Functional analysis of metabolic circuits and drug response in *Candida albicans*

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Abstract

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Candida albicans, an opportunistic pathogenic fungus, is a leading cause of nosocomial infections. In last two decades, the rise of antifungal resistance calls for better understanding of genetic networks responsible for mechanisms of resistance. Genetic analyses have been fundamental in unveiling cell adaptation mechanisms and transcriptional rewiring. High-throughput data from the Gene Replacement and Conditional Expression 1.0 collection of gene inactivations identified strains resistant to the common antifungal fluconazole, including *RAP1, ERG3* and *HCS1*. Using CRISPR-Cas9, we generated targeted single and double deletion mutants of these genes and studied their response to fluconazole treatment. Our study reveals functional diversity in strains lacking *RAP1*. RAP1 deficiency also causes colony size heterogeneity and morphological changes to pseudohyphae. We also utilized an activated transcription factor library to perform a high-throughput screenings with different stressors including pH, heavy metal tolerance, and fluconazole. We were able to find interesting phenotypic responses such as four TFs whose activation conferred resistance to hydroxyurea, eight involved in pH response and six in temperature response. Moreover, one strain with transcription factor Adr1 activated showed multidrug resistance, and we identified a change in function rewiring from control of ergosterol biosynthesis in *Candida albicans* to control of alcohol and fatty acid metabolism in *Saccharmocyes cerevisiae*. Our findings thus highlight two genes with involvement in fluconazole response which can provide new insight to the transcriptional regulation of *C. albicans* and potentially direct more effective treatments.

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Contribution of Authors

I am the principal author of this thesis and responsible for all conception and experimentation from this thesis project, under supervision of Dr. Malcolm Whiteway.

Appendix 1: The Adr1 transcription factor directs regulation of the ergosterol pathway and azole resistance in *Candida albicans*.

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I primarily collaborated with the first author of Shrivastava et al., 2023, Dr. Manjari Shrivastava. Shrivastava constructed the VPR activated transcription factor library and was the primary investigator of this project through its conceptualization and data analysis.

This project was supervised by Dr. Malcolm Whiteway. Other assistance and contribution of co-authors is listed below:

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List of Abbreviations

After Wash	AW
Ampicillin	AMP
Arginine	ARG
Aspect ratio	AR
Base Pairs	bp
Before Wash	BW
Calcofluor White	CFW
Candida Genome Database	CGD
Clustered Regularly Interspaced Short Palindromic Repeats	CRISPR
Coding Sequence	CDS
Colony Forming Unit	CFU
Double-Strand Break	DSB
Gene of Interest	GOI
Gene Replacement and Conditional Expression	GRACE
Luria-Bertani	LB
High-throughput screening	HTS
Histidine	HIS
Homologous-Directed Repair	HDR
Hydroxyurea	HU
N-Acetylglucosamine	GlcNAc
Phosphate-Buffered Saline	PBS
Polymerase Chain Reaction	PCR
Room Temperature	RT
Single guide RNA	sgRNA
Transcription Factors	TFs
Wild Type	WT
Yeast extract, Peptone, Dextrose	YPD
N-Acetylglucosamine	GlcNAc
Restriction Enzyme	RE
Saccharomyces Genome Database	SGD

Sodium Chloride	NaCl
Synthