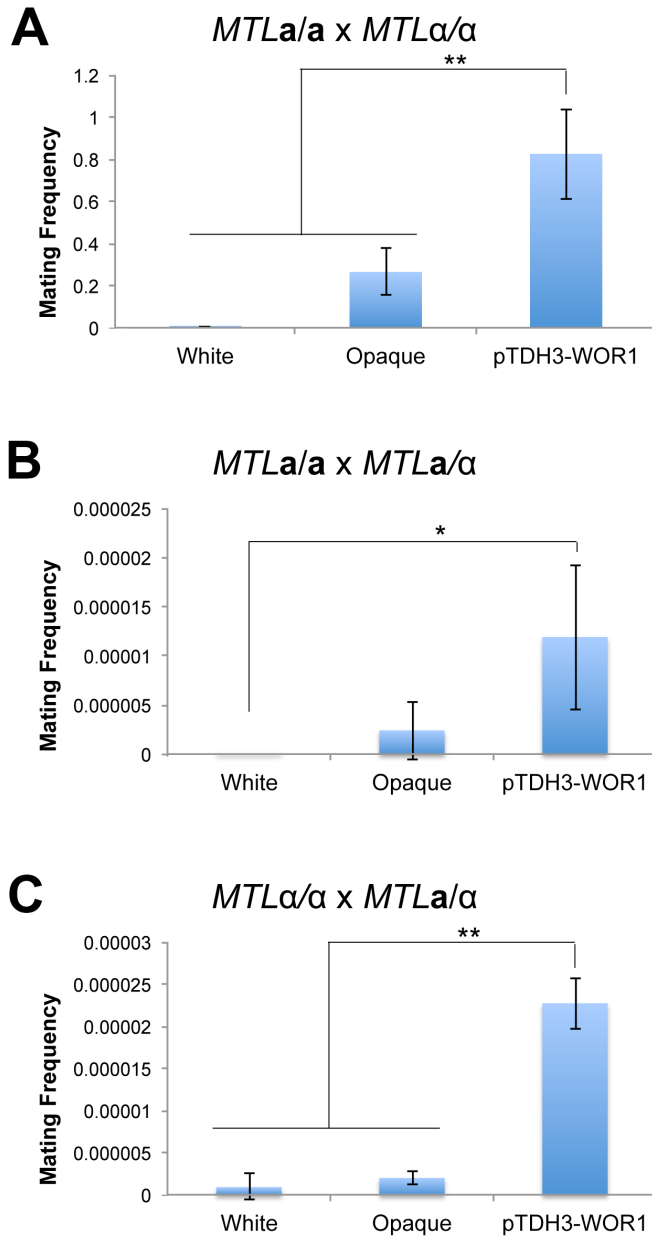


Figure B4. Mating of *C. tropicalis* a, α , and a/ α cell types is regulated by *WOR1* expression. Mating frequency of (A) a x α (CAY1503 x CAY1505), (B) a x a/ α (CAY1503 x CAY1511), and (C) α x a/ α (CAY1505 x CAY1513) white, opaque, or *pTDH3-WOR1* cells. Experiments were performed by co-incubating indicated strains on Spider medium for 1 day at room temperature, and then plating cells to selective media to quantify mating frequency. ** $p < 0.01$, * $p < 0.05$. Error bars indicate SD.

B.3 Supplemental Tables

Table B1. Clinical isolates examined in this study

<i>CAY#</i>	<i>Clinical Bank</i>	<i>MAT</i>	<i>Source</i>	<i>Other Information</i>
2446	20662.089	a/alpha	ATCC	Country: South Africa
2447	20663.022	a/alpha	ATCC	Country: USA
2448	20663.049	a/alpha	ATCC	Country: Malaysia
2449	20663.084	a/a	ATCC	Country: Canada
2450	20669.006	a/a	ATCC	Country: Australia
2451	20672.044	a/alpha	ATCC	Country: USA
2452	20674.056	a/alpha	ATCC	Country: Hungary
2453	20678.086	a/alpha	ATCC	Country: USA
2454	20681.019	a/alpha	ATCC	Country: United Kingdom
2455	20681.046	a/alpha	ATCC	Country: Czech Republic
2456	20682.027	a/alpha	ATCC	Country: Venezuela
2457	20682.071	a/alpha	ATCC	Country: China
2458	20683.028	a/alpha	ATCC	Country: Korea
2459	20683.060	a/alpha	ATCC	Country: Israel
2460	20685.088	a/alpha	ATCC	Country: Spain
2461	20686.023	a/a	ATCC	Country: Spain
2462	20692.084	a/alpha	ATCC	Country: USA
2463	20695.040	a/alpha	ATCC	Country: Colombia
2464	20695.042	alpha/alpha	ATCC	Country: Colombia
2465	20695.071	a/alpha	ATCC	Country: Canada
2466	20704.044	alpha/alpha	ATCC	Country: Slovakia
2467	20710.050	alpha/alpha	ATCC	Country: Slovakia
2468	20710.093	a/alpha	ATCC	Country: Chile
2469	20710.096	a/alpha	ATCC	Country: Chile
2470	20714.043	a/alpha	ATCC	Country: United Kingdom
2471	20714.061	a/alpha	ATCC	Country: Mexico
2472	20717.043	a/a	ATCC	Country: Czech Republic
2473	20717.060	a/alpha	ATCC	Country: Ecuador
2474	20717.087	a/alpha	ATCC	Country: Ecuador
2475	20720.061	alpha/alpha	ATCC	Country: USA
2476	20720.072	a/alpha	ATCC	Country: Hungary
2477	20722.013	a/alpha	ATCC	Country: USA
2478	20722.078	a/alpha	ATCC	Country: Spain
2479	20724.001	a/alpha	ATCC	Country: Spain
2480	20724.025	a/alpha	ATCC	Country:
2481	20724.051	a/alpha	ATCC	Country: USA
2482	20726.011	a/alpha	ATCC	Country: USA
2483	20727.049	a/alpha	ATCC	Country: Hungary
2484	20727.076	a/alpha	ATCC	Country: Hungary

2485	20731.017	a/alpha	ATCC	Country: USA
2486	20731.021	a/alpha	ATCC	Country: Korea
2487	20731.034	a/alpha	ATCC	Country: Korea
2488	20731.093	a/a	ATCC	Country: Israel
2489	20733.026	a/alpha	ATCC	Country: Israel
2490	20734.056	a/alpha	ATCC	Country: Italy
2491	20734.073	a/alpha	ATCC	Country: Italy
2492	20734.096	alpha/alpha	ATCC	Country: Korea
2493	20735.010	a/alpha	ATCC	Country: Korea
2494	20736.070	a/alpha	ATCC	Country: USA
2495	20740.003	a/alpha	ATCC	Country: Brasil
2518	NCCLS39	a/a	Aberdeen	Location:
2519	CDC45/1	a/alpha	Aberdeen	Location:
2520	J930561/2	a/a	Aberdeen	Location:
2521	J920533	a/a	Aberdeen	Location: vagina
2522	J930222	a/alpha	Aberdeen	Location: fingernail
2523	NCCLS71	a/alpha	Aberdeen	Location:
2524	J930582/3	a/alpha	Aberdeen	Location: chameleon intestine
2525	J930949	a/a	Aberdeen	Location: mouth
2526	L823	a/alpha	Aberdeen	Location: sputum
2527	J932716	a/alpha	Aberdeen	Location: vagina
2528	L601	a/alpha	Aberdeen	Location: pus
2529	J940670B	a/alpha	Aberdeen	Location: mouth
2530	J940788	a/alpha	Aberdeen	Location: mouth
2531	J941836	a/alpha	Aberdeen	Location:
2532	J950226	a/alpha	Aberdeen	Location:
2533	J941810	a/alpha	Aberdeen	Location:
2534	J941839	a/alpha	Aberdeen	Location:
2535	J951358	a/alpha	Aberdeen	Location:
2536	J942104	a/alpha	Aberdeen	Location:
2537	CDC44	alpha/alpha	Aberdeen	Location:
2538	J980157	alpha/alpha	Aberdeen	Location: abdominal biopsy
2539	J980160	alpha/alpha	Aberdeen	Location: bronchial aspirate
2540	J980184	a/alpha	Aberdeen	Location: sputum
2541	J980162	a/alpha	Aberdeen	Location: BAL
2542	J981212	a/alpha	Aberdeen	Location: vagina
2543	J981352	a/alpha	Aberdeen	Location: vagina
2544	J990332/2	a/alpha	Aberdeen	Location: vagina
2545	J981379	a/alpha	Aberdeen	Location: vagina
2546	J942105	a/alpha	Aberdeen	Location:
2547	J941019T	a/alpha	Aberdeen	Location: mouth
2548	81/022	a/alpha	Aberdeen	Location:
2549	81/056	a/alpha	Aberdeen	Location: nail
2550	81/031	a/alpha	Aberdeen	Location:
2551	83/002	a/alpha	Aberdeen	Location:

2552	75/036	a/alpha	Aberdeen	Location: mouth
2553	L470	a/alpha	Aberdeen	Location: sputum
2554	L474	a/alpha	Aberdeen	Location: vagina
2555	L391	a/a	Aberdeen	Location: faeces
2556	81/024	a/alpha	Aberdeen	Location:
2557	73/108	a/alpha	Aberdeen	Location: mouth
2558	75/035	a/alpha	Aberdeen	Location: mouth
2559	A700639	a/alpha	Aberdeen	Location: tracheal aspirate
2560	AM2005/0546	a/alpha	Aberdeen	Location:
2561	AM2005/0267	a/a	Aberdeen	Location:
2562	AM2002/0111	a/a	Aberdeen	Location: oral
2563	AM2004/0061	a/alpha	Aberdeen	Location:
2564	AM2004/0062	a/alpha	Aberdeen	Location:
2565	AM2007/0110	a/alpha	Aberdeen	Location: CSU
2566	AM2007/0111	a/alpha	Aberdeen	Location: IV catheter
2567	b30441/6/04	a/alpha	Aberdeen	Location: blood
2568	b30588/6/04	a/alpha	Aberdeen	Location: IV catheter
2569	b31083/6/04	a/a	Aberdeen	Location:
2570	b31084/6/04	a/a	Aberdeen	Location: sputum
2571	b31514/6/04	a/a	Aberdeen	Location:
2572	b31595/6/04	a/alpha	Aberdeen	Location: sputum
2573	b31597/6/04	a/alpha	Aberdeen	Location:
2574	b30349/7/04	a/alpha	Aberdeen	Location: blood
2575	b30488/7/04	a/alpha	Aberdeen	Location: BAL
2576	b30604/7/04	a/alpha	Aberdeen	Location: sputum
2577	b30613/7/04	a/alpha	Aberdeen	Location: blood
2578	b31581/7/04	a/alpha	Aberdeen	Location: urine
2579	b31586/7/04	a/alpha	Aberdeen	Location: CVP Tip (femoral)
2580	AM2004/0088	a/alpha	Aberdeen	Location: blood
2581	AM2004/0089	a/alpha	Aberdeen	Location: blood
2582	WC04-200748	a/a	Aberdeen	Location:
2583	WC02-203296	a/alpha	Aberdeen	Location:
2584	WC02-203295	a/alpha	Aberdeen	Location:
2585	L634	a/alpha	Aberdeen	Location:
2586	WC04-200749	a/a	Aberdeen	Location:
2587	GUI4448	a/alpha	Aberdeen	Location: soil (beach)
2588	GUI720	a/alpha	Aberdeen	Location: bronchial secretion
2589	GUI743	a/alpha	Aberdeen	Location:
2590	GUI3626A	a/alpha	Aberdeen	Location: sputum
2591	SCS501148	a/a	Aberdeen	Location: blood
2592	SCS74663	a/alpha	Aberdeen	Location: blood
2593	AM2005/0088	a/alpha	Aberdeen	Location: urine
2594	AM2005/0089	a/alpha	Aberdeen	Location: urine
2595	AM2005/0090	a/alpha	Aberdeen	Location: blood
2596	AM2005/0092	a/alpha	Aberdeen	Location: peritoneum

2597	AM2005/0093	a/a	Aberdeen	Location: urine
2598	AM2005/0094	a/alpha	Aberdeen	Location: urine
2599	AM2005/0543	a/alpha	Aberdeen	Location: blood
2600	AM2005/0272	a/a	Aberdeen	Location:
2601	AM2005/0270	a/alpha	Aberdeen	Location:
2602	AM2005/0282	a/alpha	Aberdeen	Location:
2603	AM2005/0569	a/alpha	Aberdeen	Location:
2604	AM2005/0276	a/alpha	Aberdeen	Location:
2605	AM2004/0090	a/a	Aberdeen	Location:
2606	AM2005/0297	a/alpha	Aberdeen	Location:
2607	AM2005/0280	a/a	Aberdeen	Location:
2608	AM2005/0257	a/alpha	Aberdeen	Location: blood
2609	AM2005/0275	a/alpha	Aberdeen	Location: sputum
2610	AM2005/0287	a/alpha	Aberdeen	Location: faeces
2611	AM2005/0289	a/alpha	Aberdeen	Location: urine
2612	AM2005/0290	a/alpha	Aberdeen	Location: sputum
2613	AM2005/0291	a/alpha	Aberdeen	Location: BAL
2614	AM2005/0292	a/a	Aberdeen	Location: sputum
2615	AM2007/0112	a/alpha	Aberdeen	Location: blood
2616	SCS2446	a/alpha	Aberdeen	Location: blood
2617	SCS122443V	a/alpha	Aberdeen	Location: blood
Switching a/alpha strains				

Table B2. List of *C. tropicalis* strains used in this study. Phenotype not determined unless noted.

Strain(s)	Genotype	MTL
CAY1502	<i>his1/his1::SAT1</i>	a/a
CAY1503	<i>arg4/arg4::SAT1</i>	a/a
CAY3376	<i>arg4/arg4::SAT1</i> (white)	a/a
CAY3378	<i>arg4/arg4::SAT1</i> (opaque)	a/a
CAY1504	<i>arg4/arg4::SAT1</i> (white)	a/a
CAY2275	<i>arg4/arg4::SAT1</i> (opaque)	a/a
CAY1505	<i>his1/his1::SAT1</i>	α/α
CAY3391	<i>his1/his1::SAT1</i> (white)	α/α
CAY3392	<i>his1/his1::SAT1</i> (opaque)	α/α
CAY1509	<i>arg4/arg4::SAT1</i>	α/α
CAY1511	<i>his1/his1::SAT1</i> (white)	a/a
CAY4048	<i>his1/his1::SAT1</i> (opaque)	a/a
CAY1513	<i>arg4/arg4::SAT1</i>	a/a
CAY4049	<i>arg4/arg4::SAT1</i> (white)	a/a
CAY4050	<i>arg4/arg4::SAT1</i> (opaque)	a/a
CAY2206	<i>arg4/arg4 wor1/wor1::SAT1</i>	a/a
CAY2342	<i>his1/his1 wor1/wor1::SAT1</i>	α/α
CAY4043	<i>his1/his1 wor1/wor1::SAT1</i>	a/a
CAY4012	<i>arg4/arg4 wor1/wor1::SAT1</i>	a/a
CAY3853	<i>arg4/arg4 pTDH3/pTDH3-WOR1::SAT1</i>	a/a
CAY3976	<i>his1/his1 pTDH3/pTDH3-WOR1::SAT1</i>	α/α
CAY4045	<i>his1/his1 pTDH3/pTDH3-WOR1::SAT1</i>	a/a
CAY3978	<i>arg4/arg4 pTDH3/pTDH3-WOR1::SAT1</i>	a/a
CAY4289	<i>arg4/arg4 a2/a2::SAT1</i>	a/a
CAY4253/CAY4254	<i>his1/his1 a2/a2::SAT1</i>	a/a
CAY4251/CAY4252	<i>arg4/arg4 a2/a2::SAT1</i>	a/a
CAY4290	<i>his1/his1 α1/α1::SAT1</i>	α/α
CAY4258/CAY4259	<i>his1/his1 α1/α1::SAT1</i>	a/a
CAY4256/CAY4257	<i>arg4/arg4 α1/α1::SAT1</i>	a/a
CAY4286	<i>arg4/arg4 MTLα-3'::SAT1</i>	a/a

Table B3. Oligonucleotides used in this study. Underlined sequences denote restriction sites.

<i>Oligo</i>	<i>Name</i>	<i>Sequence</i>
1	<i>WOR1</i> (ApaI) -900	GGAGCGGGGGCCCTACTACATTAGTTGTTCCCTG
2	<i>WOR1</i> (XhoI) 0	GCCGGCCTCGAGATAATCTAGACGCCGACATG
3	<i>WOR1</i> (SacII) +2100	GGAGCGCCGCGGGGTAACCTACAGGTGACGAATC
4	<i>WOR1</i> (SacI) +3000	GGCCGCGAGCTCTTACCCACAAGTGTGTCTAG
5	<i>WOR1</i> 5' check -950	GAATCTAAGGCAACAAACGATTTC
6	<i>WOR1</i> 3' check +3100	GGTGATTAATAATCACATTCTCGG
7	<i>WOR1</i> 5' ORF +200	GACTGATGGAATTTCTTGGTC
8	<i>WOR1</i> 3' ORF +550	GCAGCTGTCCATAAACATGG
9	<i>HIS1</i> (ApaI) -900	GGAGCGGGGGCCACCTCAAGAGATGAATGACA
10	<i>HIS1</i> (XhoI) 0	GCCGGCCTCGAGACTCTAACTATGATTGTATTC
11	<i>HIS1</i> (SacII) +900	GGAGCGCCGCGGAGTTTCATCTGTATAATAGTACA
12	<i>HIS1</i> (SacI) +1800	GGCCGCGAGCTCTCCACACATTCATAAATGTTCA
13	<i>HIS1</i> 5' check -950	CCAAGGACAACACTCGTGCAGT
14	<i>HIS1</i> 3' check +1900	CAAATTGATCACTTCTCTAGTCGA
15	<i>HIS1</i> 5' ORF +200	AGGGTAATTGTGATTGGGTA
16	<i>HIS1</i> 3' ORF +600	CTTGGAAGAAACCAAGTGAG
17	<i>ARG4</i> (ApaI) -900	GGAGCGGGGGCCCTGAATGTGTTGGTGGTGATG
18	<i>ARG4</i> (XhoI) 0	GCCGGCCTCGAGGACATTGTTGTTAGAATAAGTT
19	<i>ARG4</i> (NotI) +1400	GGAGCGCGCGCCGCACATTCATTCTCCTTCCCTCTC
20	<i>ARG4</i> (SacII) +2300	GGCCGCCCGCGGAATATTGATTGTTGATTGTTG
21	<i>ARG4</i> 5' check -950	GACGTTGTTTACCCTTGGTATC
22	<i>ARG4</i> 3' check +2400	TTGTGGAATGTAATTTGCACAC
23	<i>ARG4</i> 5' ORF +200	TCAGGATTAGAAGAAATTCGTG
24	<i>ARG4</i> 3' ORF +600	TGGACTTTGATTAACCTCTGGT
25	<i>MTLa2</i> (ApaI) -900	GGAGCGGGGGCCACCATGATTTTGAACATCTGGAA
26	<i>MTLa2</i> (XhoI) 0	GCCGGCCTCGAGATCTTGTTGCTTTTGATCGTTTT
27	<i>MTLa2</i> (SacII) +700	GGAGCGCCGCGGGTAGTGCGTACGGTGTGCGAGT
28	<i>MTLa2</i> (SacI) +1600	GGCCGCGAGCTCATGCTGGCAAGGGAATTTAGTTA
29	<i>MTLa2</i> 5' check (-950)	GGGATGATTTGCCACAGGAAG
30	<i>MTLa2</i> 3' check (+1650)	CCATGGGGTAAAAAAAAAATAGAAA
31	prBT_74 (<i>MTLa2</i> 5')	GCAAGAAACATACTTTCAAAGCAG
32	prBT_75 (<i>MTLa2</i> 3')	GCCCGTTAAGTCGTTTCTTGGT
33	<i>MTLa1</i> (ApaI) -900	GGAGCGGGGGCCACGTTGTTGGATTATTACAAAAATG
34	<i>MTLa1</i> (XhoI) 0	GCCGGCCTCGAGGGCAATTGGTTTCTTTCAGTGTTA
35	<i>MTLa1</i> (SacII) +700	GGAGCGCCGCGGTCTCACTCTTTTCTGACGGTCT
36	<i>MTLa1</i> (SacI) +1550	GGCCGCGAGCTCGAGTGCTGTCATTGTTGAGAAGTT
37	<i>MTLa1</i> 5' check (-950)	AAGTCGCTCACAGAACTTTCAATC
38	<i>MTLa1</i> 3' check (+1600)	GCTAGGATTTGTCAATTATACCCA
39	prBT_68 (<i>MTLa1</i> 5')	ACTTGAATACGGAATGTTGAATGA
40	prBT_69 (<i>MTLa1</i> 3')	GGTCTAGGACCGTATATAACCAAGTCA
41	<i>TDH3</i> (ApaI) -950	GGAGCGGGGGCCGTTAGTCGTTATGGAGGACA
42	<i>TDH3</i> (0)	TGTTAAAATTTAATTTGTAAGTGATTGATT
43	<i>WOR1</i> (0)	AAATCAAATCACTTACAAATTAATTTTAACATCCATGTCGGCG TCTAGATTATCATC
44	<i>WOR1</i> (XhoI) +2500	GCCGGCCTCGAGGTCATATACCCAACACCGAAC
45	prBT_72 (<i>MTLa1</i> 5')	GGATAAAGAGAGCTTAAGTTCAGAAGAG
46	prBT_73 (<i>MTLa1</i> 3')	AAGTATCATCTGTCTTATCTGATTCTTTC
47	prBT_70 (<i>MTLa2</i>)	TAAAACATTAAGCATAGAGGACAAAGAA

48	prBT_71 (<i>MTLα2</i>)	AACTTCAAATGCAAAATGTAAAACATAC
49	<i>ACT1</i> 5Q	GGTGATGGTGTTACCCACGTT
50	<i>ACT1</i> 3Q	TGTAACCACGTTTCAGACAAGATCTTC
51	<i>WOR1</i> 5Q	CCGTCTAATGTTATACCTGCATCAA
52	<i>WOR1</i> 3Q	TTCGTCGTACTTATGGTAATTGTTTTCT
53	<i>MTLa1</i> (0)	GAAAGTCAATCAATCACTGTTTTGTACTAAAGGCCATGCTTAAA GCTCAAAAGGAAGAG
54	<i>MTLa1</i> (XhoI) +450	GCCGGCCTCGAGCTAAGTATCATCTGTCTTATCTGA
55	<i>MTLα2</i> (0)	GAAAGTCAATCAATCACTGTTTTGTACTAAAGGCCATGATTAGT CACTGGGATACCCTT
56	<i>MTLα2</i> (XhoI) +600	GCCGGCCTCGAGTCATTGTGACAATAATTGAGCAAG
57	<i>MTL</i> flank for (ApaI)	GGAGCGGGGCCCACACGACACAGTCCAACAGCAGA
58	<i>MTL</i> flank rev (HindIII)	GGCCGCAAGCTTATCACTTCGACCTATTTCTTTTAAAAC
59	<i>MTL</i> flank 5' chk (FS5-1)	AGAACCCGTATGATTTTACGAAAAG
60	pSFS2a <i>SAT</i> rev (FS5-2)	CTCAGGGATCACCGAAAT
61	M13 (-21) for (FS5-3)	TGTAAAACGACGGCCAGT
62	<i>MTLα</i> 3' check (FS5-4)	GTGACGCCATATTTTCTGGTGC
63	<i>MTLa</i> 3' check (FS5-5)	GCAAGAAACATACTTTCAAAGCAG

Table B4. Plasmids used in this study.

<i>Plasmid</i>	<i>Name</i>	<i>Oligos used for cloning</i>	<i>Enzyme(s) used for restriction digest of DNA prior to transformation</i>
pRB29	<i>WOR1</i> deletion plasmid	1/2, 3/4	<i>Apa</i> I/ <i>Sac</i> I
pRB55	<i>HIS1</i> deletion plasmid	9/10, 11/12	<i>Apa</i> I/ <i>Sac</i> I
pRB59	<i>ARG4</i> deletion plasmid	17/18, 19/20	<i>Apa</i> I/ <i>Sac</i> II
pRB288	<i>MTLa2</i> deletion plasmid	25/26, 27/28	<i>Apa</i> I/ <i>Sac</i> I
pRB289	<i>MTL</i> α 1 deletion plasmid	33/34, 35/36	<i>Apa</i> I/ <i>Sac</i> I
pRB235	<i>pTDH3-WOR1</i> plasmid	41/42, 43/44, 41/44 for fusion	<i>Sma</i> I
pRB290	<i>MTL</i> marker plasmid	57/58	<i>EcoR</i> I

Table B5. List of α white-specific genes.

α White Specific Gene	Relative Fold Change	<i>C. albicans</i> Ortholog	Gene Name	White-opaque fold change in Ca
CTRG_00813	-759.19	N/A		N/A
CTRG_00731	-201.54	orf19.4366		N/A
CTRG_01245	-170.21	orf19.1461		N/A
CTRG_03259	-123.62	N/A		N/A
CTRG_00040	-111.79	orf19.4679	AGP2	White, 11.3
CTRG_00051	-98.22	N/A		N/A
CTRG_00471	-86.38	orf19.3369	MOH1	N/A
CTRG_00468	-74.26	N/A		N/A
CTRG_03341	-72.22	orf19.4886		N/A
CTRG_00284	-49.15	orf19.2693	GST2	N/A
CTRG_00722	-44.66	orf19.4372		N/A
CTRG_00590	-44.12	orf19.5843	SRR1	N/A
CTRG_00268	-36.44	N/A		N/A
CTRG_00255	-35.66	orf19.2738	SUL2	N/A
CTRG_00723	-35.21	N/A		N/A
CTRG_04391	-30.43	orf19.6017		N/A
CTRG_00267	-30.41	N/A		N/A
CTRG_05962	-30.29	orf19.7284	ASR2	N/A
CTRG_05963	-30.27	orf19.7283		N/A
CTRG_00142	-28.79	orf19.2762	AHP1	N/A
CTRG_02543	-28.63	orf19.251		N/A
CTRG_02106	-27.41	orf19.5625		N/A
CTRG_00467	-27.24	orf19.335		N/A
CTRG_04140	-25.41	N/A		N/A
CTRG_00815	-25.29	orf19.2846		White, 4.9
CTRG_04184	-24.97	N/A		N/A
CTRG_01290	-24.31	N/A		N/A
CTRG_03937	-24.16	orf19.3803	MNN22	N/A
CTRG_05130	-23.82	N/A		N/A
CTRG_01691	-23.42	orf19.1862		White, 23.1
CTRG_01140	-23.00	orf19.2048		White, 28.1
CTRG_01210	-21.92	orf19.851		White, 3.3
CTRG_03661	-21.42	orf19.5025	MET3	White, 6.2
CTRG_00285	-21.13	orf19.2691		N/A
CTRG_02101	-20.61	orf19.6793		N/A
CTRG_03800	-20.52	orf19.2897	SOU2	N/A
CTRG_04122	-20.14	orf19.2296		N/A
CTRG_01270	-18.77	N/A		N/A

CTRG_00389	-18.68	orf19.513		N/A
CTRG_01286	-18.65	orf19.842	ASR3	N/A
CTRG_00394	-18.46	orf19.539	LAP3	Opaque, 47.8
CTRG_00158	-18.13	orf19.2770		N/A
CTRG_00458	-17.80	orf19.2810	AAP1	Opaque, 12.9
CTRG_03271	-17.38	orf19.4773	AOX2	N/A
CTRG_01813	-17.35	orf19.2170	PHM7	N/A
CTRG_00902	-17.35	N/A		N/A
CTRG_01048	-16.96	orf19.7502		N/A
CTRG_01093	-16.88	orf19.2114		N/A
CTRG_05042	-16.76	orf19.6489	FMP45	White, 3.8
CTRG_05423	-16.45	orf19.5763		N/A
CTRG_00976	-16.43	N/A		N/A
CTRG_05162	-16.09	N/A		N/A
CTRG_00037	-16.00	N/A		N/A
CTRG_00280	-15.89	N/A		N/A
CTRG_00254	-15.78	orf19.2737		N/A
CTRG_03993	-15.15	orf19.4438	RME1	N/A
CTRG_00754	-15.10	orf19.4379	PRP13	N/A
CTRG_03296	-15.05	orf19.1830		Opaque, 13.8
CTRG_01076	-14.98	orf19.1923	RRN3	N/A
CTRG_01887	-14.46	orf19.2241	PST1	N/A
CTRG_00157	-14.06	N/A		N/A
CTRG_00580	-13.89	N/A		N/A
CTRG_02719	-13.69	orf19.5592		N/A
CTRG_00809	-13.64	orf19.2848		N/A
CTRG_00408	-12.96	orf19.535	RBR1	Opaque, 22.3
CTRG_01812	-12.92	orf19.2172	ARA1	N/A
CTRG_03292	-12.73	N/A		N/A
CTRG_00156	-12.63	orf19.2768	AMS1	N/A
CTRG_00162	-12.53	N/A		N/A
CTRG_04602	-12.50	orf19.4526	HSP30	N/A
CTRG_05940	-12.28	orf19.7310		White, 18.5
CTRG_04252	-12.04	N/A		N/A
CTRG_00660	-11.88	orf19.3131	OYE32	N/A
CTRG_03620	-11.85	orf19.4970		N/A
CTRG_03991	-11.80	N/A		N/A
CTRG_03352	-11.65	orf19.4873		Opaque, 9.7
CTRG_00526	-11.55	orf19.6316		N/A
CTRG_00265	-11.38	N/A		N/A
CTRG_00286	-11.36	orf19.1313	CDR3	Opaque, 2.9

CTRG_01777	-11.31	N/A		N/A
CTRG_03611	-11.22	N/A		N/A
CTRG_00749	-11.06	orf19.4391		N/A
CTRG_01353	-10.93	orf19.5826		N/A
CTRG_02048	-10.83	orf19.5348	TPS3	N/A
CTRG_01884	-10.72	orf19.2244		N/A
CTRG_04037	-10.65	orf19.2898		White, 6.5
CTRG_00738	-10.63	orf19.4380.1		N/A
CTRG_01886	-10.25	N/A		N/A
CTRG_00501	-10.14	orf19.2726		White, 5.6
CTRG_00356	-10.05	orf19.473	TP04	White, 3.9
CTRG_01828	-10.01	N/A		N/A
CTRG_03270	-9.85	orf19.4774	AOX1	White, 6
CTRG_01237	-9.82	orf19.1449		N/A
CTRG_04893	-9.71	N/A		N/A
CTRG_01522	-9.65	orf19.5210		N/A
CTRG_01246	-9.55	orf19.1462	SMP2	N/A
CTRG_04726	-9.29	N/A		N/A
CTRG_00727	-9.14	orf19.4370		N/A
CTRG_01211	-8.97	orf19.860	BMT8	N/A
CTRG_01183	-8.88	orf19.893	PGA17	Opaque, 75.1
CTRG_02442	-8.87	orf19.244	DCG1	N/A
CTRG_01861	-8.79	orf19.3612	PST2	Opaque, 6.3
CTRG_01292	-8.46	orf19.2006		N/A
CTRG_01123	-8.46	orf19.2076		N/A
CTRG_00303	-8.45	orf19.5311		N/A
CTRG_01138	-8.41	orf19.2050		N/A
CTRG_03324	-8.39	N/A		N/A
CTRG_01247	-8.37	orf19.1464	BMT5	N/A
CTRG_05178	-8.30	orf19.6687		N/A
CTRG_02069	-8.27	orf19.1395		White, 6.8
CTRG_00604	-8.22	orf19.3885		N/A
CTRG_01581	-8.10	orf19.792		N/A
CTRG_00463	-8.09	N/A		N/A
CTRG_02376	-8.08	orf19.1653		N/A
CTRG_00302	-8.02	orf19.5308		N/A
CTRG_01831	-7.94	N/A		N/A
CTRG_03325	-7.73	N/A		N/A
CTRG_03114	-7.73	N/A		N/A
CTRG_04984	-7.69	orf19.6557		N/A
CTRG_01131	-7.66	orf19.2065		N/A

CTRG_00328	-7.64	orf19.1307		N/A
CTRG_00488	-7.56	orf19.5295		N/A
CTRG_05043	-7.54	orf19.6492		N/A
CTRG_00434	-7.50	orf19.3799		N/A
CTRG_02430	-7.49	orf19.6998	GTT1	Opaque, 11
CTRG_04959	-7.46	orf19.6574	ALK6	N/A
CTRG_00464	-7.41	N/A		N/A
CTRG_04116	-7.40	orf19.675		N/A
CTRG_00512	-7.37	orf19.552		N/A
CTRG_01966	-7.33	N/A		N/A
CTRG_04358	-7.33	N/A		N/A
CTRG_01202	-7.31	orf19.868	ADAEC	N/A
CTRG_03584	-7.26	orf19.4934	OP4	Opaque, 36.5
CTRG_05939	-7.23	orf19.7311		N/A
CTRG_05663	-7.19	N/A		N/A
CTRG_03256	-7.06	orf19.4749		N/A
CTRG_05662	-6.98	orf19.7093	HGT13	N/A
CTRG_05144	-6.98	orf19.1332	SNG4	N/A
CTRG_00960	-6.98	N/A		N/A
CTRG_00461	-6.96	N/A		N/A
CTRG_02440	-6.93	orf19.246		N/A
CTRG_00955	-6.83	orf19.3226		N/A
CTRG_00300	-6.78	N/A		N/A
CTRG_04599	-6.78	orf19.4530.1		N/A
CTRG_01827	-6.70	N/A		N/A
CTRG_06043	-6.66	N/A		N/A
CTRG_04834	-6.64	orf19.2460		N/A
CTRG_01829	-6.64	orf19.1349		N/A
CTRG_00940	-6.64	orf19.2531	CSP37	N/A
CTRG_00022	-6.62	orf19.4575		N/A
CTRG_01916	-6.46	orf19.4082	DDR48	Opaque, 9.7
CTRG_02568	-6.46	N/A		N/A
CTRG_01965	-6.45	N/A		N/A
CTRG_01885	-6.40	orf19.2242	PRB1	N/A
CTRG_01917	-6.35	N/A		N/A
CTRG_01531	-6.34	N/A		N/A
CTRG_03682	-6.31	orf19.7214		White, 6.5
CTRG_00633	-6.30	N/A		N/A
CTRG_04174	-6.26	orf19.2781		N/A
CTRG_01371	-6.25	orf19.1586	FGR22	N/A
CTRG_03823	-6.21	orf19.2882	XUT1	N/A

CTRG_01991	-6.16	orf19.1368		N/A
CTRG_05531	-6.16	orf19.6673	HEX1	N/A
CTRG_01809	-6.09	orf19.2175		N/A
CTRG_00896	-6.09	orf19.2613	ECM4	N/A
CTRG_01326	-6.06	orf19.5799		N/A
CTRG_05672	-6.06	N/A		N/A
CTRG_01504	-6.05	N/A		N/A
CTRG_00152	-5.98	N/A		N/A
CTRG_00175	-5.92	orf19.4571		N/A
CTRG_03145	-5.84	orf19.6117		N/A
CTRG_01325	-5.84	N/A		N/A
CTRG_00594	-5.83	orf19.6649	BRF1	N/A
CTRG_00172	-5.81	orf19.4568	ZCF25	N/A
CTRG_04298	-5.78	orf19.6077		N/A
CTRG_05222	-5.73	orf19.5158		N/A
CTRG_02545	-5.71	orf19.258		N/A
CTRG_02540	-5.69	orf19.348	SKN2	N/A
CTRG_00480	-5.69	orf19.1324	RAD2	N/A
CTRG_03085	-5.68	orf19.6420	PGA13	Opaque, 12.9
CTRG_02877	-5.68	orf19.3405	ZCF18	N/A
CTRG_05029	-5.66	orf19.6919		N/A
CTRG_01141	-5.65	orf19.2047		N/A
CTRG_00559	-5.65	orf19.651	LYP1	N/A
CTRG_04919	-5.61	orf19.7085		White, 7.8
CTRG_06181	-5.60	orf19.5431		N/A
CTRG_05165	-5.60	orf19.7200		N/A
CTRG_00074	-5.52	orf19.4167		N/A
CTRG_05163	-5.46	N/A		N/A
CTRG_00042	-5.43	orf19.4682	HGT17	N/A
CTRG_02594	-5.43	orf19.313	DAL4	N/A
CTRG_00301	-5.43	orf19.5307	JEN2	Opaque, 12
CTRG_00021	-5.41	orf19.4577		N/A
CTRG_00117	-5.39	orf19.367	CNH1	N/A
CTRG_00656	-5.39	orf19.457		N/A
CTRG_00996	-5.38	orf19.3269	GSL2	N/A
CTRG_00126	-5.36	orf19.5651		N/A
CTRG_05143	-5.35	orf19.1333	SNG3	N/A
CTRG_00096	-5.30	N/A		N/A
CTRG_00651	-5.28	orf19.3854		N/A
CTRG_05231	-5.27	orf19.5686		White, 3.4
CTRG_04658	-5.26	N/A		N/A

CTRG_01180	-5.23	orf19.896	CHK1	N/A
CTRG_00795	-5.20	orf19.2425	HGT18	N/A
CTRG_00655	-5.20	orf19.454	SFL1	N/A
CTRG_05894	-5.19	orf19.6601		N/A
CTRG_00714	-5.16	orf19.4356	HGT3	N/A
CTRG_00631	-5.14	orf19.3874		N/A
CTRG_02361	-5.10	N/A		N/A
CTRG_01303	-5.10	orf19.5782		N/A
CTRG_00733	-5.08	orf19.4365		N/A
CTRG_00177	-5.08	orf19.4657		N/A
CTRG_00677	-5.05	orf19.1852		N/A
CTRG_05972	-5.04	orf19.5612	BMT4	N/A
CTRG_02558	-5.01	orf19.278		White, 6.1
CTRG_02544	-4.97	orf19.255	ZCF1	N/A
CTRG_05236	-4.96	orf19.5683		N/A
CTRG_00771	-4.96	orf19.2396	IFR2	N/A
CTRG_00087	-4.94	orf19.4185		N/A
CTRG_03308	-4.93	N/A		N/A
CTRG_04135	-4.86	orf19.4783		N/A
CTRG_02325	-4.83	orf19.5863		N/A
CTRG_00174	-4.81	orf19.4570		N/A
CTRG_00647	-4.79	orf19.6638	PTC4	N/A
CTRG_00219	-4.78	orf19.4610		N/A
CTRG_00368	-4.77	N/A		N/A
CTRG_04803	-4.76	orf19.3040	EHT1	White, 10
CTRG_00788	-4.75	orf19.2414		N/A
CTRG_04722	-4.73	orf19.5203		N/A
CTRG_00649	-4.73	orf19.6640	TPS1	N/A
CTRG_04849	-4.71	orf19.6899		N/A
CTRG_05434	-4.71	orf19.3214		N/A
CTRG_02900	-4.70	orf19.3508		N/A
CTRG_05890	-4.69	orf19.6592		N/A
CTRG_04203	-4.69	orf19.6229	CAT1	N/A
CTRG_05993	-4.69	orf19.4317	GRE3	N/A
CTRG_04826	-4.68	N/A		N/A
CTRG_01356	-4.68	orf19.5824		N/A
CTRG_04442	-4.68	orf19.3330.3	POX18	N/A
CTRG_00658	-4.66	orf19.459	ADP1	N/A
CTRG_01817	-4.66	orf19.2165		White, 3.7
CTRG_00344	-4.66	orf19.1314		N/A
CTRG_01865	-4.65	N/A		N/A

CTRG_00456	-4.65	orf19.323		N/A
CTRG_00238	-4.63	orf19.4595		N/A
CTRG_02460	-4.63	orf19.7002		N/A
CTRG_06185	-4.63	orf19.5426		N/A
CTRG_01086	-4.61	orf19.2107	MUQ1	N/A
CTRG_00642	-4.59	orf19.6632	ACO2	N/A
CTRG_00682	-4.58	orf19.699		N/A
CTRG_04993	-4.57	orf19.5167	IFM1	N/A
CTRG_01276	-4.56	orf19.1494	RAD23	N/A
CTRG_01212	-4.56	orf19.856	IFK2	N/A
CTRG_00900	-4.55	orf19.2616	UGT51C1	N/A
CTRG_06050	-4.55	orf19.3910		N/A
CTRG_04226	-4.54	N/A		N/A
CTRG_03859	-4.53	orf19.1239		Opaque, 43.6
CTRG_04225	-4.48	N/A		N/A
CTRG_05978	-4.44	N/A		N/A
CTRG_04127	-4.42	orf19.4771		White, 4.3
CTRG_04152	-4.42	orf19.2792	IST2	N/A
CTRG_02509	-4.41	N/A		N/A
CTRG_06203	-4.40	orf19.6993	GAP2	N/A
CTRG_03683	-4.38	N/A		N/A
CTRG_00001	-4.37	orf19.5667	MNR2	N/A
CTRG_03999	-4.34	orf19.5076	PFY1	N/A
CTRG_00343	-4.32	orf19.2701		Opaque, 8.7
CTRG_03597	-4.31	orf19.3007.2		N/A
CTRG_01390	-4.27	orf19.1564		N/A
CTRG_03094	-4.25	orf19.6398		N/A
CTRG_01821	-4.24	orf19.1354	UCF1	N/A
CTRG_05512	-4.23	orf19.6660		N/A
CTRG_04424	-4.21	orf19.3310		N/A
CTRG_01323	-4.19	N/A		N/A
CTRG_02329	-4.19	N/A		N/A
CTRG_06206	-4.19	orf19.6996		Opaque, 4.5
CTRG_04679	-4.18	orf19.2968		N/A
CTRG_00689	-4.17	N/A		N/A
CTRG_01352	-4.16	N/A		N/A
CTRG_04801	-4.16	orf19.3021		N/A
CTRG_00304	-4.16	orf19.5312	MET4	N/A
CTRG_00176	-4.15	orf19.4658		N/A
CTRG_02618	-4.14	orf19.6396		N/A
CTRG_00355	-4.12	orf19.597		N/A

CTRG_03648	-4.12	orf19.4980	HSP70	White, 10.4
CTRG_00027	-4.11	orf19.4662	RLM1	N/A
CTRG_05164	-4.10	orf19.7199		N/A
CTRG_02576	-4.08	orf19.918	CDR11	N/A
CTRG_02521	-4.07	orf19.5992	WOR2	Opaque, 4.4
CTRG_00200	-4.06	orf19.4629		N/A
CTRG_04447	-4.06	orf19.3338		N/A
CTRG_00216	-4.04	N/A		N/A
CTRG_01154	-4.03	orf19.2036		N/A
CTRG_04355	-4.03	orf19.6053	CIS2	N/A
CTRG_01764	-4.02	orf19.2248	ARE2	N/A
CTRG_05345	-4.01	orf19.1979	GIT3	White, 15.6
CTRG_00311	-4.01	orf19.1595		N/A
CTRG_04833	-3.99	orf19.2461	PRN4	Opaque, 6.3
CTRG_01908	-3.98	orf19.35		N/A
CTRG_01808	-3.97	orf19.225		N/A
CTRG_01759	-3.96	orf19.2252		N/A
CTRG_01825	-3.96	orf19.1348		N/A
CTRG_00798	-3.95	orf19.2863		N/A
CTRG_02443	-3.95	orf19.243	OXR1	N/A
CTRG_00432	-3.95	orf19.2681	RBT7	N/A
CTRG_01251	-3.94	orf19.1468		N/A
CTRG_01474	-3.93	orf19.3563		N/A
CTRG_02332	-3.89	orf19.345		N/A
CTRG_02319	-3.89	orf19.5879		N/A
CTRG_00653	-3.89	orf19.3848		N/A
CTRG_00646	-3.88	orf19.6637		White, 4.7
CTRG_03969	-3.88	orf19.6196		N/A
CTRG_05146	-3.88	orf19.6706	GYP7	Opaque, 5.4
CTRG_01923	-3.88	N/A		N/A
CTRG_02802	-3.87	orf19.5514		N/A
CTRG_04144	-3.87	orf19.5070		N/A
CTRG_05372	-3.87	orf19.1950		N/A
CTRG_03601	-3.83	orf19.4953		N/A
CTRG_05496	-3.82	N/A		N/A
CTRG_04555	-3.81	orf19.411		N/A
CTRG_02330	-3.81	N/A		N/A
CTRG_03153	-3.80	orf19.5102	PLB5	N/A
CTRG_05559	-3.75	N/A		N/A
CTRG_00920	-3.74	orf19.173		N/A
CTRG_00339	-3.74	orf19.2695	UBR1	N/A

CTRG_00694	-3.73	orf19.6621	MHP1	N/A
CTRG_01119	-3.72	orf19.2079	PHHB	N/A
CTRG_02765	-3.71	orf19.1078	HBR2	N/A
CTRG_01744	-3.69	orf19.2268	RCK2	N/A
CTRG_00779	-3.69	orf19.2401		N/A
CTRG_00290	-3.69	orf19.2703		N/A
CTRG_01553	-3.67	N/A		N/A
CTRG_01527	-3.66	orf19.5204		N/A
CTRG_01411	-3.66	orf19.1534		N/A
CTRG_05342	-3.65	orf19.1976	TRX2	N/A
CTRG_00217	-3.63	N/A		N/A
CTRG_03676	-3.62	N/A		N/A
CTRG_03430	-3.62	orf19.4791		N/A
CTRG_04546	-3.60	orf19.419		N/A
CTRG_06058	-3.58	orf19.3902		Opaque, 53
CTRG_04134	-3.58	orf19.4781	GRP1	N/A
CTRG_05951	-3.56	orf19.7296		N/A
CTRG_01173	-3.56	orf19.899		N/A
CTRG_05523	-3.55	orf19.6680	FGR27	N/A
CTRG_05406	-3.55	orf19.5602	BMT6	N/A
CTRG_03266	-3.53	orf19.4777	DAK2	N/A
CTRG_05015	-3.51	orf19.6482		N/A
CTRG_01725	-3.51	orf19.1890		N/A
CTRG_01594	-3.50	orf19.4506	LYS22	N/A
CTRG_05405	-3.50	orf19.4229		N/A
CTRG_01085	-3.50	orf19.2106		N/A
CTRG_01657	-3.49	orf19.3546	PEX2	N/A
CTRG_00786	-3.49	orf19.2411		N/A
CTRG_01724	-3.47	orf19.1891	41000	N/A
CTRG_01508	-3.46	orf19.4119	SPO72	N/A
CTRG_06061	-3.45	orf19.4251	ZCF22	N/A
CTRG_02328	-3.43	orf19.5862	AFP99	N/A
CTRG_00457	-3.40	orf19.2809	CTN3	N/A
CTRG_01458	-3.29	orf19.3577.1		N/A

Table B6. List of α /opaque-specific genes.

α /Opaque Specific Gene	Relative Fold Change	<i>C. albicans</i> Ortholog	Gene Name	White-opaque fold change in Ca
CTRG_00477	63.53	orf19.1327	RBT1	N/A
CTRG_03527	49.24	N/A		N/A
CTRG_04257	30.36	orf19.1822	UME6	N/A
CTRG_01028	28.12	N/A		N/A
CTRG_00948	15.67	orf19.2520		N/A
CTRG_03837	14.15	orf19.1264	CFL2	White, 34.5
CTRG_00686	13.10	orf19.695	RGS2	N/A
CTRG_00994	13.03	orf19.3267		N/A
CTRG_01025	12.18	orf19.7485		N/A
CTRG_00351	12.09	N/A		N/A
CTRG_01029	11.99	N/A		N/A
CTRG_00350	11.87	orf19.5302	PGA31	N/A
CTRG_01024	11.43	orf19.7486	MRPL6	N/A
CTRG_04380	11.19	orf19.6028	HGC1	Opaque, 4.4
CTRG_00939	9.73	orf19.2532	PRS	N/A
CTRG_06027	9.54	orf19.3938		N/A
CTRG_00970	9.44	orf19.3244		N/A
CTRG_00548	9.34	orf19.639.1		N/A
CTRG_00364	9.04	orf19.484	MRPL40	N/A
CTRG_01164	8.75	orf19.2023	HGT7	White, 8.8
CTRG_00853	8.46	orf19.2573	FRS1	N/A
CTRG_03735	8.37	orf19.2128		N/A
CTRG_00516	8.20	orf19.1738.1		N/A
CTRG_00470	8.06	orf19.3367		N/A
CTRG_00214	7.89	orf19.4616	POL30	N/A
CTRG_00097	7.68	N/A		N/A
CTRG_00921	7.51	orf19.172	RPC19	N/A
CTRG_03454	7.45	orf19.5279		N/A
CTRG_00931	7.45	orf19.2546	TRP2	N/A
CTRG_01061	7.36	orf19.7513	ALK2	Opaque, 5
CTRG_00975	7.30	orf19.3239	CTF18	N/A
CTRG_03165	7.29	orf19.397		N/A
CTRG_01052	7.28	orf19.1143		N/A
CTRG_04519	7.25	orf19.445		N/A
CTRG_04767	7.02	orf19.4459		Opaque, 5.4
CTRG_04802	7.01	orf19.1037		N/A
CTRG_00953	6.92	orf19.3221	CPA2	N/A
Grand	6.89	N/A		N/A

CTRG_02258	6.84	orf19.7386		N/A
CTRG_02890	6.81	orf19.685	YHM1	N/A
CTRG_00822	6.77	orf19.2837	ALG5	Opaque, 3.2
CTRG_03385	6.67	orf19.4837	DAM1	N/A
CTRG_03729	6.61	orf19.2158	NAG3	N/A
CTRG_01012	6.57	orf19.3287	BUD22	N/A
CTRG_05803	6.54	orf19.7631	SLD5	N/A
CTRG_00376	6.49	orf19.496		N/A
CTRG_03509	6.46	orf19.5235		N/A
CTRG_03070	6.42	orf19.6129	MRPL8	N/A
CTRG_02707	6.37	orf19.2654	RMS1	N/A
CTRG_01095	6.33	orf19.2116	NAT2	N/A
CTRG_05511	6.24	orf19.6662		N/A
CTRG_00903	6.20	orf19.3751		N/A
CTRG_00892	6.14	orf19.3753	SEF1	N/A
CTRG_00841	6.12	orf19.163		N/A
CTRG_00104	6.10	orf19.4176		N/A
CTRG_00789	6.08	orf19.2415		N/A
CTRG_02205	5.98	orf19.3777		N/A
CTRG_03215	5.90	orf19.4721		N/A
CTRG_05855	5.88	N/A		N/A
CTRG_02688	5.88	orf19.2639		N/A
CTRG_04132	5.81	orf19.4424	PHO100	N/A
CTRG_03460	5.79	orf19.5271		N/A
CTRG_03448	5.73	orf19.5265	KIP4	N/A
CTRG_04464	5.70	orf19.3357		N/A
CTRG_04108	5.69	orf19.668		N/A
CTRG_00324	5.61	orf19.1303		N/A
CTRG_03118	5.59	orf19.8		N/A
CTRG_00946	5.59	orf19.2524	MGE1	N/A
CTRG_00919	5.59	orf19.175		N/A
CTRG_02976	5.52	orf19.119		N/A
CTRG_00923	5.51	orf19.171	DBP2	N/A
CTRG_01166	5.49	orf19.2019		N/A
CTRG_04138	5.49	N/A		N/A
CTRG_01268	5.48	orf19.1485		N/A
CTRG_03689	5.45	orf19.7219	FTR1	N/A
CTRG_03053	5.43	orf19.6136		N/A
CTRG_05801	5.40	orf19.7634	MCD1	N/A
CTRG_03582	5.37	orf19.4932		N/A
CTRG_00981	5.37	orf19.3250		N/A

CTRG_00419	5.37	orf19.549		N/A
CTRG_05485	5.28	orf19.409		N/A
CTRG_05942	5.27	orf19.7307		N/A
CTRG_04890	5.21	orf19.7019	YML6	N/A
CTRG_00871	5.19	orf19.2594		N/A
CTRG_05917	5.18	orf19.7324	THI13	N/A
CTRG_00170	5.17	orf19.4566		N/A
CTRG_02921	5.15	orf19.3466		N/A
CTRG_01199	5.12	orf19.863		N/A
CTRG_00930	5.05	orf19.2547		N/A
CTRG_05297	5.00	orf19.3202		N/A
CTRG_03301	5.00	orf19.989		N/A
CTRG_03287	5.00	orf19.1835		N/A
CTRG_03811	5.00	orf19.2885	PRI2	N/A
CTRG_03921	4.95	orf19.3818	GOA1	N/A
CTRG_01000	4.92	orf19.3276	PWP2	N/A
CTRG_00880	4.87	orf19.2600	SPC98	N/A
CTRG_03373	4.83	orf19.4853	HCM1	N/A
CTRG_00098	4.82	N/A		N/A
CTRG_03730	4.82	orf19.2160	NAG4	N/A
CTRG_05261	4.78	orf19.947	MRP17	N/A
CTRG_02941	4.75	orf19.3646	CTR1	N/A
CTRG_05688	4.73	orf19.6828		N/A
CTRG_02808	4.70	orf19.3480		N/A
CTRG_03233	4.68	orf19.4739	MSS116	N/A
CTRG_03943	4.68	orf19.3831		N/A
CTRG_04883	4.67	orf19.7012		N/A
CTRG_03590	4.65	orf19.4941	TYE7	N/A
CTRG_04514	4.63	orf19.439		N/A
CTRG_01334	4.60	orf19.5839	PDR17	N/A
CTRG_02247	4.60	orf19.7422		N/A
CTRG_01097	4.59	orf19.2119	NDT80	White, 3.7
CTRG_02769	4.59	orf19.73		N/A
CTRG_00924	4.58	orf19.169	CHO2	N/A
CTRG_01707	4.57	orf19.2214		N/A
CTRG_05603	4.57	orf19.6752		N/A
CTRG_04455	4.56	orf19.3348		N/A
CTRG_00893	4.56	orf19.2610		N/A
CTRG_05877	4.55	N/A		N/A
CTRG_03506	4.55	orf19.5230	MRPS9	N/A
CTRG_02893	4.53	orf19.688		N/A

CTRG_04341	4.51	orf19.2998	TSR2	N/A
CTRG_02907	4.50	orf19.3501		N/A
CTRG_00198	4.50	orf19.4631	ERG251	N/A
CTRG_05483	4.49	orf19.406	ERG1	N/A
CTRG_06159	4.48	orf19.7664		N/A
CTRG_04302	4.48	orf19.6073	HMX1	Opaque, 7.2
CTRG_01026	4.48	orf19.7488		N/A
CTRG_02237	4.46	orf19.7409.1		N/A
CTRG_04781	4.45	orf19.3010		N/A
CTRG_04487	4.44	orf19.760		N/A
CTRG_02757	4.43	orf19.1087		N/A
CTRG_02366	4.41	orf19.1662		N/A
CTRG_00760	4.41	orf19.2382		N/A
CTRG_01754	4.40	orf19.2259		N/A
CTRG_00825	4.39	orf19.2832	INN1	N/A
CTRG_00414	4.37	orf19.542	HXK2	White, 8
CTRG_06234	4.36	orf19.5505	HIS7	N/A
CTRG_03084	4.31	orf19.6417	TSR1	N/A
CTRG_03285	4.31	orf19.2352		N/A
CTRG_03189	4.30	orf19.1109		N/A
CTRG_04800	4.28	orf19.3022		N/A
CTRG_06131	4.27	orf19.4018		N/A
CTRG_00881	4.22	orf19.2601	HEM1	N/A
CTRG_05282	4.21	orf19.923	THR1	N/A
CTRG_04457	4.20	orf19.3350	MRP20	N/A
CTRG_03579	4.19	orf19.4929		N/A
CTRG_03989	4.19	orf19.5064	MRPL3	N/A
CTRG_00144	4.18	orf19.2754		N/A
CTRG_05919	4.16	N/A		N/A
CTRG_04651	4.14	orf19.2934		N/A
CTRG_02801	4.13	orf19.5515		N/A
CTRG_00378	4.12	orf19.498		N/A
CTRG_00979	4.12	orf19.3249	LAG1	N/A
CTRG_00823	4.11	orf19.2834	RPD3	N/A
CTRG_05835	4.10	orf19.7599	UTP5	N/A
CTRG_03362	4.10	orf19.4862.2	PET100	N/A
CTRG_03387	4.10	orf19.4835		N/A
CTRG_04728	4.10	orf19.1065	SSA2	N/A
CTRG_03311	4.09	orf19.4896		N/A
CTRG_05320	4.08	orf19.3171	ACH1	N/A
CTRG_03908	4.08	orf19.4418	FMT1	N/A

CTRG_05166	4.07	orf19.7203		N/A
CTRG_03972	4.07	orf19.1232	VRG4	N/A
CTRG_02731	4.01	orf19.5747		N/A
CTRG_04244	3.99	orf19.6266		N/A
CTRG_04902	3.99	orf19.7065	PSF3	N/A
CTRG_03355	3.99	orf19.4870	DBP3	N/A
CTRG_04960	3.97	orf19.6565		N/A
CTRG_01448	3.97	orf19.828		N/A
CTRG_03971	3.95	orf19.1233	ADE4	N/A
CTRG_00828	3.92	orf19.2829		N/A
CTRG_00962	3.91	orf19.3231	CDC27	N/A
CTRG_00521	3.90	orf19.551		N/A
CTRG_05195	3.89	orf19.7194		N/A
CTRG_03827	3.88	orf19.1277		N/A
CTRG_03691	3.87	orf19.7222	PAM16	N/A
CTRG_04793	3.86	orf19.3034	RLI1	N/A
CTRG_01569	3.86	orf19.805		N/A
CTRG_03864	3.83	orf19.1802		N/A
CTRG_03515	3.83	orf19.5232	CSI2	N/A
CTRG_05887	3.83	orf19.6597		N/A
CTRG_04941	3.82	orf19.7112	FRP2	N/A
CTRG_00322	3.79	orf19.1300		N/A
CTRG_00885	3.77	orf19.2604		N/A
CTRG_03001	3.73	orf19.1201		N/A
CTRG_04100	3.71	orf19.657	SAM2	N/A
CTRG_00935	3.71	orf19.2541		N/A
CTRG_03178	3.68	orf19.385	GCV2	N/A
CTRG_02651	3.68	orf19.1988	CDC45	N/A
CTRG_03425	3.66	orf19.4796		N/A
CTRG_05235	3.60	orf19.5684		N/A

Table B7. List of α genes down regulated in the white state relative to Δ/Δ *wor1* mutants.

<i>Gene ID</i>	<i>Fold Change KO / W</i>	<i>C. albicans Ortholog</i>	<i>Gene Name</i>
CTRG_00036	2.37	orf19.4670	CAS5
CTRG_00401	1.82	orf19.526	NHP2
CTRG_00500	3.60	orf19.2724	
CTRG_00521	3.82	orf19.551	
CTRG_00686	3.66	orf19.695	RGS2
CTRG_00921	1.89	orf19.172	RPC19
CTRG_00923	1.95	orf19.171	DBP2
CTRG_00970	1.76	orf19.3244	
CTRG_01171	2.22	orf19.897	VPS20
CTRG_01980	1.76	orf19.1360	
CTRG_02122	1.60	orf19.5628	
CTRG_02651	1.91	orf19.1988	CDC45
CTRG_02660	1.78	orf19.3710	YHB5
CTRG_02702	2.87	orf19.2649	PCL1
CTRG_02941	1.85	orf19.3646	CTR1
CTRG_03165	2.07	orf19.397	
CTRG_03859	3.04	orf19.1239	
CTRG_04492	1.93	orf19.755	MRPL37
CTRG_04504	1.63	orf19.58	RRP6
CTRG_04757	1.97	orf19.4479	
CTRG_04798	1.75	orf19.3026	MAS1
CTRG_05067	1.79	orf19.7050	NOP15
CTRG_05261	1.57	orf19.947	MRP17
CTRG_05262	1.72	orf19.946	MET14
CTRG_05403	1.60	orf19.4553	
CTRG_05878	1.66	orf19.7552	
CTRG_05981	1.90	orf19.4299	MSW1
CTRG_06027	1.67	orf19.3938	

Table B8. List of α genes up regulated in the white state relative to Δ/Δ *wor1* mutants.

<i>Gene ID</i>	<i>Fold Change - KO / W</i>	<i>C. albicans Ortholog</i>	<i>Gene Name</i>
CTRG_00038	0.34		
CTRG_00077	0.21		
CTRG_00289	0.42		
CTRG_00296	0.27		
CTRG_00330	0.42		
CTRG_00368	0.45		
CTRG_00379	0.54	orf19.499	
CTRG_00409	0.41		
CTRG_00457	0.52	orf19.289	CTN3
CTRG_00478	0.53	orf19.1321	HWP1
CTRG_00508	0.37	orf19.2671	
CTRG_00569	0.50	orf19.5288	IFE2
CTRG_00628	0.48	orf19.3871	DAD3
CTRG_00656	0.59	orf19.457	UBP2
CTRG_00658	0.62	orf19.459	ADP1
CTRG_00811	0.39		
CTRG_00858	0.37	orf19.2581	
CTRG_00863	0.27		
CTRG_00882	0.61	orf19.268	ADH5
CTRG_00915	0.62	orf19.3742	
CTRG_01001	0.60	orf19.3278	GSY1
CTRG_01002	0.65	orf19.3283	OAR1
CTRG_01142	0.38	orf19.246	POT1-2
CTRG_01170	0.29		
CTRG_01207	0.29	orf19.855	
CTRG_01497	0.46	orf19.415	
CTRG_01692	0.31		
CTRG_01878	0.52	orf19.492	
CTRG_01904	0.48		
CTRG_01949	0.49	orf19.368	MSH3
CTRG_01984	0.39		
CTRG_02003	0.45	orf19.1382	YEA4
CTRG_02102	0.52	orf19.5615	AYR2
CTRG_02376	0.54	orf19.1653	
CTRG_02420	0.38		
CTRG_02528	0.55	orf19.5974	ATG9
CTRG_02532	0.31	orf19.5978	FZD6

CTRG_02545	0.43	orf19.258	
CTRG_02550	0.52	orf19.271	ADH4
CTRG_02613	0.24		
CTRG_02661	0.51	orf19.3487	
CTRG_02690	0.36	orf19.2641	ARP1
CTRG_02802	0.63	orf19.5514	
CTRG_02926	0.57		
CTRG_03057	0.52	orf19.6133	PIF1
CTRG_03098	0.55	orf19.6431	
CTRG_03207	0.39		
CTRG_03235	0.52	orf19.4742	
CTRG_03307	0.45	orf19.488	YFW5
CTRG_03323	0.29	orf19.491	
CTRG_03345	0.39	orf19.4884	WOR1
CTRG_03352	0.41	orf19.4873	
CTRG_03398	0.30		
CTRG_03492	0.31	orf19.1167	TCD1
CTRG_03557	0.49	orf19.6351	
CTRG_03602	0.44		
CTRG_03688	0.18		
CTRG_03751	0.49	orf19.3113	
CTRG_03922	0.39	orf19.3813	
CTRG_04133	0.56	orf19.478	
CTRG_04166	0.40		
CTRG_04167	0.51		
CTRG_04261	0.39		
CTRG_04421	0.41	orf19.338	STB5
CTRG_04478	0.61	orf19.769	IFE1
CTRG_04584	0.63		
CTRG_04602	0.38	orf19.4526	HSP3
CTRG_04816	0.44	orf19.2481	
CTRG_04884	0.38	orf19.713	
CTRG_04904	0.18		
CTRG_05026	0.62	orf19.6476	
CTRG_05172	0.47	orf19.653	
CTRG_05368	0.41	orf19.1955	SHR5
CTRG_05451	0.36		
CTRG_05697	0.30	orf19.7456	
CTRG_05845	0.41		
CTRG_05944	0.58		
CTRG_05990	0.59	orf19.4286	

Table B9. List of α genes down regulated in the opaque state relative to *WOR1* overexpressing strains.

<i>Gene ID</i>	<i>Fold Change – KO/O</i>	<i>C. albicans ortholog</i>	<i>Gene Name</i>
CTRG_00015	2.33	orf19.5643	ECM7
CTRG_00016	9.89	orf19.5645	MET15
CTRG_00017	2.21	orf19.4580	
CTRG_00021	2.48	orf19.4577	
CTRG_00029	2.87	orf19.4665	
CTRG_00042	3.65	orf19.4682	HGT17
CTRG_00056	2.79	orf19.4698	PTC8
CTRG_00062	3.38	orf19.4706	
CTRG_00073	2.73		
CTRG_00074	2.40	orf19.4167	
CTRG_00090	4.40		
CTRG_00093	2.28	orf19.4182	
CTRG_00094	2.31	orf19.4183	MUC1
CTRG_00111	3.72	orf19.5633	
CTRG_00117	2.69	orf19.367	CNH1
CTRG_00126	4.11	orf19.5651	
CTRG_00142	3.06	orf19.2762	AHP1
CTRG_00156	2.82	orf19.2768	AMS1
CTRG_00158	3.98	orf19.2770	
CTRG_00164	2.35	orf19.2772	HOS3
CTRG_00182	2.47	orf19.4649	ZCF27
CTRG_00183	3.17	orf19.4647	HAP3
CTRG_00200	3.02	orf19.4629	
CTRG_00211	3.13	orf19.4618	FBA1
CTRG_00213	2.71	orf19.4617	
CTRG_00216	3.55		
CTRG_00217	2.25		
CTRG_00231	2.88	orf19.4607	
CTRG_00238	3.05	orf19.4595	
CTRG_00242	2.15	orf19.4592	HSX11
CTRG_00248	2.59	orf19.2731	
CTRG_00254	8.41	orf19.2737	
CTRG_00255	43.93	orf19.2738	SUL2
CTRG_00258	2.44		
CTRG_00265	38.74		
CTRG_00267	75.70		
CTRG_00268	41.83		

CTRG_00280	3.21		
CTRG_00284	3.68	orf19.2693	GST2
CTRG_00285	7.36	orf19.2691	
CTRG_00286	21.15	orf19.1313	CDR3
CTRG_00290	3.46	orf19.2703	
CTRG_00300	6.56		
CTRG_00301	2.88	orf19.5307	JEN2
CTRG_00303	3.40	orf19.5311	
CTRG_00336	3.34		
CTRG_00345	2.14	orf19.1317	OSH3
CTRG_00356	6.55	orf19.473	TP04
CTRG_00368	2.31		
CTRG_00383	2.36	orf19.505	SRV2
CTRG_00394	6.57	orf19.539	LAP3
CTRG_00402	2.93	orf19.524	
CTRG_00406	6.82		
CTRG_00407	4.97	orf19.532	RBR2
CTRG_00413	2.38	orf19.541	
CTRG_00422	3.48	orf19.606	
CTRG_00424	3.54	orf19.334	
CTRG_00432	3.41	orf19.2681	RBT7
CTRG_00434	2.17	orf19.3799	
CTRG_00458	2.65	orf19.2810	AAP1
CTRG_00461	3.92		
CTRG_00463	5.24		
CTRG_00464	4.82		
CTRG_00467	6.17	orf19.335	
CTRG_00468	9.60		
CTRG_00471	9.21	orf19.3369	MOH1
CTRG_00488	2.59	orf19.5295	
CTRG_00506	4.58	orf19.3364	
CTRG_00512	23.06	orf19.552	
CTRG_00513	3.47	orf19.553	
CTRG_00515	12.01	orf19.1741	DIT1
CTRG_00517	2.71	orf19.1738	UGP1
CTRG_00533	2.36	orf19.6305	
CTRG_00537	2.37	orf19.633	
CTRG_00557	2.51	orf19.649	
CTRG_00566	3.38	orf19.1010	MNT3
CTRG_00577	2.50	orf19.5852	
CTRG_00578	2.26	orf19.3519	SUA72

CTRG_00583	4.98	orf19.3515	
CTRG_00584	2.66		
CTRG_00590	7.08	orf19.5843	SRR1
CTRG_00593	2.60	orf19.6650	
CTRG_00597	3.25	orf19.6643	MCT1
CTRG_00601	3.06	orf19.3888	PGI1
CTRG_00604	3.21	orf19.3885	
CTRG_00606	2.61	orf19.726	PPZ1
CTRG_00608	3.58	orf19.723	BCR1
CTRG_00611	2.75	orf19.719	
CTRG_00634	2.44	orf19.6624	
CTRG_00635	2.80	orf19.6625	
CTRG_00638	3.78		
CTRG_00649	2.68	orf19.6640	TPS1
CTRG_00655	3.49	orf19.454	SFL1
CTRG_00672	2.17	orf19.1857	
CTRG_00673	2.79	orf19.1856	
CTRG_00689	4.00		
CTRG_00691	4.34		
CTRG_00694	3.17	orf19.6621	MHP1
CTRG_00722	5.84	orf19.4372	
CTRG_00723	15.08		
CTRG_00728	2.10	orf19.4369	
CTRG_00731	66.45	orf19.4366	
CTRG_00733	3.76	orf19.4365	
CTRG_00736	2.13	orf19.4381	VTC3
CTRG_00740	2.60		
CTRG_00741	4.86	orf19.4384	HXT5
CTRG_00743	2.01	orf19.4400	
CTRG_00749	3.52	orf19.4391	
CTRG_00752	4.74		
CTRG_00753	2.36	orf19.4362	
CTRG_00775	2.31	orf19.2397. 3	
CTRG_00860	17.82		
CTRG_00868	2.92		
CTRG_00874	5.37	orf19.2583	
CTRG_00914	4.94	orf19.3740	PGA23
CTRG_00996	2.57	orf19.3269	GSL2
CTRG_01020	2.16	orf19.7479	NTH1
CTRG_01048	15.06	orf19.7502	
CTRG_01107	2.24	orf19.2094	PDR6

CTRG_01119	5.09	orf19.2079	PHHB
CTRG_01123	2.48	orf19.2076	
CTRG_01125	6.66		
CTRG_01127	2.53	orf19.2069	SMF3
CTRG_01131	5.32	orf19.2065	
CTRG_01149	2.19	orf19.2040	
CTRG_01154	4.54	orf19.2036	
CTRG_01175	4.95	orf19.903	GPM1
CTRG_01176	2.48	orf19.904	
CTRG_01180	3.02	orf19.896	CHK1
CTRG_01181	2.82	orf19.895	HOG1
CTRG_01187	2.43	orf19.882	HSP78
CTRG_01189	2.55	orf19.880	
CTRG_01196	2.66	orf19.872	
CTRG_01208	13.23	orf19.849	
CTRG_01210	4.86	orf19.851	
CTRG_01211	2.44	orf19.860	BMT8
CTRG_01212	9.52	orf19.856	IFK2
CTRG_01214	2.60	orf19.1994	
CTRG_01216	2.84	orf19.1996	CHA1
CTRG_01218	2.80	orf19.2000	
CTRG_01226	2.38	orf19.1991	
CTRG_01227	2.29	orf19.1990	SNX4
CTRG_01228	2.52	orf19.1989	DCW1
CTRG_01230	2.06	orf19.1442	PLB4.5
CTRG_01231	2.67	orf19.1444	
CTRG_01246	2.25	orf19.1462	SMP2
CTRG_01251	2.50	orf19.1468	
CTRG_01255	2.02	orf19.1474	SLA1
CTRG_01256	3.02	orf19.1476	
CTRG_01270	5.24		
CTRG_01272	2.18	orf19.1490	MSB2
CTRG_01279	2.07	orf19.1496	
CTRG_01280	2.84	orf19.1499	CTF1
CTRG_01283	2.63	orf19.846	
CTRG_01291	2.63	orf19.2005	REG1
CTRG_01305	2.09	orf19.909	STP4
CTRG_01321	3.67	orf19.5797	PLC2
CTRG_01323	3.36		
CTRG_01358	2.87	orf19.5821	
CTRG_01360	2.33	orf19.5818	SUR2

CTRG_01366	2.01	orf19.5812	
CTRG_01435	3.23	orf19.847	YIM1
CTRG_01443	5.10	orf19.822	HSP21
CTRG_01460	2.26	orf19.3575	CDC19
CTRG_01474	2.31	orf19.3563	
CTRG_01492	2.43		
CTRG_01493	2.48		
CTRG_01522	3.55	orf19.5210	
CTRG_01526	2.31	orf19.5205	
CTRG_01539	4.75		
CTRG_01541	2.03	orf19.6877	PNG2
CTRG_01550	2.54		
CTRG_01602	2.22	orf19.4496	
CTRG_01603	1.90	orf19.4495	NDH51
CTRG_01630	1.97	orf19.4127	
CTRG_01656	2.40	orf19.3537	
CTRG_01736	2.02	orf19.2277	TPK2
CTRG_01774	4.93	orf19.23	RTA3
CTRG_01824	2.16	orf19.1350	
CTRG_01827	3.62		
CTRG_01828	4.93		
CTRG_01829	3.54	orf19.1349	
CTRG_01852	62.85		
CTRG_01861	3.29	orf19.3612	PST2
CTRG_01885	3.29	orf19.2242	PRB1
CTRG_01901	4.91	orf19.2237	SPR1
CTRG_01908	2.60	orf19.35	
CTRG_01911	1.99		
CTRG_01913	2.16	orf19.28	
CTRG_01918	2.22	orf19.4084	KIS1
CTRG_01921	11.40		
CTRG_01923	13.68		
CTRG_01924	2.20	orf19.4072	IFF6
CTRG_01925	3.38	orf19.4076	MET10
CTRG_01926	3.45		
CTRG_01952	2.34	orf19.220	PIR1
CTRG_01955	2.59	orf19.215	
CTRG_01991	2.60	orf19.1368	
CTRG_02002	2.23	orf19.1383	
CTRG_02034	2.78	orf19.1751	SPT23
CTRG_02036	2.50	orf19.5365	

CTRG_02048	3.15	orf19.5348	TPS3
CTRG_02053	2.18	orf19.5342	
CTRG_02064	2.86	orf19.1760	RAS1
CTRG_02092	2.01	orf19.1777	
CTRG_02101	4.23	orf19.6793	
CTRG_02106	31.25	orf19.5625	
CTRG_02108	3.48	orf19.5622	GLC3
CTRG_02120	2.08	orf19.5630	APA2
CTRG_02152	2.10	orf19.1684	
CTRG_02153	2.04	orf19.1686	
CTRG_02161	2.48	orf19.1693	CAS4
CTRG_02184	3.31	orf19.1728	
CTRG_02186	2.20		
CTRG_02259	2.36	orf19.7385	
CTRG_02277	3.33		
CTRG_02298	2.96	orf19.5902	RAS2
CTRG_02416	2.28	orf19.1607	ALR1
CTRG_02420	4.82		
CTRG_02484	7.73		
CTRG_02521	4.53	orf19.5992	WOR2
CTRG_02602	3.40	orf19.342	BMT7
CTRG_02662	2.21	orf19.6282	
CTRG_02721	2.71	orf19.5723	POX1
CTRG_02768	2.95	orf19.1075	
CTRG_02869	2.44	orf19.929	
CTRG_02898	2.83	orf19.691	GPD2
CTRG_02904	3.64		
CTRG_03013	2.43	orf19.1189	
CTRG_03134	2.46	orf19.2356	CRZ2
CTRG_03253	3.37	orf19.4752	MSN4
CTRG_03273	2.56		
CTRG_03296	3.90	orf19.1830	
CTRG_03345	18.90	orf19.4884	WOR1
CTRG_03430	2.51	orf19.4791	
CTRG_03601	2.42	orf19.4953	
CTRG_03602	3.06		
CTRG_03611	4.25		
CTRG_03621	5.22	orf19.4972	
CTRG_03661	28.97	orf19.5025	MET3
CTRG_03688	3.61		
CTRG_03752	3.14	orf19.3112	ZRT1

CTRG_03859	3.76	orf19.1239	
CTRG_03883	3.08	orf19.3794	CSR1
CTRG_03886	5.79	orf19.3793	
CTRG_03910	2.45	orf19.2593	BIO2
CTRG_03969	4.04	orf19.6196	
CTRG_04040	8.37	orf19.2903	AG01
CTRG_04092	2.21	orf19.2308	
CTRG_04116	3.67	orf19.675	
CTRG_04122	2.68	orf19.2296	
CTRG_04184	2.70		
CTRG_04234	1.88	orf19.6257	GLT1
CTRG_04235	1.89		
CTRG_04259	4.73	orf19.6277	
CTRG_04334	2.78	orf19.2990	XOG1
CTRG_04391	6.36	orf19.6017	
CTRG_04483	5.43	orf19.752	
CTRG_04601	2.03	orf19.4528	
CTRG_04602	6.02	orf19.4526	HSP30
CTRG_04613	3.51	orf19.3664	HSP31
CTRG_04658	3.79		
CTRG_04725	2.37	orf19.1067	GPM2
CTRG_04744	3.01	orf19.1048	IFD6
CTRG_04745	7.84		
CTRG_04755	6.15	orf19.4481	MFALPHA
CTRG_04839	3.10	orf19.2454	PHO87
CTRG_04840	2.22	orf19.2452	
CTRG_04841	3.51	orf19.2451	PGA45
CTRG_04901	2.35	orf19.7032	
CTRG_04919	6.50	orf19.7085	
CTRG_04926	2.25	orf19.7078	
CTRG_04964	2.41	orf19.6570	NUP
CTRG_04975	2.89	orf19.7140	
CTRG_04977	3.91	orf19.7148	TPO2
CTRG_05015	2.88	orf19.6482	
CTRG_05034	2.57	orf19.6544	LPI9
CTRG_05042	3.45	orf19.6489	FMP45
CTRG_05063	2.76	orf19.7053	GAC1
CTRG_05065	3.30		
CTRG_05110	3.67		
CTRG_05115	2.73	orf19.6540	PFK2
CTRG_05131	4.10	orf19.1344	

CTRG_05150	2.64	orf19.6684	PNC1
CTRG_05164	3.18	orf19.7199	
CTRG_05262	8.13	orf19.946	MET14
CTRG_05331	4.07	orf19.1964	
CTRG_05372	3.22	orf19.1950	
CTRG_05378	2.03	orf19.1944	GPR1
CTRG_05438	2.00	orf19.6656	DUR3
CTRG_05441	2.72	orf19.6654	
CTRG_05583	2.43	orf19.6717	
CTRG_05752	3.88	orf19.5614	
CTRG_05762	2.57	orf19.5552	
CTRG_05766	3.78	orf19.5557	MNN4-4
CTRG_05796	3.11	orf19.7676	XYL2
CTRG_05862	2.22		
CTRG_06050	2.99	orf19.3910	
CTRG_06054	5.83		
CTRG_06058	11.96	orf19.3902	
CTRG_06083	3.74		
CTRG_06094	2.16	orf19.3967	PFK1
CTRG_06100	2.55		
CTRG_06101	2.20		
CTRG_06185	2.53	orf19.5426	
CTRG_06206	3.41	orf19.6996	
CTRG_06224	2.22	orf19.1331	HSM3
CTRG_06229	1.99		

Table B10. List of α genes up regulated in the opaque state relative to *WOR1* overexpressing strains.

<i>Gene ID</i>	<i>Fold Change - KO/O</i>	<i>C. albicans Ortholog</i>	<i>Gene Name</i>
CTRG_00233	0.16		
CTRG_00299	0.22	orf19.5305	RHD3
CTRG_00339	0.28	orf19.2695	UBR1
CTRG_00350	0.29	orf19.5302	PGA31
CTRG_00351	0.30		
CTRG_00477	0.09	orf19.1327	RBT1
CTRG_00478	0.32	orf19.1321	HWP1
CTRG_00660	0.29	orf19.3131	OYE32
CTRG_00662	0.14		
CTRG_00680	0.21	orf19.1847	ARO10
CTRG_00686	0.24	orf19.695	RGS2
CTRG_00711	0.15	orf19.467	
CTRG_00725	0.15		
CTRG_00822	0.35	orf19.2837	ALG5
CTRG_00828	0.36	orf19.2829	
CTRG_00841	0.30	orf19.163	
CTRG_00842	0.38	orf19.164	
CTRG_00860	0.35		
CTRG_00881	0.38	orf19.2601	HEM1
CTRG_00908	0.24	orf19.3732	ERG25
CTRG_00919	0.44	orf19.175	
CTRG_00924	0.34	orf19.169	CHO2
CTRG_00939	0.43	orf19.2532	PRS
CTRG_00953	0.19	orf19.3221	CPA2
CTRG_00962	0.30	orf19.3231	CDC27
CTRG_00986	0.48	orf19.3259	
CTRG_00989	0.24	orf19.3266	
CTRG_00993	0.45	orf19.3282	BMT3
CTRG_00997	0.37	orf19.3272	
CTRG_01021	0.25	orf19.7481	MDH1
CTRG_01024	0.35	orf19.7486	MRPL6
CTRG_01025	0.33	orf19.7485	
CTRG_01028	0.05		
CTRG_01029	0.21		
CTRG_01041	0.35		
CTRG_01052	0.42	orf19.1143	
CTRG_01070	0.10	orf19.7522	

CTRG_01071	0.24	orf19.7523	MKC1
CTRG_01076	0.31	orf19.1923	RRN3
CTRG_01113	0.39	orf19.2084	CDH1
CTRG_01164	0.14	orf19.2023	HGT7
CTRG_01206	0.10	orf19.854	UGA11
CTRG_01207	0.34	orf19.855	
CTRG_01290	0.41		
CTRG_01775	0.37	orf19.24	RTA2
CTRG_01788	0.35		
CTRG_01968	0.40	orf19.204	
CTRG_02178	0.38	orf19.1719	SGA1
CTRG_02193	0.11		
CTRG_02237	0.42	orf19.7409. 1	
CTRG_02283	0.26		
CTRG_02374	0.27	orf19.1655	PXP2
CTRG_02398	0.46	orf19.1623	CAP1
CTRG_02464	0.29		
CTRG_02480	0.44	orf19.5921	
CTRG_02481	0.47	orf19.5924	ZCF31
CTRG_02529	0.42	orf19.5975	TRY4
CTRG_02561	0.24	orf19.276	
CTRG_02659	0.45	orf19.3707	YHB1
CTRG_02711	0.20		
CTRG_02734	0.36	orf19.5586	
CTRG_02834	0.37	orf19.3442	
CTRG_02836	0.42	orf19.3443	OYE2
CTRG_02846	0.23	orf19.3432	
CTRG_02917	0.41	orf19.3460	
CTRG_02972	0.38	orf19.111	CAN2
CTRG_03070	0.39	orf19.6129	MRPL8
CTRG_03121	0.28	orf19.11	
CTRG_03123	0.45	orf19.6146	CLG1
CTRG_03165	0.46	orf19.397	
CTRG_03215	0.33	orf19.4721	
CTRG_03231	0.34	orf19.4737	TPO3
CTRG_03257	0.47	orf19.5094	BUL1
CTRG_03270	0.30	orf19.4774	AOX1
CTRG_03299	0.37		
CTRG_03338	0.44	orf19.986	GLY1
CTRG_03401	0.40		
CTRG_03403	0.37		

CTRG_03418	0.44	orf19.4802	FTH1
CTRG_03419	0.40	orf19.6888	
CTRG_03420	0.39	orf19.4795	
CTRG_03425	0.50	orf19.4796	
CTRG_03452	0.35	orf19.5269	
CTRG_03454	0.35	orf19.5279	
CTRG_03475	0.24	orf19.1150	
CTRG_03514	0.39	orf19.5234	RBD1
CTRG_03527	0.06		
CTRG_03548	0.49	orf19.6357	
CTRG_03553	0.51	orf19.3755	
CTRG_03676	0.29		
CTRG_03683	0.26		
CTRG_03729	0.26	orf19.2158	NAG3
CTRG_03787	0.32		
CTRG_03791	0.25		
CTRG_03797	0.34		
CTRG_03827	0.43	orf19.1277	
CTRG_03837	0.18	orf19.1264	CFL2
CTRG_03840	0.38	orf19.1267. 1	
CTRG_03950	0.15	orf19.742	ALD6
CTRG_04031	0.12	orf19.2496	ATO2
CTRG_04130	0.40		
CTRG_04135	0.40	orf19.4783	
CTRG_04144	0.32	orf19.5070	
CTRG_04257	0.29	orf19.1822	UME6
CTRG_04293	0.43	orf19.6084	
CTRG_04294	0.36	orf19.6083	
CTRG_04304	0.34		
CTRG_04354	0.28	orf19.4551	CTN1
CTRG_04376	0.32		
CTRG_04464	0.43	orf19.3357	
CTRG_04471	0.47	orf19.6066	
CTRG_04545	0.28	orf19.422	SPT20
CTRG_04587	0.42	orf19.4543	UGA2
CTRG_04615	0.07	orf19.3668	HGT2
CTRG_04617	0.35	orf19.3670	GAL1
CTRG_04702	0.33	orf19.6844	ICL1
CTRG_04802	0.36	orf19.1037	
CTRG_04803	0.35	orf19.3040	EHT1
CTRG_04905	0.23		

CTRG_04927	0.34	orf19.7094	HGT12
CTRG_04939	0.30		
CTRG_04941	0.35	orf19.7112	FRP2
CTRG_05165	0.32	orf19.7200	
CTRG_05306	0.34	orf19.3190	HAL9
CTRG_05365	0.22		
CTRG_05388	0.22	orf19.5565	
CTRG_05482	0.38	orf19.5113	ADH2
CTRG_05511	0.23	orf19.6662	
CTRG_05581	0.40	orf19.6713	
CTRG_05629	0.42		
CTRG_05630	0.06	orf19.6782	BMT1
CTRG_05631	0.13		
CTRG_05664	0.35	orf19.6817	FCR1
CTRG_05708	0.37	orf19.7446	OPI3
CTRG_05760	0.30		
CTRG_05791	0.25	orf19.7668	MAL2
CTRG_05827	0.16	orf19.7586	CHT3
CTRG_05830	0.44	orf19.7593	
CTRG_05834	0.49	orf19.7598	
CTRG_05855	0.14		
CTRG_05877	0.37		
CTRG_05887	0.40	orf19.6597	
CTRG_05890	0.30	orf19.6592	
CTRG_05908	0.43	orf19.6606	
CTRG_05917	0.22	orf19.7324	THI13
CTRG_05926	0.44	orf19.7337	
CTRG_05928	0.31		
CTRG_06030	0.50	orf19.3934	CAR1
CTRG_06032	0.48	orf19.3931	SFC1
CTRG_06039	0.36	orf19.3925	
CTRG_06158	0.30		
CTRG_06203	0.38	orf19.6993	GAP2
CTRG_06232	0.43		
CTRG_06249	0.37		
CTRG_06250	0.24		
CTRG_06253	0.47	orf19.6921	
Grand	0.31		

Table B11. *a2* and $\alpha 1$ genes are essential for *a* and α cell mating, respectively. Mating frequency was quantified for wild-type opaque strains and two independent isolates of each mutant. N.D. indicates no mating was detected in these crosses.

	$\mathbf{a} \times \alpha$	$\mathbf{a} \times \mathbf{a}/\alpha$	$\alpha \times \mathbf{a}/\alpha$
WT Opaque	0.18	2.78×10^{-6}	2.56×10^{-6}
$\Delta \mathbf{a2}$ in $\mathbf{a}$	1.16×10^{-7}	N.D.	---
$\Delta \alpha 1$ in α	3.42×10^{-7}	---	N.D.
$\Delta \mathbf{a2}$ in $\mathbf{a}/\alpha$	---	6.59×10^{-6}	N.D.
$\Delta \alpha 1$ in $\mathbf{a}/\alpha$	---	N.D.	4.54×10^{-6}

B.4 References

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2. Butler G, Rasmussen MD, Lin MF, Santos MA, Sakthikumar S, et al. (2009) Evolution of pathogenicity and sexual reproduction in eight *Candida* genomes. *Nature* 459: 657-662.
3. Tsong AE, Miller MG, Raisner RM, Johnson AD (2003) Evolution of a combinatorial transcriptional circuit: a case study in yeasts. *Cell* 115: 389-399.
4. Porman AM, Alby K, Hirakawa MP, Bennett RJ (2011) Discovery of a phenotypic switch regulating sexual mating in the opportunistic fungal pathogen *Candida tropicalis*. *Proc Natl Acad Sci U S A* 108: 21158-21163.

**Appendix C: Supplemental Information for “Transcriptional
Regulation of a White-Intermediate-Opaque Tristable Switch in
Candida tropicalis”**

C.1 Supplemental Figures

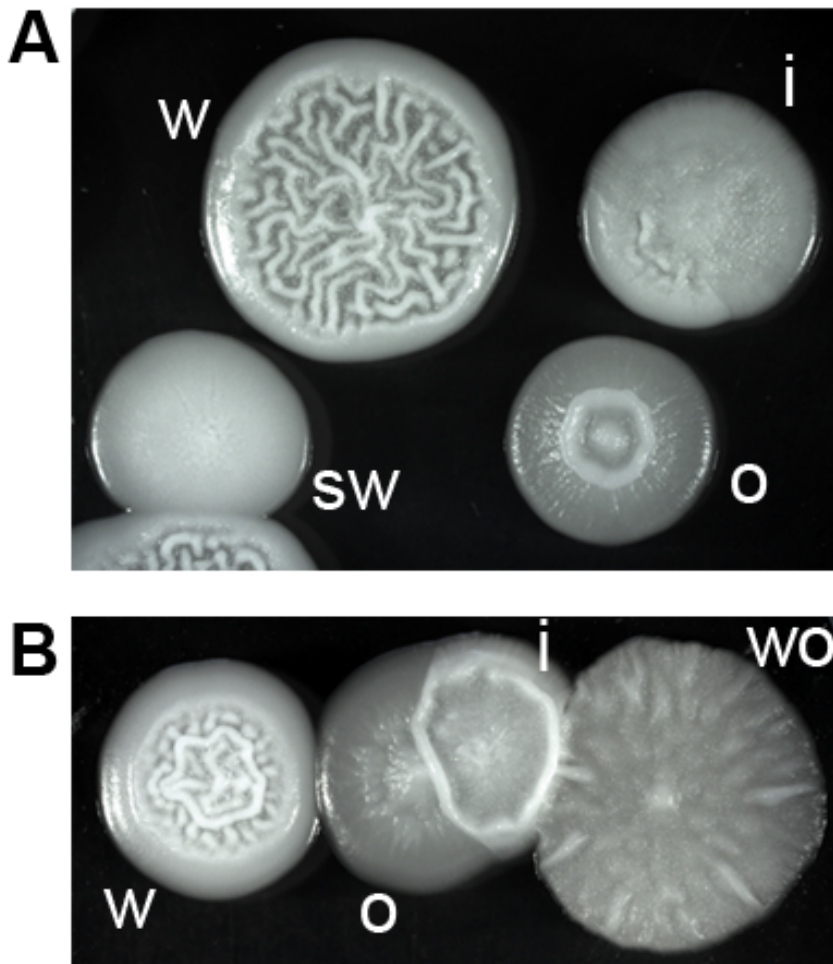


Figure C1. Colony morphology of the white, opaque, and intermediate states. (A) Colony morphology of white (w), smooth white (sw), opaque (o), and intermediate (i) colonies from *MTLa/a* strain CAY1503 (CAY3371, CAY3372, CAY3373, CAY3381, respectively). (B) Colony morphology of white (w), opaque (o), intermediate (i), and wrinkled opaque (wo) phenotypes from *MTLa/a* strain CAY1505 (CAY3051, CAY3053, CAY3393, and CAY6023, respectively). Colonies were grown at 30°C for 7 days on Spider media.

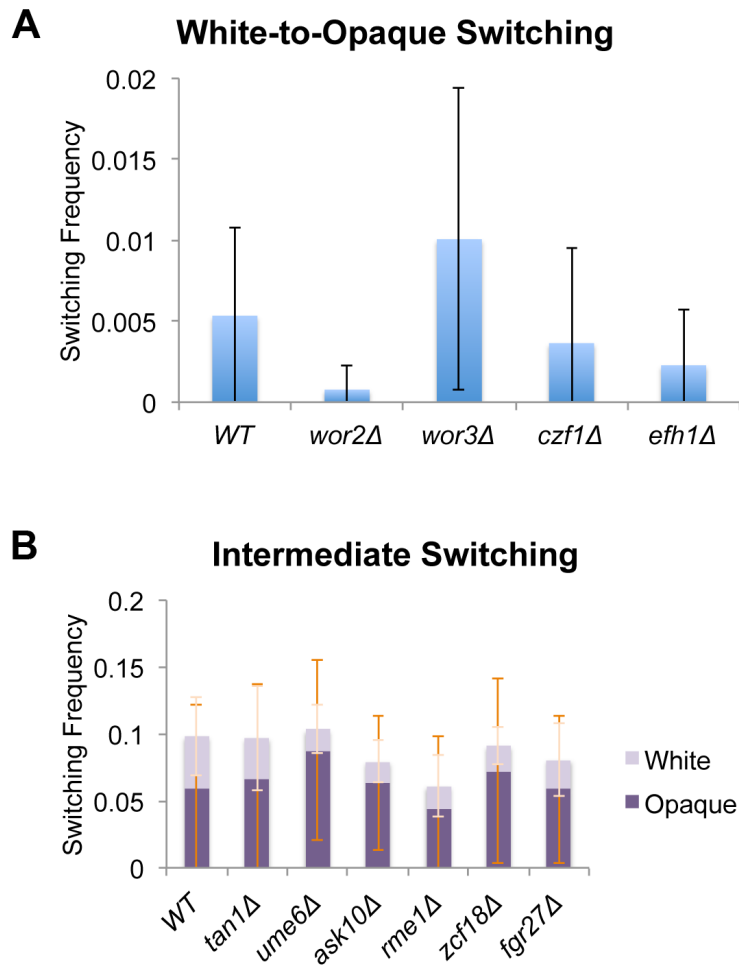


Figure C2. Switching frequencies of *C. tropicalis* deletion mutants.
(A) White-to-opaque switching rates of mutants lacking *C. albicans* white-opaque regulators. **(B)** Intermediate-to-opaque and intermediate-to-white switching rates of mutants lacking differentially expressed transcription factors from *C. tropicalis*.

C.2 Supplemental Tables

Table C1. Global comparison of gene expression in white, opaque, and intermediate states. log(fold change) and corrected P-values are reported for each pairwise comparison. Transcripts per million are also reported for each of two replicates on white cells (W), opaque cells (O), and intermediate cells (I).

Data posted online in an Excel file:

<https://onedrive.live.com/redir?resid=34AB4D33156B1437!115&authkey=!AFtND0NBzM0bfUA&ithint=file%2c.xlsx>

Table C2. White-specific gene list.

<i>Gene</i>	<i>Gene Name</i>	<i>Ca Ortholog</i>	<i>logFC Op</i>	<i>logFC Int</i>
CTRG_00882	ADH5	orf19.2608	6.969320166	3.534535202
CTRG_05127			6.512769079	5.042171723
CTRG_01821	UCF1	orf19.1354	5.815641465	2.791070601
CTRG_03962		orf19.4450.1	5.490694416	3.081501124
CTRG_03991			5.440079197	5.133125896
CTRG_00749		orf19.4391	5.107272832	2.327127861
CTRG_01386			4.796607962	4.48960724
CTRG_06232			4.710927466	3.471147471
CTRG_02376		orf19.1653	4.610610184	3.145284721
CTRG_00580			4.585756836	3.482960698
CTRG_04602	HSP30	orf19.4526	4.567208698	3.303059275
CTRG_04816		orf19.2481	4.530183359	2.70348503
CTRG_02917		orf19.3460	4.523983421	2.761531147
CTRG_02844	TRY5	orf19.3434	4.505136087	2.398730593
CTRG_06081	MAL31	orf19.3981	4.499977463	1.670250804
CTRG_05940		orf19.7310	4.449176571	2.159398603
CTRG_02835	FRP6	orf19.3441	4.277862419	2.254723539
CTRG_05162			4.093542375	2.625405207
CTRG_05482	ADH2	orf19.5113	3.927083235	2.460038808
CTRG_00604		orf19.3885	3.926980034	2.933297982
CTRG_04307	ENA2	orf19.6070	3.861415732	2.482052446
CTRG_00569	IFE2	orf19.5288	3.842768737	2.69225289
CTRG_00457	CTN3	orf19.2809	3.832563406	2.16061927
CTRG_02659	YHB1	orf19.3707	3.734010129	2.508285624
CTRG_04184			3.731098481	2.43469379
CTRG_02662		orf19.6282	3.70474051	2.524332265
CTRG_03258	ASR1	orf19.2344	3.650393145	3.776342446
CTRG_01691		orf19.1862	3.642961193	1.776610424
CTRG_02701	BUB3	orf19.2655	3.611034632	2.028265488
CTRG_00142	AHP1	orf19.2762	3.579483841	2.435354347
CTRG_01885	PRB1	orf19.2242	3.577949513	1.709615567
CTRG_00040	AGP2	orf19.4679	3.553822457	2.198128298
CTRG_01062	PCK1	orf19.7514	3.550285275	1.891180197
CTRG_02708			3.504424611	1.859650321
CTRG_05042	FMP45	orf19.6489	3.475423647	2.180539538
CTRG_02611	HEM13	orf19.2803	3.46337866	2.767906011
CTRG_05065			3.457429613	3.310510516
CTRG_01536	OSM1	orf19.6882	3.45056274	2.75150751

CTRG_01777			3.403677182	2.695009194
CTRG_02539	RSN1	orf19.347	3.377015253	1.928968044
CTRG_01581		orf19.792	3.374622958	1.772159619
CTRG_02765	HBR2	orf19.1078	3.35337076	1.819880618
CTRG_04359	ASM3	orf19.6037	3.339645388	1.874789728
CTRG_01697		orf19.1867	3.32442432	2.095416302
CTRG_02935		orf19.3655	3.305556062	2.185157784
CTRG_00051			3.298930202	2.226509983
CTRG_01698	RNR22	orf19.1868	3.239396641	2.26527641
CTRG_02802		orf19.5514	3.233661653	2.806588933
CTRG_04525		orf19.449	3.210905753	1.777435352
CTRG_05894		orf19.6601	3.187551935	1.995717618
CTRG_02333		orf19.346	3.176373803	1.750648439
CTRG_04355	CIS2	orf19.6053	3.169725023	1.935146349
CTRG_00559	LYP1	orf19.651	3.118029573	1.594535006
CTRG_04203	CAT1	orf19.6229	3.10511105	2.1581187
CTRG_03121		orf19.11	3.102535557	1.939338226
CTRG_00731		orf19.4366	3.093996468	2.332826001
CTRG_05665		orf19.6816	2.989180823	1.809530315
CTRG_03826	PDC11	orf19.2877	2.97925618	1.835246796
CTRG_05478	CFL5	orf19.1930	2.941690573	2.59976932
CTRG_04578	DA01	orf19.3065	2.91779023	1.844141324
CTRG_04122		orf19.2296	2.91700996	2.021109278
CTRG_05514		orf19.6658	2.901634024	1.817749161
CTRG_04182			2.90085416	1.879962349
CTRG_01257		orf19.1477	2.884521385	2.067917828
CTRG_00914	PGA23	orf19.3740	2.876495198	1.814138144
CTRG_04972		orf19.7131	2.864459614	2.019735243
CTRG_00902			2.843686626	1.901030497
CTRG_02869		orf19.929	2.838532488	1.585320298
CTRG_00198	ERG251	orf19.4631	2.838248974	2.117000074
CTRG_03915			2.810126367	2.013247036
CTRG_05284	HMS1	orf19.921	2.802326092	1.860447616
CTRG_00268			2.777268642	1.973038694
CTRG_00265			2.770552755	1.674079501
CTRG_05994		orf19.4316	2.74290309	1.664649312
CTRG_00267			2.71804153	1.849112221
CTRG_03866		orf19.1800	2.711518038	1.649380546
CTRG_00273		orf19.1421	2.663523801	1.668615501
CTRG_04847		orf19.6898	2.641025844	1.968577737
CTRG_04002	CDR4	orf19.5079	2.632060534	2.504637783

CTRG_04424		orf19.3310	2.623940703	1.589825341
CTRG_06001	PUT1	orf19.4274	2.602338145	1.976086886
CTRG_03488		orf19.1162	2.600608386	1.977168774
CTRG_00162			2.596782176	1.656906819
CTRG_05477	CFL4	orf19.1932	2.583364596	2.223352288
CTRG_02851		orf19.3425	2.557826486	1.851980006
CTRG_05862			2.540793795	2.27116702
CTRG_03760		orf19.3105	2.540329445	2.008095566
CTRG_04892	GPH1	orf19.7021	2.539338067	1.820004247
CTRG_00649	TPS1	orf19.6640	2.521796684	1.610485334
CTRG_03695		orf19.7227	2.50602222	2.029866709
CTRG_01751		orf19.2262	2.493433597	1.767043101
CTRG_00458	AAP1	orf19.2810	2.488376799	1.80067965
CTRG_04391		orf19.6017	2.457064342	1.906651004
CTRG_00648		orf19.6639	2.435701754	2.181812821
CTRG_01385	HOM2	orf19.1559	2.41581207	1.837230558
CTRG_04234	GLT1	orf19.6257	2.403996106	1.900230347
CTRG_01888		orf19.3151	2.366876503	1.591936161
CTRG_05855			2.366481349	1.894575829
CTRG_04529		orf19.55	2.340489711	1.781963894
CTRG_03601		orf19.4953	2.309178781	1.857720244
CTRG_03952		orf19.4449	2.301986825	1.645183772
CTRG_02530		orf19.5976	2.297584136	1.749158718
CTRG_03151			2.29121231	1.60266021
CTRG_00890	PHO113	orf19.2619	2.281135014	1.611540995
CTRG_04804		orf19.3041	2.279090031	1.868920979
CTRG_05178		orf19.6687	2.271120039	2.626114334
CTRG_01828			2.196403241	2.354358385
CTRG_05258	IFG3	orf19.944	2.174289355	1.948902686
CTRG_03259			2.165468215	2.400161555
CTRG_04808	STE3	orf19.2492	2.16237713	2.372751699
CTRG_05203		orf19.5125	2.147016623	1.739071935
CTRG_01202	ADAEC	orf19.868	2.144637347	1.708739817
CTRG_04897		orf19.7027	2.138083037	1.722316239
CTRG_03611			2.094191348	1.878738271
CTRG_01365		orf19.5813	2.064256403	1.602403865
CTRG_04437		orf19.3325	2.055642881	1.602660719
CTRG_03590	TYE7	orf19.4941	2.055266019	2.427067548
CTRG_03827		orf19.1277	2.04732429	1.681384723
CTRG_02983	EBP1	orf19.125	2.037943648	2.572497287
CTRG_05397		orf19.5605	2.022641017	1.703303421

CTRG_04648		orf19.3679	2.00427768	1.744079011
CTRG_06233		orf19.5504	1.967039287	2.052470592
CTRG_00655	SFL1	orf19.454	1.907084943	1.654972673
CTRG_06088	PUT2	orf19.3974	1.877493513	1.751175847
CTRG_05963		orf19.7283	1.841382757	1.648958182
CTRG_05607		orf19.6747	1.826401014	1.662050198
CTRG_01212	IFK2	orf19.856	1.826071713	1.651646437
CTRG_01931		orf19.5334	1.802747912	1.990945387
CTRG_00284	GST2	orf19.2693	1.78373807	1.901067553

Table C3. Opaque-specific gene list.

<i>Gene</i>	<i>Gene Name</i>	<i>Ca Ortholog</i>	<i>logFC Wh</i>	<i>logFC Int</i>
CTRG_03584	OP4	orf19.4934	8.913061058	10.44932021
CTRG_02412	MDR1	orf19.5604	4.394050746	2.898172055
CTRG_04977	TPO2	orf19.7148	3.00212112	3.444508121
CTRG_01140		orf19.2048	2.617337349	2.996626814

Table C4. White-repressed gene list.

<i>Gene</i>	<i>Gene Name</i>	<i>Ca Ortholog</i>	<i>logFC Op</i>	<i>logFC Int</i>
CTRG_03147		orf19.2624	-5.554944849	-4.349215234
CTRG_03744		orf19.3088	-5.164134922	-4.11998128
CTRG_05917	THI13	orf19.7324	-5.017642615	-4.5111372
CTRG_03530		orf19.5220	-4.4191151	-3.527496422
CTRG_05878		orf19.7552	-4.017785632	-3.173528024
CTRG_00375		orf19.494	-3.995132735	-3.260182375
CTRG_00718	KRE1	orf19.4377	-3.818816278	-4.253407526
CTRG_04917		orf19.7088	-3.803855823	-2.759798196
CTRG_03554	CHR1	orf19.3756	-3.766815918	-2.991898537
CTRG_05780	LTV1	orf19.7650	-3.764199009	-2.902258298
CTRG_05883		orf19.7546	-3.738088553	-2.854846669
CTRG_03387		orf19.4835	-3.646662688	-2.720897703
CTRG_06159		orf19.7664	-3.610604897	-3.142579289
CTRG_05835	UTP5	orf19.7599	-3.599852517	-2.808286069
CTRG_03243		orf19.4760	-3.574216022	-2.874083219
CTRG_01133		orf19.2059	-3.53775459	-3.140436975
CTRG_05808		orf19.7624	-3.493874828	-2.681278345
CTRG_02386		orf19.1642	-3.485964175	-2.953782427
CTRG_04982	UTP18	orf19.7154	-3.466713707	-2.475809678
CTRG_01790	MAK16	orf19.5500	-3.422158861	-2.956524668
CTRG_03137		orf19.2362	-3.385092063	-2.635526432
CTRG_03084	TSR1	orf19.6417	-3.374619575	-2.393684824
CTRG_03729	NAG3	orf19.2158	-3.368373872	-2.871160908
CTRG_03502	CDC6	orf19.5242	-3.349969391	-2.963480804
CTRG_03283	RPA190	orf19.1839	-3.347676351	-2.072572194
CTRG_01684	RCL1	orf19.1886	-3.316533591	-2.817338252
CTRG_02797		orf19.5517	-3.309982133	-2.862312555
CTRG_05884		orf19.6589	-3.277443484	-2.455671738
CTRG_03086		orf19.6418	-3.2671868	-2.55040181
CTRG_01012	BUD22	orf19.3287	-3.244197656	-2.818962352
CTRG_03409	YTM1	orf19.4815	-3.239976506	-2.465477632
CTRG_00612	RRN11	orf19.718	-3.218470304	-2.797742233
CTRG_03107	RIO2	orf19.6369	-3.1993479	-2.444590807
CTRG_00848		orf19.2564	-3.198735015	-2.771157047
CTRG_02886		orf19.3393	-3.19050155	-2.708009075
CTRG_03612		orf19.4962	-3.186161058	-2.566767963
CTRG_03428		orf19.4793	-3.176174833	-2.387424747
CTRG_05181		orf19.7197	-3.169331594	-2.194487661

CTRG_04905			-3.16515529	-2.099525163
CTRG_02335			-3.13878122	-2.441313526
CTRG_00525		orf19.6316.4	-3.12186137	-2.293345338
CTRG_05175	ENP1	orf19.5507	-3.118845216	-2.162470244
CTRG_05813		orf19.7618	-3.117639028	-2.368803492
CTRG_05067	NOP15	orf19.7050	-3.109672832	-2.095218386
CTRG_05147			-3.107509583	-2.092374042
CTRG_04091		orf19.2309	-3.106092427	-2.736244598
CTRG_04703		orf19.6845	-3.10543857	-2.925041595
CTRG_03281			-3.087828931	-2.335966071
CTRG_00744		orf19.4399	-3.086614574	-2.592934407
CTRG_01076	RRN3	orf19.1923	-3.073249658	-2.478595996
CTRG_03177	DIP2	orf19.5106	-3.068903815	-2.273437662
CTRG_03311		orf19.4896	-3.058304853	-2.339679665
CTRG_03977		orf19.5049	-3.05596415	-2.724869361
CTRG_05956		orf19.7291	-3.055494832	-2.335442598
CTRG_00715		orf19.4355	-3.047564368	-2.78595915
CTRG_01532		orf19.6886	-3.013838812	-2.581657942
CTRG_04810	FAL1	orf19.2488	-3.006287013	-2.517361827
CTRG_03097		orf19.6432	-3.002624891	-2.168988654
CTRG_04878		orf19.7042	-2.995146491	-1.615431348
CTRG_04606		orf19.3665	-2.99128739	-2.468281392
CTRG_03448	KIP4	orf19.5265	-2.990596239	-2.364646396
CTRG_04208		orf19.6234	-2.985266689	-2.643161776
CTRG_05688		orf19.6828	-2.94935819	-2.5919979
CTRG_02334	NOP14	orf19.5959	-2.944361149	-2.549106516
CTRG_04259		orf19.6277	-2.934454189	-1.838703141
CTRG_00195		orf19.4634	-2.928219578	-2.51661887
CTRG_00492	HCA4	orf19.2712	-2.925984745	-2.407932188
CTRG_05887		orf19.6597	-2.919438217	-2.307316332
CTRG_03196	RIX7	orf19.4219	-2.912026244	-2.155028392
CTRG_00885		orf19.2604	-2.899409816	-2.4531661
CTRG_02726	NOG2	orf19.5732	-2.895710154	-2.224889147
CTRG_00855		orf19.2575	-2.886773924	-2.546308418
CTRG_02170		orf19.1708	-2.883030933	-2.487305668
CTRG_05619		orf19.6766	-2.871468473	-2.324777705
CTRG_03515	CSI2	orf19.5232	-2.865336458	-2.102823993
CTRG_03183		orf19.3220	-2.850592006	-2.087814691
CTRG_04651		orf19.2934	-2.835964076	-2.289501702
CTRG_03240		orf19.4748	-2.830112903	-2.205473916
CTRG_06178	UTP8	orf19.5436	-2.828810127	-2.38429159

CTRG_05877			-2.823829507	-2.52785234
CTRG_00354	IMP4	orf19.603	-2.814546303	-2.38572791
CTRG_03547		orf19.6356	-2.811635322	-2.076656205
CTRG_06000	UTP13	orf19.4268	-2.792302749	-2.248743785
CTRG_02247		orf19.7422	-2.7910024	-2.416322149
CTRG_05186		orf19.7182	-2.790566407	-1.929727769
CTRG_05179	ENP2	orf19.6686	-2.788990898	-1.882380213
CTRG_06223			-2.785084835	-2.603689374
CTRG_03238	JIP5	orf19.4746	-2.7821569	-2.027584742
CTRG_00764		orf19.2386	-2.774737257	-2.385497151
CTRG_02755		orf19.1091	-2.774140702	-2.452434983
CTRG_01649		orf19.3539	-2.763588195	-2.462211804
CTRG_05594		orf19.6730	-2.762065498	-2.501007775
CTRG_05871	DPB2	orf19.7564	-2.755718082	-2.392074647
CTRG_00168		orf19.4563	-2.755308855	-2.30419524
CTRG_02686		orf19.4143	-2.755001406	-2.409914931
CTRG_05404	SPB1	orf19.76	-2.750836541	-2.232158792
CTRG_00282	NAN1	orf19.2688	-2.744426953	-2.292791165
CTRG_04844		orf19.6463	-2.739426079	-1.688059272
CTRG_06092		orf19.3970	-2.736866114	-2.213431272
CTRG_02947		orf19.86	-2.710990566	-2.554506547
CTRG_00827	RRP9	orf19.2830	-2.709726243	-2.24324733
CTRG_04148		orf19.5066	-2.705433762	-2.351339433
CTRG_04992		orf19.5168	-2.702191972	-1.819959469
CTRG_00923	DBP2	orf19.171	-2.691379181	-2.068153307
CTRG_04086		orf19.2314	-2.683901219	-2.221264445
CTRG_02676	SPB4	orf19.6298	-2.676772844	-2.272575254
CTRG_05921	ELF1	orf19.7332	-2.673139874	-1.782903189
CTRG_04080		orf19.2320	-2.668665976	-2.170280207
CTRG_05541			-2.664504889	-2.08585973
CTRG_00592	DBP8	orf19.6652	-2.663240152	-2.320512459
CTRG_01616		orf19.154	-2.649515997	-2.438926532
CTRG_03692		orf19.7223	-2.64869107	-2.087742374
CTRG_01866	UTP15	orf19.3609	-2.644257324	-2.178645148
CTRG_00742	YVH1	orf19.4401	-2.641409761	-2.478941385
CTRG_03613		orf19.4963	-2.63839159	-1.976921026
CTRG_01011		orf19.7460	-2.633167065	-2.367895228
CTRG_00800	SRP40	orf19.2859	-2.632704129	-2.145650306
CTRG_03380		orf19.4844	-2.625798212	-1.919265687
CTRG_03607		orf19.4960	-2.624234697	-1.902392995
CTRG_00358		orf19.475	-2.620909079	-2.257109375

CTRG_01999		orf19.1388	-2.620733595	-2.217609326
CTRG_02414		orf19.1609	-2.617394526	-2.071897527
CTRG_01289		orf19.839	-2.606438835	-2.283298583
CTRG_01103	MPP10	orf19.1915	-2.59864822	-2.084735631
CTRG_03943		orf19.3831	-2.596654284	-2.163449733
CTRG_03684		orf19.7215	-2.595852695	-1.674001227
CTRG_00826	RPC31	orf19.2831	-2.591833326	-2.375048074
CTRG_01871	RRP15	orf19.563	-2.591568297	-2.131606057
CTRG_02653		orf19.3704	-2.583891455	-2.275088551
CTRG_01718	NOC4	orf19.1902	-2.581488324	-2.221660191
CTRG_03355	DBP3	orf19.4870	-2.568438761	-1.79513242
CTRG_03321			-2.565274052	-2.494342999
CTRG_00905		orf19.3724	-2.563952552	-2.140039714
CTRG_00233			-2.561054029	-2.117078172
CTRG_05725		orf19.3781	-2.554857494	-1.913011996
CTRG_01455		orf19.813	-2.550863856	-1.863557704
CTRG_04074		orf19.2330	-2.540191105	-2.157373046
CTRG_01524		orf19.5207	-2.540143241	-2.218367172
CTRG_02014	PUS7	orf19.1753	-2.539835647	-2.096093533
CTRG_03623	DIM1	orf19.5010	-2.538746764	-1.796686678
CTRG_03707		orf19.7243	-2.535053451	-2.300534623
CTRG_04210		orf19.6236	-2.534387416	-2.166185924
CTRG_03215		orf19.4721	-2.532234307	-2.016474586
CTRG_03059	FRE7	orf19.6139	-2.523662166	-2.129653183
CTRG_00107		orf19.5678	-2.521409462	-2.199856122
CTRG_03113		orf19.1815	-2.518036112	-1.679977692
CTRG_02290		orf19.5905	-2.51773809	-2.200377303
CTRG_04624	ABP140	orf19.3676	-2.505408101	-2.225605582
CTRG_00307	ECM1	orf19.5299	-2.501115404	-2.092795889
CTRG_02384		orf19.1646	-2.499295959	-2.051182834
CTRG_00495	SAS10	orf19.2717	-2.487644988	-2.030461426
CTRG_03895		orf19.6862	-2.483462326	-2.162840045
CTRG_04504	RRP6	orf19.58	-2.482153426	-2.0686574
CTRG_05209		orf19.5134	-2.477551655	-1.717262443
CTRG_05297		orf19.3202	-2.47692188	-2.025182598
CTRG_01000	PWP2	orf19.3276	-2.474510981	-2.0774535
CTRG_03876		orf19.1791	-2.461896876	-2.095531091
CTRG_01026		orf19.7488	-2.458940955	-1.88744785
CTRG_03668	SIM1	orf19.5032	-2.449885475	-1.641461293
CTRG_01328		orf19.5802	-2.436728264	-2.14320418
CTRG_00625	RPL7	orf19.3867	-2.435869776	-2.00284424

CTRG_05830		orf19.7593	-2.43009149	-2.499314489
CTRG_00473	DOT4	orf19.3370	-2.42980012	-1.829073577
CTRG_03106		orf19.6371	-2.428636483	-1.839763614
CTRG_04416		orf19.3303	-2.423641068	-2.105262703
CTRG_00734	SGD1	orf19.4363	-2.419569818	-1.925011987
CTRG_00510	RPF1	orf19.2667	-2.414025258	-2.076330058
CTRG_01668	NSA1	orf19.2185	-2.413881669	-2.141760685
CTRG_01842	RRP8	orf19.3630	-2.413735699	-2.023530301
CTRG_05822		orf19.7581	-2.407765103	-1.720362368
CTRG_00663	NMD3	orf19.706	-2.398967222	-1.887571851
CTRG_04719	NOP4	orf19.5198	-2.39296446	-1.892517556
CTRG_00733		orf19.4365	-2.39013484	-2.164915375
CTRG_05918		orf19.7330	-2.388095232	-2.303352468
CTRG_00514	DIT2	orf19.554	-2.376604636	-1.631744483
CTRG_02809	NIP7	orf19.3478	-2.375741953	-2.100128822
CTRG_04757		orf19.4479	-2.371587165	-2.037508774
CTRG_03462	MUP1	orf19.5280	-2.370499079	-1.633905996
CTRG_00679		orf19.1849	-2.357995542	-1.96738299
CTRG_00317		orf19.5038	-2.352873068	-1.894106415
CTRG_04853		orf19.6903	-2.349537691	-1.61310318
CTRG_01117		orf19.2090	-2.34608161	-1.951118297
CTRG_05528			-2.345527649	-1.981468213
CTRG_04341	TSR2	orf19.2998	-2.34009712	-1.942927792
CTRG_04138			-2.333543242	-2.567578119
CTRG_04503	REI1	orf19.59	-2.332480418	-2.04185513
CTRG_04393	RRS1	orf19.6014	-2.323622337	-1.982738914
CTRG_04866		orf19.6907	-2.322735493	-1.72056619
CTRG_04131		orf19.4779	-2.313358556	-1.876635332
CTRG_05468		orf19.1287	-2.304631551	-2.084032195
CTRG_04103	KRR1	orf19.661	-2.298437156	-1.929574401
CTRG_03202	LEU3	orf19.4225	-2.29075809	-1.655379866
CTRG_01815		orf19.2167	-2.285137776	-1.905897373
CTRG_03657		orf19.5020	-2.281359483	-1.688436214
CTRG_02079		orf19.1404	-2.280764399	-1.947510674
CTRG_02394	UTP4	orf19.1633	-2.280728197	-1.835903962
CTRG_00595	SDA1	orf19.6648	-2.28027657	-1.79591065
CTRG_04705		orf19.6847	-2.279024734	-1.977820833
CTRG_03709		orf19.7245	-2.274419173	-1.79450754
CTRG_03224		orf19.4730	-2.273024936	-1.782446069
CTRG_05579	UTP9	orf19.6710	-2.267426553	-1.980356169
CTRG_00602		orf19.3887	-2.26683599	-2.22191872

CTRG_05811		orf19.7620	-2.252110794	-2.001264931
CTRG_01650	MAK5	orf19.3540	-2.241454515	-1.808038604
CTRG_04192		orf19.2788	-2.235863368	-2.106496111
CTRG_04921	DCC1	orf19.7083	-2.224831177	-1.800789252
CTRG_02788		orf19.5541	-2.211629474	-1.769140769
CTRG_03526			-2.209642689	-2.018342767
CTRG_01147		orf19.2042	-2.203985569	-1.9129614
CTRG_03143	ASF1	orf19.3715	-2.197415196	-1.922202679
CTRG_05332	BUD23	orf19.1966	-2.195978673	-1.873138373
CTRG_00252	SEN2	orf19.2735	-2.193062904	-1.81043427
CTRG_03287		orf19.1835	-2.182192148	-1.749697278
CTRG_06202	SOF1	orf19.5407	-2.169864079	-1.946848603
CTRG_00081	RLP24	orf19.4191	-2.1685358	-1.79811489
CTRG_02124			-2.155504068	-2.069379595
CTRG_01846		orf19.3626	-2.153587574	-1.830358441
CTRG_06085		orf19.3978	-2.150916206	-1.83735301
CTRG_05942		orf19.7307	-2.14307699	-1.589338689
CTRG_02919		orf19.3463	-2.141649502	-1.654380614
CTRG_02156		orf19.1687	-2.139308399	-1.640456853
CTRG_03816	POP3	orf19.7657	-2.12744398	-1.960607128
CTRG_01983	SMM1	orf19.1362	-2.123090524	-1.754619376
CTRG_00494	RPC53	orf19.2715	-2.111666352	-1.761592771
CTRG_04412	ZPR1	orf19.3300	-2.107919309	-1.650608095
CTRG_02312		orf19.5884	-2.106873463	-1.733969154
CTRG_03412		orf19.4812	-2.104395696	-1.6732279
CTRG_06078	HAS1	orf19.3962	-2.098220304	-1.635523148
CTRG_06182	BUD21	orf19.5430	-2.097319479	-1.776576595
CTRG_03758		orf19.3107	-2.095857728	-1.990125827
CTRG_01877	PES1	orf19.4093	-2.087787044	-1.70239577
CTRG_05193	OGG1	orf19.7190	-2.086915307	-1.590716017
CTRG_00246	HGH1	orf19.4587	-2.084636829	-1.725913384
CTRG_04024	BMS1	orf19.2504	-2.074798617	-1.621363184
CTRG_04305	CDC13	orf19.6072	-2.073927738	-2.262557602
CTRG_04082		orf19.2319	-2.071618833	-1.653509891
CTRG_01727	RPA12	orf19.2287	-2.069710621	-1.900135281
CTRG_00433		orf19.3798	-2.056098726	-1.738227535
CTRG_01753		orf19.2260	-2.052034349	-1.761992099
CTRG_04417		orf19.3304	-2.0503191	-1.862366245
CTRG_04372	CNS1	orf19.6052	-2.047041072	-1.732591061
CTRG_05801	MCD1	orf19.7634	-2.046163105	-2.010518153
CTRG_02117		orf19.5704	-2.044947171	-1.798387037

CTRG_02675		orf19.6297	-2.040996405	-1.731494872
CTRG_00472			-2.035741005	-1.770866359
CTRG_04811		orf19.2487	-2.027872157	-1.668307146
CTRG_00763	KT112	orf19.2385	-2.008256441	-1.78232964
CTRG_02204		orf19.3778	-1.999985774	-1.687655057
CTRG_03563		orf19.6343	-1.998252717	-1.647148938
CTRG_04363	RPO41	orf19.6041	-1.987096514	-1.668814269
CTRG_02157			-1.972502914	-2.209310491
CTRG_02524		orf19.5987	-1.965066891	-1.689445362
CTRG_04536		orf19.429	-1.9608564	-1.671648216
CTRG_03851		orf19.1250	-1.956544012	-1.702774351
CTRG_01305	STP4	orf19.909	-1.95557522	-1.612486661
CTRG_01525		orf19.5206	-1.950618763	-1.695175307
CTRG_00812		orf19.2847	-1.950058599	-1.618871123
CTRG_05574		orf19.6175	-1.942585756	-1.723566686
CTRG_00512		orf19.552	-1.934766751	-1.612159922
CTRG_03134	CRZ2	orf19.2356	-1.927298502	-2.50158923
CTRG_02921		orf19.3466	-1.907382932	-1.658600364
CTRG_02449		orf19.7369	-1.888751648	-1.720950057
CTRG_04257	UME6	orf19.1822	-1.870923516	-1.634933022
CTRG_00132		orf19.4582	-1.861782733	-1.593557077
CTRG_04302	HMX1	orf19.6073	-1.837150918	-2.082519281
CTRG_04106	NEP1	orf19.665	-1.820473198	-1.674976234
CTRG_01627		orf19.182	-1.819229012	-1.646997372
CTRG_04388	NRM1	orf19.6022	-1.765611227	-2.081629669
CTRG_06077	HYS2	orf19.3960	-1.747623935	-1.778005886
CTRG_02775		orf19.5523	-1.722797303	-1.729249102
CTRG_05980		orf19.4301	-1.713113653	-1.786181225
CTRG_05398		orf19.5607	-1.70566474	-1.68073787
CTRG_01314		orf19.5785	-1.700618191	-2.01950044
CTRG_02892		orf19.687	-1.690172833	-1.796418472
CTRG_01132	SOD4	orf19.2062	-1.677721388	-1.629807822
CTRG_01372	ZRT2	orf19.1585	-1.621167735	-2.058225535
CTRG_02294	TEC1	orf19.5908	-1.610343516	-1.654675029
CTRG_05354		orf19.580	-1.610269789	-1.590202393
CTRG_02274			-1.604643529	-1.763473996

Table C5. Opaque-repressed gene list.

<i>Gene</i>	<i>Gene Name</i>	<i>Ca Ortholog</i>	<i>logFC Wh</i>	<i>logFC Int</i>
CTRG_02956		orf19.95	-1.645842608	-1.598149143
CTRG_00662			-2.028011714	-1.904004922
CTRG_03787			-2.367237758	-1.867800901
CTRG_06032	SFC1	orf19.3931	-2.914907785	-1.682614941
CTRG_01660	WH11	orf19.3548.1	-2.960944119	-2.724939924
CTRG_03292			-3.231975168	-2.630847026
CTRG_01861	PST2	orf19.3612	-3.267186731	-2.146599468
CTRG_02708			-3.504424611	-1.629173432
CTRG_01062	PCK1	orf19.7514	-3.550285275	-1.643068222
CTRG_01885	PRB1	orf19.2242	-3.577949513	-1.852390261
CTRG_01691		orf19.1862	-3.642961193	-1.850175169
CTRG_06113	ADH1	orf19.3997	-3.739340093	-2.394832112
CTRG_00457	CTN3	orf19.2809	-3.832563406	-1.656945713
CTRG_00408	RBR1	orf19.535	-3.834580282	-2.843705271
CTRG_04111		orf19.670.2	-4.027618632	-2.573464627
CTRG_02835	FRP6	orf19.3441	-4.277862419	-2.007717891
CTRG_05940		orf19.7310	-4.449176571	-2.275316592
CTRG_06081	MAL31	orf19.3981	-4.499977463	-2.811765005
CTRG_00749		orf19.4391	-5.107272832	-2.76665644
CTRG_03962		orf19.4450.1	-5.490694416	-2.394288616
CTRG_01821	UCF1	orf19.1354	-5.815641465	-3.00897989
CTRG_00882	ADH5	orf19.2608	-6.969320166	-3.419440249

Table C6. Intermediate-repressed gene list.

<i>Gene</i>	<i>Gene Name</i>	<i>Ca Ortholog</i>	<i>logFC Wh</i>	<i>logFC Op</i>
CTRG_01852	YWP1	orf19.3618	-4.202525786	-3.19957951

Table C7. White-specific GO terms.

<i>GO term</i>	<i>Cluster frequency</i>	<i>Background frequency</i>	<i>P-value</i>	<i>FDR</i>
oxidation-reduction process	30 out of 105 genes, 28.6%	732 out of 12983 background genes, 5.6%	2.18E-11	0.00%
single-organism metabolic process	40 out of 105 genes, 38.1%	2428 out of 12983 background genes, 18.7%	0.00094	0.00%
cellular amino acid catabolic process	6 out of 105 genes, 5.7%	61 out of 12983 background genes, 0.5%	0.0035	0.00%
energy reserve metabolic process	4 out of 105 genes, 3.8%	18 out of 12983 background genes, 0.1%	0.00421	0.00%
alpha-amino acid metabolic process	9 out of 105 genes, 8.6%	186 out of 12983 background genes, 1.4%	0.00724	0.00%
alpha-amino acid catabolic process	5 out of 105 genes, 4.8%	46 out of 12983 background genes, 0.4%	0.01234	0.00%
carboxylic acid metabolic process	14 out of 105 genes, 13.3%	496 out of 12983 background genes, 3.8%	0.01673	0.00%
oxoacid metabolic process	14 out of 105 genes, 13.3%	506 out of 12983 background genes, 3.9%	0.02077	0.00%
organic acid metabolic process	14 out of 105 genes, 13.3%	507 out of 12983 background genes, 3.9%	0.02122	0.00%
glutamate biosynthetic process	3 out of 105 genes, 2.9%	10 out of 12983 background genes, 0.1%	0.02198	0.00%
proline catabolic process to glutamate	2 out of 105 genes, 1.9%	2 out of 12983 background genes, 0.0%	0.02401	0.00%
alanine metabolic process	2 out of 105 genes, 1.9%	2 out of 12983 background genes, 0.0%	0.02401	0.00%
pyruvate family amino acid metabolic process	2 out of 105 genes, 1.9%	2 out of 12983 background genes, 0.0%	0.02401	0.00%
amino-acid betaine metabolic process	3 out of 105 genes, 2.9%	11 out of 12983 background genes, 0.1%	0.03004	0.00%
carnitine metabolic process	3 out of 105 genes, 2.9%	11 out of 12983 background genes, 0.1%	0.03004	0.00%
single-organism process	58 out of 105 genes, 55.2%	4801 out of 12983 background genes, 37.0%	0.03982	0.00%
small molecule biosynthetic process	11 out of 105 genes, 10.5%	353 out of 12983 background genes, 2.7%	0.04904	0.00%
organic acid catabolic process	6 out of 105 genes, 5.7%	98 out of 12983 background genes, 0.8%	0.05216	0.00%
carboxylic acid catabolic process	6 out of 105 genes, 5.7%	98 out of 12983 background genes, 0.8%	0.05216	0.00%
organic acid biosynthetic process	9 out of 105 genes, 8.6%	240 out of 12983 background genes, 1.8%	0.05232	0.00%
carboxylic acid biosynthetic process	9 out of 105 genes, 8.6%	240 out of 12983 background genes, 1.8%	0.05232	0.00%
dicarboxylic acid biosynthetic process	3 out of 105 genes, 2.9%	14 out of 12983 background genes, 0.1%	0.06512	0.18%
glutamate metabolic process	3 out of 105 genes, 2.9%	14 out of 12983 background genes, 0.1%	0.06512	0.17%
cellular amino acid metabolic process	10 out of 105 genes, 9.5%	306 out of 12983 background genes, 2.4%	0.06846	0.17%
hexose catabolic process to ethanol	2 out of 105 genes, 1.9%	3 out of 12983 background genes, 0.0%	0.07165	0.40%

glucose catabolic process to ethanol	2 out of 105 genes, 1.9%	3 out of 12983 background genes, 0.0%	0.07165	0.38%
cellular response to chemical stimulus	19 out of 105 genes, 18.1%	968 out of 12983 background genes, 7.5%	0.09464	0.37%
dicarboxylic acid metabolic process	4 out of 105 genes, 3.8%	39 out of 12983 background genes, 0.3%	0.09942	0.43%

Table C8. Opaque-specific GO terms.

<i>GO term</i>	<i>Cluster frequency</i>	<i>Background frequency</i>	<i>P-value</i>	<i>FDR</i>
drug transport	2 out of 4 genes, 50.0%	84 out of 12983 background genes, 0.6%	0.00296	2.00%

Table C9. White-repressed GO terms.

<i>GO term</i>	<i>Cluster frequency</i>	<i>Background frequency</i>	<i>P-value</i>	<i>FDR</i>
ribosome biogenesis	127 out of 263 genes, 48.3%	314 out of 12983 background genes, 2.4%	7.20E-139	0.00%
ncRNA processing	121 out of 263 genes, 46.0%	315 out of 12983 background genes, 2.4%	4.68E-128	0.00%
ribonucleoprotein complex biogenesis	129 out of 263 genes, 49.0%	393 out of 12983 background genes, 3.0%	3.48E-127	0.00%
ncRNA metabolic process	125 out of 263 genes, 47.5%	384 out of 12983 background genes, 3.0%	4.23E-122	0.00%
rRNA metabolic process	102 out of 263 genes, 38.8%	224 out of 12983 background genes, 1.7%	3.52E-115	0.00%
RNA processing	130 out of 263 genes, 49.4%	495 out of 12983 background genes, 3.8%	6.86E-114	0.00%
rRNA processing	99 out of 263 genes, 37.6%	212 out of 12983 background genes, 1.6%	7.28E-113	0.00%
RNA metabolic process	146 out of 263 genes, 55.5%	885 out of 12983 background genes, 6.8%	4.22E-99	0.00%
nucleic acid metabolic process	153 out of 263 genes, 58.2%	1248 out of 12983 background genes, 9.6%	3.47E-85	0.00%
cellular component biogenesis	134 out of 263 genes, 51.0%	956 out of 12983 background genes, 7.4%	1.91E-79	0.00%
ribosomal small subunit biogenesis	64 out of 263 genes, 24.3%	111 out of 12983 background genes, 0.9%	2.04E-78	0.00%
nucleobase-containing compound metabolic process	154 out of 263 genes, 58.6%	1568 out of 12983 background genes, 12.1%	1.70E-71	0.00%
maturation of SSU-rRNA	56 out of 263 genes, 21.3%	92 out of 12983 background genes, 0.7%	5.39E-70	0.00%
cellular aromatic compound metabolic process	156 out of 263 genes, 59.3%	1671 out of 12983 background genes, 12.9%	1.67E-69	0.00%
cellular nitrogen compound metabolic process	159 out of 263 genes, 60.5%	1757 out of 12983 background genes, 13.5%	2.44E-69	0.00%
heterocycle metabolic process	156 out of 263 genes, 59.3%	1683 out of 12983 background genes, 13.0%	4.81E-69	0.00%
maturation of SSU-rRNA from tricistronic rRNA transcript (SSU-rRNA, 5.8S rRNA, LSU-rRNA)	53 out of 263 genes, 20.2%	82 out of 12983 background genes, 0.6%	3.76E-68	0.00%
organic cyclic compound metabolic process	156 out of 263 genes, 59.3%	1762 out of 12983 background genes, 13.6%	4.10E-66	0.00%
nitrogen compound metabolic process	160 out of 263 genes, 60.8%	2005 out of 12983 background genes, 15.4%	8.85E-62	0.00%
gene expression	142 out of 263 genes, 54.0%	1515 out of 12983 background genes, 11.7%	2.31E-61	0.00%
maturation of 5.8S rRNA	46 out of 263 genes, 17.5%	75 out of 12983 background genes, 0.6%	4.37E-57	0.00%

maturation of 5.8S rRNA from tricistronic rRNA transcript (SSU-rRNA, 5.8S rRNA, LSU-rRNA)	46 out of 263 genes, 17.5%	75 out of 12983 background genes, 0.6%	4.37E-57	0.00%
cellular component organization or biogenesis	149 out of 263 genes, 56.7%	2123 out of 12983 background genes, 16.4%	2.46E-48	0.00%
ribosomal large subunit biogenesis	43 out of 263 genes, 16.3%	88 out of 12983 background genes, 0.7%	3.42E-47	0.00%
cellular macromolecule metabolic process	165 out of 263 genes, 62.7%	2768 out of 12983 background genes, 21.3%	8.87E-46	0.00%
cleavage involved in rRNA processing	37 out of 263 genes, 14.1%	65 out of 12983 background genes, 0.5%	1.28E-43	0.00%
macromolecule metabolic process	166 out of 263 genes, 63.1%	2909 out of 12983 background genes, 22.4%	1.71E-43	0.00%
endonucleolytic cleavage in ITS1 to separate SSU-rRNA from 5.8S rRNA and LSU-rRNA from tricistronic rRNA transcript (SSU-rRNA, 5.8S rRNA, LSU-rRNA)	31 out of 263 genes, 11.8%	39 out of 12983 background genes, 0.3%	1.80E-43	0.00%
endonucleolytic cleavage involved in rRNA processing	32 out of 263 genes, 12.2%	43 out of 12983 background genes, 0.3%	2.87E-43	0.00%
endonucleolytic cleavage of tricistronic rRNA transcript (SSU-rRNA, 5.8S rRNA, LSU-rRNA)	32 out of 263 genes, 12.2%	43 out of 12983 background genes, 0.3%	2.87E-43	0.00%
RNA phosphodiester bond hydrolysis	37 out of 263 genes, 14.1%	69 out of 12983 background genes, 0.5%	2.88E-42	0.00%
RNA phosphodiester bond hydrolysis, endonucleolytic	32 out of 263 genes, 12.2%	46 out of 12983 background genes, 0.4%	1.13E-41	0.00%
RNA 5'-end processing	28 out of 263 genes, 10.6%	33 out of 12983 background genes, 0.3%	1.23E-40	0.00%
ncRNA 5'-end processing	27 out of 263 genes, 10.3%	32 out of 12983 background genes, 0.2%	5.74E-39	0.00%
rRNA 5'-end processing	27 out of 263 genes, 10.3%	32 out of 12983 background genes, 0.2%	5.74E-39	0.00%
endonucleolytic cleavage to generate mature 5'-end of SSU-rRNA from (SSU-rRNA, 5.8S rRNA, LSU-rRNA)	26 out of 263 genes, 9.9%	31 out of 12983 background genes, 0.2%	2.64E-37	0.00%
endonucleolytic	24 out of 263	29 out of 12983	5.42E-34	0.00%

cleavage in 5'-ETS of tricistronic rRNA transcript (SSU-rRNA, 5.8S rRNA, LSU-rRNA)	genes, 9.1%	background genes, 0.2%		
nucleic acid phosphodiester bond hydrolysis	39 out of 263 genes, 14.8%	124 out of 12983 background genes, 1.0%	1.97E-33	0.00%
cellular metabolic process	174 out of 263 genes, 66.2%	3963 out of 12983 background genes, 30.5%	2.36E-30	0.00%
primary metabolic process	170 out of 263 genes, 64.6%	3888 out of 12983 background genes, 29.9%	7.22E-29	0.00%
organic substance metabolic process	174 out of 263 genes, 66.2%	4082 out of 12983 background genes, 31.4%	1.40E-28	0.00%
RNA modification	32 out of 263 genes, 12.2%	99 out of 12983 background genes, 0.8%	1.82E-27	0.00%
metabolic process	182 out of 263 genes, 69.2%	4668 out of 12983 background genes, 36.0%	1.56E-25	0.00%
cellular process	206 out of 263 genes, 78.3%	6024 out of 12983 background genes, 46.4%	8.10E-24	0.00%
maturation of LSU-rRNA	19 out of 263 genes, 7.2%	35 out of 12983 background genes, 0.3%	6.62E-21	0.00%
maturation of LSU-rRNA from tricistronic rRNA transcript (SSU-rRNA, 5.8S rRNA, LSU-rRNA)	18 out of 263 genes, 6.8%	31 out of 12983 background genes, 0.2%	1.87E-20	0.00%
tRNA modification	22 out of 263 genes, 8.4%	69 out of 12983 background genes, 0.5%	3.64E-18	0.00%
tRNA processing	24 out of 263 genes, 9.1%	105 out of 12983 background genes, 0.8%	3.51E-16	0.00%
RNA methylation	15 out of 263 genes, 5.7%	34 out of 12983 background genes, 0.3%	2.17E-14	0.00%
tRNA metabolic process	27 out of 263 genes, 10.3%	169 out of 12983 background genes, 1.3%	3.60E-14	0.00%
ribosome localization	15 out of 263 genes, 5.7%	44 out of 12983 background genes, 0.3%	2.24E-12	0.00%
establishment of ribosome localization	15 out of 263 genes, 5.7%	44 out of 12983 background genes, 0.3%	2.24E-12	0.00%
ribosomal subunit export from nucleus	15 out of 263 genes, 5.7%	44 out of 12983 background genes, 0.3%	2.24E-12	0.00%
ribonucleoprotein complex localization	15 out of 263 genes, 5.7%	44 out of 12983 background genes, 0.3%	2.24E-12	0.00%
ribonucleoprotein complex export from nucleus	15 out of 263 genes, 5.7%	44 out of 12983 background genes, 0.3%	2.24E-12	0.00%
rRNA-containing ribonucleoprotein complex export from nucleus	15 out of 263 genes, 5.7%	44 out of 12983 background genes, 0.3%	2.24E-12	0.00%
ribosome assembly	16 out of 263 genes, 6.1%	53 out of 12983 background genes, 0.4%	2.40E-12	0.00%
methylation	18 out of 263	81 out of 12983	1.67E-11	0.00%

	genes, 6.8%	background genes, 0.6%		
ribosomal large subunit assembly	12 out of 263 genes, 4.6%	29 out of 12983 background genes, 0.2%	8.76E-11	0.00%
macromolecule methylation	17 out of 263 genes, 6.5%	79 out of 12983 background genes, 0.6%	1.57E-10	0.00%
ribonucleoprotein complex subunit organization	22 out of 263 genes, 8.4%	149 out of 12983 background genes, 1.1%	1.64E-10	0.00%
ribonucleoprotein complex assembly	21 out of 263 genes, 8.0%	141 out of 12983 background genes, 1.1%	4.91E-10	0.00%
nuclear export	18 out of 263 genes, 6.8%	109 out of 12983 background genes, 0.8%	3.68E-09	0.00%
tRNA methylation	9 out of 263 genes, 3.4%	19 out of 12983 background genes, 0.1%	2.37E-08	0.00%
nucleocytoplasmic transport	21 out of 263 genes, 8.0%	175 out of 12983 background genes, 1.3%	3.40E-08	0.00%
nuclear transport	21 out of 263 genes, 8.0%	176 out of 12983 background genes, 1.4%	3.79E-08	0.00%
rRNA modification	9 out of 263 genes, 3.4%	23 out of 12983 background genes, 0.2%	1.95E-07	0.00%
ribosomal large subunit export from nucleus	9 out of 263 genes, 3.4%	25 out of 12983 background genes, 0.2%	4.72E-07	0.00%
positive regulation of transcription from RNA polymerase I promoter	7 out of 263 genes, 2.7%	12 out of 12983 background genes, 0.1%	5.72E-07	0.00%
establishment of organelle localization	15 out of 263 genes, 5.7%	98 out of 12983 background genes, 0.8%	6.71E-07	0.00%
organelle assembly	16 out of 263 genes, 6.1%	115 out of 12983 background genes, 0.9%	7.88E-07	0.00%
macromolecule modification	42 out of 263 genes, 16.0%	800 out of 12983 background genes, 6.2%	6.88E-06	0.00%
organelle localization	15 out of 263 genes, 5.7%	125 out of 12983 background genes, 1.0%	2.04E-05	0.00%
rRNA methylation	6 out of 263 genes, 2.3%	12 out of 12983 background genes, 0.1%	3.31E-05	0.00%
regulation of transcription from RNA polymerase I promoter	8 out of 263 genes, 3.0%	30 out of 12983 background genes, 0.2%	6.19E-05	0.00%
polyadenylation-dependent RNA catabolic process	6 out of 263 genes, 2.3%	18 out of 12983 background genes, 0.1%	0.0006	0.00%
cellular macromolecular complex assembly	24 out of 263 genes, 9.1%	415 out of 12983 background genes, 3.2%	0.0024	0.00%
snoRNA metabolic process	7 out of 263 genes, 2.7%	35 out of 12983 background genes, 0.3%	0.00326	0.00%
rRNA base methylation	4 out of 263 genes, 1.5%	7 out of 12983 background genes, 0.1%	0.00332	0.00%
nuclear polyadenylation-	4 out of 263 genes, 1.5%	7 out of 12983 background genes, 0.1%	0.00332	0.00%

dependent CUT catabolic process				
tRNA catabolic process	5 out of 263 genes, 1.9%	14 out of 12983 background genes, 0.1%	0.00343	0.00%
nuclear polyadenylation-dependent tRNA catabolic process	5 out of 263 genes, 1.9%	14 out of 12983 background genes, 0.1%	0.00343	0.00%
snRNA catabolic process	3 out of 263 genes, 1.1%	3 out of 12983 background genes, 0.0%	0.00497	0.00%
snoRNA catabolic process	3 out of 263 genes, 1.1%	3 out of 12983 background genes, 0.0%	0.00497	0.00%
nuclear polyadenylation-dependent snoRNA catabolic process	3 out of 263 genes, 1.1%	3 out of 12983 background genes, 0.0%	0.00497	0.00%
nuclear polyadenylation-dependent snRNA catabolic process	3 out of 263 genes, 1.1%	3 out of 12983 background genes, 0.0%	0.00497	0.00%
macromolecular complex assembly	25 out of 263 genes, 9.5%	467 out of 12983 background genes, 3.6%	0.00581	0.00%
snRNA metabolic process	6 out of 263 genes, 2.3%	26 out of 12983 background genes, 0.2%	0.0065	0.00%
assembly of large subunit precursor of preribosome	4 out of 263 genes, 1.5%	8 out of 12983 background genes, 0.1%	0.00655	0.00%
transcription of nuclear large rRNA transcript from RNA polymerase I promoter	5 out of 263 genes, 1.9%	16 out of 12983 background genes, 0.1%	0.00725	0.00%
polyadenylation-dependent ncRNA catabolic process	5 out of 263 genes, 1.9%	17 out of 12983 background genes, 0.1%	0.0101	0.02%
nuclear ncRNA surveillance	5 out of 263 genes, 1.9%	17 out of 12983 background genes, 0.1%	0.0101	0.02%
nuclear polyadenylation-dependent ncRNA catabolic process	5 out of 263 genes, 1.9%	17 out of 12983 background genes, 0.1%	0.0101	0.02%
CUT catabolic process	4 out of 263 genes, 1.5%	9 out of 12983 background genes, 0.1%	0.0116	0.04%
CUT metabolic process	4 out of 263 genes, 1.5%	9 out of 12983 background genes, 0.1%	0.0116	0.04%
mitochondrial RNA processing	4 out of 263 genes, 1.5%	9 out of 12983 background genes, 0.1%	0.0116	0.04%
pre-replicative complex assembly involved in cell cycle DNA replication	5 out of 263 genes, 1.9%	20 out of 12983 background genes, 0.2%	0.02409	0.06%
pre-replicative complex assembly	5 out of 263 genes, 1.9%	20 out of 12983 background genes, 0.2%	0.02409	0.06%
pre-replicative	5 out of 263	20 out of 12983	0.02409	0.06%

complex assembly involved in nuclear cell cycle DNA replication	genes, 1.9%	background genes, 0.2%		
cellular component assembly	28 out of 263 genes, 10.6%	612 out of 12983 background genes, 4.7%	0.02979	0.06%
cell cycle DNA replication	6 out of 263 genes, 2.3%	34 out of 12983 background genes, 0.3%	0.03314	0.06%
nuclear mRNA surveillance of mRNA 3'-end processing	3 out of 263 genes, 1.1%	5 out of 12983 background genes, 0.0%	0.04823	0.08%
mitochondrial RNA metabolic process	5 out of 263 genes, 1.9%	23 out of 12983 background genes, 0.2%	0.04974	0.08%
peptidyl-histidine modification	4 out of 263 genes, 1.5%	13 out of 12983 background genes, 0.1%	0.06174	0.12%
RNA surveillance	5 out of 263 genes, 1.9%	26 out of 12983 background genes, 0.2%	0.09252	0.17%
nuclear RNA surveillance	5 out of 263 genes, 1.9%	26 out of 12983 background genes, 0.2%	0.09252	0.17%
nuclear polyadenylation-dependent mRNA catabolic process	3 out of 263 genes, 1.1%	6 out of 12983 background genes, 0.0%	0.09502	0.21%
polyadenylation-dependent mRNA catabolic process	3 out of 263 genes, 1.1%	6 out of 12983 background genes, 0.0%	0.09502	0.20%
ncRNA 3'-end processing	6 out of 263 genes, 2.3%	41 out of 12983 background genes, 0.3%	0.09839	0.20%

Table C10. Opaque-repressed GO terms.

<i>GO term</i>	<i>Cluster frequency</i>	<i>Background frequency</i>	<i>P-value</i>	<i>FDR</i>
carbon utilization	2 out of 18 genes, 11.1%	16 out of 12983 background genes, 0.1%	0.01297	34.00%

Table C11. Strains used in this study. *TDH3* and *MAL2* strains were generated from transformation of white cells (CAY4599) or opaque cells (CAY4600) as noted in phenotype.

Strain(s)	Phenotype	Genotype	MTL	Strain Background
CAY675		WT	a/α	ATCC 34139 ST-120
CAY2449		WT	a/a	ATCC20663.084
CAY2569		WT	a/a	b31083/6/04
CAY2582		WT	a/a	WC04-200748
CAY2591		WT	a/a	SCS501148
CAY2597		WT	a/a	AM2005/0093
CAY1503/1504		<i>arg4::SAT1/arg4::SAT1^R</i>	a/a	ATCC 34139 ST-120
CAY3371/3384	White	<i>arg4::SAT1/arg4::SAT1^R</i>	a/a	ATCC 34139 ST-120
CAY3372/3385	Smooth white	<i>arg4::SAT1/arg4::SAT1^R</i>	a/a	ATCC 34139 ST-120
CAY3381/3389	Intermediate	<i>arg4::SAT1/arg4::SAT1^R</i>	a/a	ATCC 34139 ST-120
CAY3373/3386	Opaque	<i>arg4::SAT1/arg4::SAT1^R</i>	a/a	ATCC 34139 ST-120
CAY3374	Wrinkled opaque	<i>arg4::SAT1/arg4::SAT1^R</i>	a/a	ATCC 34139 ST-120
CAY1505/1506		<i>his1::SAT1/his1::SAT1^R</i>	α/α	ATCC 34139 ST-120
CAY3051/3391	White	<i>his1::SAT1/his1::SAT1^R</i>	α/α	ATCC 34139 ST-120
CAY3393/3394	Intermediate	<i>his1::SAT1/his1::SAT1^R</i>	α/α	ATCC 34139 ST-120
CAY3053/3392	Opaque	<i>his1::SAT1/his1::SAT1^R</i>	α/α	ATCC 34139 ST-120
CAY6023	Wrinkled opaque	<i>his1::SAT1/his1::SAT1^R</i>	α/α	ATCC 34139 ST-120
CAY4599	White	AM2005/0093	a/a	ATCC 34139 ST-120
CAY4600	Opaque	AM2005/0093	a/a	ATCC 34139 ST-120
CAY4787		<i>arg4/arg4::FRT</i> <i>his1/his1::FRT</i>	a/a	ATCC 34139 ST-120
CAY2824/2825		<i>arg4/arg4/his1/his1::FRT</i> <i>wor2::ARG4/wor2::HIS1</i>	a/a	ATCC 34139 ST-120
CAY2868/2870		<i>arg4/arg4/his1/his1::FRT</i> <i>wor3::ARG4/wor3::HIS1</i>	a/a	ATCC 34139 ST-120
CAY3601/3604		<i>arg4/arg4/his1/his1::FRT</i> <i>czf1::ARG4/czf1::HIS1</i>	a/a	ATCC 34139 ST-120
CAY4508/4509		<i>arg4/arg4/his1/his1::FRT</i> <i>efh1::ARG4/efh1::HIS1</i>	a/a	ATCC 34139 ST-120
CAY4927/4928		<i>arg4/arg4/his1/his1::FRT</i> <i>tan1::ARG4/tan1::HIS1</i>	a/a	ATCC 34139 ST-120
CAY5131/5132		<i>arg4/arg4/his1/his1::FRT</i> <i>rme1::ARG4/rme1::HIS1</i>	a/a	ATCC 34139 ST-120
CAY4931/4932		<i>arg4/arg4/his1/his1::FRT</i> <i>zcf18::ARG4/zcf18::HIS1</i>	a/a	ATCC 34139 ST-120
CAY4934/4935		<i>arg4/arg4/his1/his1::FRT</i> <i>ask10::ARG4/ask10::HIS1</i>	a/a	ATCC 34139 ST-120

CAY5145/5161		<i>arg4/arg4/his1/his1::FRT fgr27::ARG4/fgr27::HIS1</i>	a/a	ATCC 34139 ST-120
CAY4938/4939		<i>arg4/arg4/his1/his1::FRT ume6::ARG4/ume6::HIS1</i>	a/a	ATCC 34139 ST-120
CAY5608/5609	White	<i>TDH3-GFP::SAT1^R</i>	a/a	AM2005/0093
CAY5606/5608	White	<i>TDH3-TDH3::SAT1^R</i>	a/a	AM2005/0093
CAY5592/5593	White	<i>TDH3-WOR1::SAT1^R</i>	a/a	AM2005/0093
CAY5594/5595	White	<i>TDH3-WOR2::SAT1^R</i>	a/a	AM2005/0093
CAY5596/5597	White	<i>TDH3-WOR3::SAT1^R</i>	a/a	AM2005/0093
CAY5598/5600	White	<i>TDH3-CZF1::SAT1^R</i>	a/a	AM2005/0093
CAY5602/5603	White	<i>TDH3-EFH1::SAT1^R</i>	a/a	AM2005/0093
CAY5586/5587	White	<i>TDH3-AHR1::SAT1^R</i>	a/a	AM2005/0093
CAY5650/5651	White	<i>TDH3-CTRG_03621::SAT1^R</i>	a/a	AM2005/0094
CAY5578/5579	White	<i>TDH3-TAN1::SAT1^R</i>	a/a	AM2005/0093
CAY5590/5591	White	<i>TDH3-NDT802::SAT1^R</i>	a/a	AM2005/0093
CAY5582/5583	White	<i>TDH3-ASK10::SAT1^R</i>	a/a	AM2005/0093
CAY5580/5581	White	<i>TDH3-ZCF18::SAT1^R</i>	a/a	AM2005/0093
CAY5584/5585	White	<i>TDH3-ZCF22::SAT1^R</i>	a/a	AM2005/0093
CAY5588/5589	White	<i>TDH3-FGR27::SAT1^R</i>	a/a	AM2005/0093
CAY5648/5649	Opaque	<i>TDH3-GFP::SAT1^R</i>	a/a	AM2005/0093
CAY5646/5648	Opaque	<i>TDH3-TDH3::SAT1^R</i>	a/a	AM2005/0094
CAY5625/5627	Opaque	<i>TDH3-WOR1::SAT1^R</i>	a/a	AM2005/0093
CAY5628/5630	Opaque	<i>TDH3-WOR2::SAT1^R</i>	a/a	AM2005/0093
CAY5632/5633	Opaque	<i>TDH3-WOR3::SAT1^R</i>	a/a	AM2005/0093
CAY5634/5636	Opaque	<i>TDH3-CZF1::SAT1^R</i>	a/a	AM2005/0093
CAY5638/5640	Opaque	<i>TDH3-EFH1::SAT1^R</i>	a/a	AM2005/0093
CAY5652/5653	Opaque	<i>TDH3-AHR1::SAT1^R</i>	a/a	AM2005/0093
CAY5612/5613	Opaque	<i>TDH3-CTRG_03621::SAT1^R</i>	a/a	AM2005/0094
CAY5610/5611	Opaque	<i>TDH3-TAN1::SAT1^R</i>	a/a	AM2005/0093
CAY5622/5624	Opaque	<i>TDH3-NDT802::SAT1^R</i>	a/a	AM2005/0093
CAY5616/5617	Opaque	<i>TDH3-ASK10::SAT1^R</i>	a/a	AM2005/0093
CAY5614/5615	Opaque	<i>TDH3-ZCF18::SAT1^R</i>	a/a	AM2005/0093
CAY5618/5619	Opaque	<i>TDH3-ZCF22::SAT1^R</i>	a/a	AM2005/0093
CAY5620/5621	Opaque	<i>TDH3-FGR27::SAT1^R</i>	a/a	AM2005/0093
CAY5196/5197	White	<i>MAL2-YFP::SAT1^R</i>	a/a	AM2005/0093
CAY5234/5235	White	<i>MAL2-WOR1::SAT1^R</i>	a/a	AM2005/0093
CAY5236/5237	White	<i>MAL2-WOR2::SAT1^R</i>	a/a	AM2005/0093
CAY5187/5188	White	<i>MAL2-WOR3::SAT1^R</i>	a/a	AM2005/0093
CAY5189/5190	White	<i>MAL2-CZF1::SAT1^R</i>	a/a	AM2005/0093
CAY5193/5194	White	<i>MAL2-EFH1::SAT1^R</i>	a/a	AM2005/0093

CAY5322/5323	White	<i>MAL2-AHR1::SAT1^R</i>	a/a	AM2005/0093
CAY5220/5221	White	<i>MAL2-TAN1::SAT1^R</i>	a/a	AM2005/0093
CAY5181/5182	White	<i>MAL2-UME6::SAT1^R</i>	a/a	AM2005/0093
CAY5232/5233	White	<i>MAL2-NDT802::SAT1^R</i>	a/a	AM2005/0093
CAY5172/5173	White	<i>MAL2-ASK10::SAT1^R</i>	a/a	AM2005/0093
CAY5164/5165	White	<i>MAL2-RME1::SAT1^R</i>	a/a	AM2005/0093
CAY5168/5169	White	<i>MAL2-ZCF18::SAT1^R</i>	a/a	AM2005/0093
CAY5176/5177	White	<i>MAL2-ZCF22::SAT1^R</i>	a/a	AM2005/0093
CAY5226/5227	White	<i>MAL2-FGR27::SAT1^R</i>	a/a	AM2005/0093
CAY5893/5894	White	<i>MAL2-CTRG_03621::SAT1^R</i>	a/a	AM2005/0093
CAY5198/5199	Opaque	<i>MAL2-YFP::SAT1^R</i>	a/a	AM2005/0093
CAY5321/5322	Opaque	<i>MAL2-WOR1::SAT1^R</i>	a/a	AM2005/0093
CAY5323/5324	Opaque	<i>MAL2-WOR2::SAT1^R</i>	a/a	AM2005/0093
CAY5238/5240	Opaque	<i>MAL2-WOR3::SAT1^R</i>	a/a	AM2005/0093
CAY5191/5192	Opaque	<i>MAL2-CZF1::SAT1^R</i>	a/a	AM2005/0093
CAY5242/5243	Opaque	<i>MAL2-EFH1::SAT1^R</i>	a/a	AM2005/0093
CAY5224/5225	Opaque	<i>MAL2-AHR1::SAT1^R</i>	a/a	AM2005/0093
CAY5162/5163	Opaque	<i>MAL2-TAN1::SAT1^R</i>	a/a	AM2005/0093
CAY5230/5309	Opaque	<i>MAL2-UME6::SAT1^R</i>	a/a	AM2005/0093
CAY5317/5318	Opaque	<i>MAL2-NDT802::SAT1^R</i>	a/a	AM2005/0093
CAY5174/5175	Opaque	<i>MAL2-ASK10::SAT1^R</i>	a/a	AM2005/0093
CAY5166/5167	Opaque	<i>MAL2-RME1::SAT1^R</i>	a/a	AM2005/0093
CAY5170/5171	Opaque	<i>MAL2-ZCF18::SAT1^R</i>	a/a	AM2005/0093
CAY5178/5179	Opaque	<i>MAL2-ZCF22::SAT1^R</i>	a/a	AM2005/0093
CAY5228/5229	Opaque	<i>MAL2-FGR27::SAT1^R</i>	a/a	AM2005/0093
CAY5959/5960	Opaque	<i>MAL2-CTRG_03621::SAT1^R</i>	a/a	AM2005/0093

Table C12. Plasmids used in this study.

<i>Plasmid</i>	<i>Name</i>	<i>Oligos Used or Reference</i>
pRB55	<i>HIS1</i> Deletion Plasmid	Porman <i>et al.</i> 2011
pRB59	<i>ARG4</i> Deletion Plasmid	Porman <i>et al.</i> 2011
pRB354	<i>AHR1</i> Deletion Plasmid	33/34, 35/36
pRB236	<i>TDH3-GFP</i>	93/94, 97/98
pRB367	<i>TDH3-TDH3</i>	93/95
pRB235	<i>TDH3-WOR1</i>	93/94, 99/100
pRB238	<i>TDH3-WOR2</i>	93/94, 101/102
pRB239	<i>TDH3-WOR3</i>	93/94, 103/104
pRB240	<i>TDH3-CZF1</i>	93/94, 105/106
pRB241	<i>TDH3-EFH1</i>	93/94, 107/108
pRB379	<i>TDH3-AHR1</i>	93/94, 109/110
pRB374	<i>TDH3-TAN1</i>	93/94, 111/112
pRB389	<i>TDH3-NDT802</i>	93/94, 113/114
pRB377	<i>TDH3-ASK10</i>	93/94, 115/116
pRB375	<i>TDH3-ZCF18</i>	93/94, 117/118
pRB378	<i>TDH3-ZCF22</i>	93/94, 119/120
pRB445	<i>TDH3-FGR27</i>	93/94, 121/122
pRB324	<i>MAL2-YFP</i>	123/124,
pRB399	<i>MAL2-WOR1</i>	123/124, 128/129
pRB412	<i>MAL2-WOR2</i>	123/124, 130/131
pRB413	<i>MAL2-WOR3</i>	123/124, 132/133
pRB414	<i>MAL2-CZF1</i>	123/124, 134/135
pRB415	<i>MAL2-EFH1</i>	123/124, 136/137
pRB408	<i>MAL2-AHR1</i>	123/124, 138/139
pRB403	<i>MAL2-TAN1</i>	123/124, 140/141
pRB410	<i>MAL2-UME6</i>	123/124, 142/143
pRB411	<i>MAL2-NDT802</i>	123/124, 144/145
pRB406	<i>MAL2-ASK10</i>	123/124, 146/147
pRB404	<i>MAL2-RME1</i>	123/124, 148/149
pRB405	<i>MAL2-ZCF18</i>	123/124, 150/151
pRB407	<i>MAL2-ZCF22</i>	123/124, 152/153
pRB409	<i>MAL2-FGR27</i>	123/124, 154/155
pRB427	<i>MAL2-CTRG_03621</i>	123/124, 156/157

Table C13. Oligonucleotides used in this study. Restriction sites are underlined.

<i>Oligo</i>	<i>Name</i>	<i>Sequence</i>
1	Ct WOR2 5' fwd (-700)	ACTTATAGTTATATAAAATCCGACAT
2	Ct WOR2 5' rev (0)	cacggcgcgccctagcagcggCTGTTGCTGATTAGGATTACTG
3	Ct WOR2 3' fwd (+800)	gtcagcgggccgcatccctgcTACCAGAGGTGTAAATCCGATG
4	Ct WOR2 3' rev (+1300)	TAATTTGTTCTAAAGTTTCATTTTAAT
5	Ct WOR2 5' check (-750)	TCAACTGCAACTAAATACATTCC
6	Ct WOR2 3' check (+1350)	TTACAATCATTTGTCTGAAAATTTTC
7	Ct WOR2 5' orf (+200)	ACGTTCTCATAGGTCCTGGTCA
8	Ct WOR2 3' orf (+600)	TTAGGAGGTAATATTCCTGAAG
9	Ct WOR3 5' fwd (-500)	TCTCCATCTGACACCAAAATAGA
10	Ct WOR3 5' rev (0)	cacggcgcgccctagcagcggGGTATTAGGTCTATAGTAATTAGC
11	Ct WOR3 3' fwd (+1730)	gtcagcgggccgcatccctgcAGTTGAATAAATGTATGGGACAA
12	Ct WOR3 3' rev (+2230)	TGTGCTCCTCCTCCCGCTTTTA
13	Ct WOR3 5' check (-550)	GACCCCTAATATCTAGAACAATT
14	Ct WOR3 3' check (+2280)	TGCGGTGGTACAAATACCCCACT
15	Ct WOR3 5' orf (+200)	TCTCAACAACCAGGAGTATATG
16	Ct WOR3 3' orf (+600)	AGAAGATGCAGATGAAATACTA
17	Ct CZF1 5' fwd (-500)	CAATCAACTTGCCACACTCAT
18	Ct CZF1 5' rev (0)	cacggcgcgccctagcagcggGATTTGTGTAAATATTATGCTAAATC
19	Ct CZF1 3' fwd (+1180)	gtcagcgggccgcatccctgcGAACCAAAGGAGAACAATGGTATA
20	Ct CZF1 3' rev (+1680)	TGTTGGTTCTGCTGAGCTCTCCT
21	Ct CZF1 5' check (-550)	TGTATTCTGATCTATTGGCGTTAA
22	Ct CZF1 3' check (+1720)	TGGGCGACTGTGATTTTCAGAAT
23	Ct CZF1 5' orf (+200)	AAATGTTCCAGTTCCAGGCCCT
24	Ct CZF1 3' orf (+600)	GGCAACAGGATATTGTTGAACA
25	Ct EFH1 5' fwd (-550)	GCTATTGACTTACCAGTTTCCT
26	Ct EFH1 5' rev (0)	cacggcgcgccctagcagcggTTATAAATCGAGCAATGTATTTTC
27	Ct EFH1 3' fwd (+3000)	gtcagcgggccgcatccctgcACTTTGGATCTTTACTTTTTATATG
28	Ct EFH1 3' rev (+2550)	GAGGTCCAGGAATTCAACATAT
29	Ct EFH1 5' check (-600)	ATCTATATTCGGTTTATGAGTTCTT
30	Ct EFH1 3' check (+2600)	ATGCCAATAAATGAACCAGTTAAA
31	Ct EFH1 5' orf +200	CAACTACAACCTCAACAACAAT
32	Ct EFH1 3' orf +600	TATGTACTTGAAGTTGCATTAGGA
33	Ct Ahr1 (ApaI) -500	ggagcggggcccTTCCCTTGGATAGACTGGAACA
34	Ct Ahr1 (XhoI) 0	gccggcctcgagCTGAGGGTTGACAGGTTTAGT
35	Ct Ahr1 (SacII) +2700	ggagcgccgaggTTTTGCCACGACGTGTCTTT
36	Ct Ahr1 (SacI) +3250	ggccgagagctcACTGTTTGTGTCTCTCTGAATCT
37	Ct Ahr1 5' check -600	ACTGCTGTTACTACTACTACTGC
38	Ct Ahr1 3' check -3300	GATTTCGATTTGGGATAGATTCA

39	Ct Ahr1 5' ORF +600	CAACAGCAACAACAGCAACC
40	Ct Ahr1 3' ORF +1000	GTTGTGGATGAGGAGGTGGT
41	Ct TAN1 5' fwd (-480)	TTCAAGATATTTCCAATAATAT
42	Ct TAN1 5' rev (0)	cacggcgcgccctagcagcggATTGTTCTTAGGTTATTGACG
43	Ct TAN1 3' fwd (+1000)	gtcagcgggccgcatccctgcTTAGCTTGGATTATTTTCAGTAG
44	Ct TAN1 3' rev (+1500)	CAAAGCCAAAAATCATGGACAC
45	Ct TAN1 5' chk (-550)	AGGTGGTAGGTAATTATCATC
46	Ct TAN1 3' chk (+1560)	ACTTCTCAAAAAGTTGTTCGTC
47	Ct Tan1 5' ORF (+200)	TCTGATAAAATCCCTGAATATTTTGA
48	Ct Tan1 3' ORF (+600)	GATTGAAATTTCTCTTTGATACTTGA
49	Ct UME6 5' fwd (-500)	CTACCACCAGACCATCTCAA
50	Ct UME6 5' rev (0)	cacggcgcgccctagcagcggTTAAGACCAAAATTTTTTTATTAAT
51	Ct UME6 3' fwd (+2000)	gtcagcgggccgcatccctgcGTTCTATTTGCCAGACTATAAGAA
52	Ct UME6 3' rev (+2500)	CTTGATTATATAGATTAGTAGAC
53	Ct UME6 5' chk (-580)	ACACGACTATCCGAAACAGG
54	Ct UME6 3' chk (+2550)	TTCAAAGTATATAGCGTTTAAA
55	Ct Ume6 5' ORF (+200)	ACATCAACAATATCTGCAACAAGT
56	Ct Ume6 3' ORF (+600)	CATAATAGATTGCGATTGGGGAT
57	Ct NDT802 5' fwd (-450)	TACGTATCAACGCTGGGATA
58	Ct NDT802 5' rev (0)	cacggcgcgccctagcagcggGATTTAGCCGCTGATGTAAT
59	Ct NDT802 3' fwd (+1500)	gtcagcgggccgcatccctgcATCTATTGCTACAATTAGTT
60	Ct NDT802 3' rev (+2000)	CTAATCGTTGCATGTATCGAT
61	Ct NDT802 5' chk (-550)	ATCAGCACTCATCATCAAAATA
62	Ct NDT802 3' chk (+2050)	AGCTAAATGTCTGAATAAGGTTA
63	Ct NDT802 5' orf	TCTCGAATTGATCGTGGGTTTG
64	Ct NDT802 3' orf	CTGAATTTGGTTGTAAATTCGCAA
65	Ct ASK10 5' fwd (-500)	TCATCAGCGGATCAACTCTA
66	Ct ASK10 5' rev (0)	cacggcgcgccctagcagcggGATTTAGCATTTGTTTAGATAA
67	Ct ASK10 3' fwd (+2500)	gtcagcgggccgcatccctgcACCTTAACGAGTATAGCATTA
68	Ct ASK10 3' rev (+3000)	TCATGAATGCTTGTGCAAC
69	Ct Ask10 5' ORF (+200)	CTTGTCAAAGAATTAGAAGCGAAT
70	Ct Ask10 3' ORF (+600)	AGTCGGTTCAAGTTTAGGGATTG
71	Ct RME1 5' fwd (-500)	ATTACCATCAAACGTTTTTGC
72	Ct RME1 5' rev (0)	cacggcgcgccctagcagcggGTTTGCAGGAAAAAGGAATTG
73	Ct RME1 3' fwd (+1550)	gtcagcgggccgcatccctgcAGTAAAGTTGTTCGTTATAATCT
74	Ct RME1 3' rev (+2000)	TCTGCTTGTATCCTTGAATT
75	Ct RME1 5' chk (-550)	AACGAATCCCCCTTTCCACT
76	Ct RME1 3' chk (+2050)	TAATGAGGACATACTTTAATC
77	Ct ZCF18 5' fwd (-500)	TCAATTGAAGAATTGTTTGTAC
78	Ct ZCF18 5' rev (0)	cacggcgcgccctagcagcggTGAAGAAGCTTGAGTCAATAC
79	Ct ZCF18 3' fwd (+1250)	gtcagcgggccgcatccctgcGCATGAGCCGAAATACACAT

80	Ct ZCF18 3' rev (+1750)	AGCACCTACTAGTGTTC
81	Ct ZCF18 5' chk (-550)	CGAACGAGGCTGAAGCCAAA
82	Ct ZCF18 3' chk (+1800)	CCGATGAATAAAGTGATTAAAC
83	Ct Zcf18 5' ORF (+200)	TGCAAGGATTGGATGCAGCT
84	Ct Zcf18 3' ORF (+600)	GCCGAGTGCTTTGAAGAATAATA
85	Ct FGR27 5' fwd (-500)	GTGAAGCGAAGGAGCGAAAT
86	Ct FGR27 5' rev (0)	cacggcgcgcctagcagcggGCCTAATGATTTCTTTATCCGT
87	Ct FGR27 3' fwd (+2650)	gtcagcggcgcgcctcctgcACGTGACTTGCGTCAGTATGG
88	Ct FGR27 3' rev (+3150)	GATTGGAATACTAAGAAGAATT
89	Ct FGR27 5' chk (-550)	TGCATGGTGAATGGCAGGAG
90	Ct FGR27 3' chk (+3200)	TGGATATTTCAATAATACTAAT
91	Ct FGR27 5' ORF (+300)	AGCATTACCAACCACCTCCA
92	Ct FGR27 3' ORF (+700)	TATTGACTTCCGACGCGTTC
93	Ct TDH3 (-950) ApaI	ggagcggggcccGTTAGTCGTTATGGAGGACA
94	Ct TDH3 (0)	TGTTAAAATTTAATTTGTAAGTGATTTGATTT
95	Ct TDH3 (+2300) XhoI	gccggcctcgagTGTGATGACATTGTATAGCTCGT
96	Ct TDH3 5' check (-1050)	
97	GFP (0)	aaatcaaatcacttacaaattaaattttaacaGGT ATGT CTAAAGGTGAA GAAT
98	GFP (+950) XhoI	gccggcctcgagTAGAGTCGGAAACCAATATCAA
99	Ct WOR1 (0)	aaatcaaatcacttacaaattaaattttaacatcc ATGT CGGCGTCTAGAT TATCATC
100	Ct WOR1 (+2500) XhoI	gccggcctcgagGTCATATACCCAACACCGAAC
101	Ct WOR2 (0)	aaatcaaatcacttacaaattaaattttaacaCAG ATGA ACTCTCCAGTT ACTT
102	Ct WOR2 (+1000) XhoI	gccggcctcgagACGGATAAACAGTTTGTGATAG
103	Ct WOR3 (0)	aaatcaaatcacttacaaattaaattttaacaACC ATGG ATCAACCCTAT TTAG
104	Ct WOR3 (+2000) XhoI	gccggcctcgagCTACAAAGTAATCATCATTCAC
105	Ct CZF1 (0)	aaatcaaatcacttacaaattaaattttaacaATC ATGAG TAATTTATCT AATC
106	Ct CZF1 (+1400) XhoI	gccggcctcgagAACAGTAACAATACAACCCATT
107	Ct EFH1 (0)	aaatcaaatcacttacaaattaaattttaacaTAA ATGA ATGAAATTATG ACAG
108	Ct EFH1 (+2100) XhoI	gccggcctcgagACTGAATAGTACATATAAAAAGT
109	Ct Ahr1 (0)	aaatcaaatcacttacaaattaaattttaacatccATC ATGG CAAAGAAAA AATTAAATCC
110	Ct Ahr1 (+2700) XhoI	gccggcctcgagAAAGACACGTCGTGGCAAAA
111	Ct Tan1 (0)	aaatcaaatcacttacaaattaaattttaacaAAT ATGGG TAAAAGAGCA GCTGA
112	Ct Tan1 (+1000) XhoI	gccggcctcgagCTACTTAATATGGCAATTGGGG
113	Ct Ndt802 (0)	aaatcaaatcacttacaaattaaattttaacaATC ATGG CCAATAGAACC AAGTC
114	Ct Ndt802 (+1600) XhoI	gccggcctcgagCAACATGACTAAACGACTGAATTTAT
115	Ct Ask10 (0)	aaatcaaatcacttacaaattaaattttaacaATC ATGG CTACATCACCT ACTAGTT

116	Ct Ask10 (+2500) XhoI	gccggcctcgagATGTGAATATAACTCCTACGTCTG
117	Ct Zcf18 (0)	aaatcaaactacttacaataattttaacaTCAATGTTGGAATTTCC CGATTTC
118	Ct Zcf18 (+1400) XhoI	gccggcctcgagGTGCAGATCTGGAAATAGGTTC
119	Ct Zcf22 (0)	aaatcaaactacttacaataattttaacatccACCATGACTGGTCTAC AGAATA
120	Ct Zcf22 (+1900) XhoI	gccggcctcgagACGAAGACTAGAAGATGTCCGA
121	Ct FGR27 (0)	aaatcaaactacttacaataattttaacaGGCATGTCATCTACTATA CATTC
122	Ct FGR27 (+2650) XhoI	gccggcctcgagTGGGTTCCATACTGACGCAA
123	Ct MAL2 (-750) KpnI	ggcgccggtaccGCTTTGGGTATTTTGTGAG
124	Ct MAL2 (0) ApaI	gccggcgggcccCATAATTAATAGTTTTGTTGAAGA
125	Ct MAL2 5' check (-800)	GCTTGAAAAATCATCAACAG
126	YFP (0) ApaI	
127		
128	Ct Wor1 (0) ApaI	gccggcgggcccATGTCGGCGTCTAGATTAT
129	Ct Wor1 (+2500) BamHI	ggcgccggtaccCCCAACACCGAACGGAAT
130	Ct Wor2 for (0) ApaI	ggcgccgggcccATGAACTCTCCAGTTACTTC
131	Ct Wor2 rev (+1000) BamHI	ggagcgggatccGAACGACATAACGAAACGGA
132	Ct Wor3 for (0) ApaI	ggcgccgggcccATGGATCAACCCTATTTAGAG
133	Ct Wor3 rev (+2000) BamHI	ggagcgggatccCTACAAAGTAATCATCATTCAC
134	Ct Czf1 for (0) ApaI	ggcgccgggcccATGAGTAATTTATCTAATCCTAAT
135	Ct Czf1 rev (+1400) BamHI	ggagcgggatccAACAGTAACAATACAACCCATT
136	Ct Efh1 for (0) ApaI	ggcgccgggcccATGAATGAAATTATGACAGCTTC
137	Ct Efh1 rev (+2100) BamHI	ggagcgggatccACTGAATAGTACATATAAAAAGT
138	Ct Ahr1 (0) ApaI	ggcgccgggcccATGGCAAAGAAAAAATTAATCC
139	Ct Ahr1 (+2700) BamHI	ggagcgggatccATGGGCAAATTAGTAGACAATA
140	Ct Tan1 for (0) ApaI	ggcgccgggcccATGGGTAAAAGAGCAGCTG
141	Ct Tan1 rev (+1000) BamHI	ggagcgggatccCTACTTAATATGGCAATTGGGG
142	Ct Ume6 (0) ApaI	ggcgccgggcccATGATTACACATATGGTTACAC
143	Ct Ume6 (+2270) BamHI	ggagcgggatccTAGTGAGTCACCAAATGAAC
144	Ct Ndt802 (0) ApaI	ggcgccgggcccATGGCCAATAGAACCAAGT
145	Ct Ndt802 (+1600) BamHI	ggagcgggatccCAACATGACTAAACGACTGAA
146	Ct Ask10 for (0) ApaI	ggcgccgggcccATGGCTACATCACCTACTAG
147	Ct Ask10 rev (+2580) BamHI	ggagcgggatccCGTTAAACATCGATGCCG
148	Ct Rme1 for (0) ApaI	ggcgccgggcccATGGTTGGTTATAACTTGATAG
149	Ct Rme1 rev (+1350) BamHI	ggagcgggatccGAATATTTGGAATATTGAGCAC
150	Ct Zcf18 for (0) ApaI	ggcgccgggcccATGTTGGAATTTCCCGATT
151	Ct Zcf18 (+1400) BamHI	ggagcgggatccGTGCAGATCTGGAAATAGGTTC
152	Ct Zcf22 for (0) ApaI	ggcgccgggcccATGACTGGTCTACAGAATAATC
153	Ct Zcf22 rev (+2000) BamHI	ggagcgggatccTCATTCGGTATTTGCAGATAC
154	Ct Fgr27 for (0) ApaI	ggcgccgggcccATGTCATCTACTATACATTGAG
155	Ct Fgr27 rev (+2660) BamHI	ggagcgggatccGGGTTCCATACTGACGCA

156	Ct 03621 for (0) ApaI	ggccgcgggcccATGGATTCGGGCAATAACC
157	Ct 03621 rev (+1900) BglII	ggagcgagatctCTGTATCTAATATGAGTTCCA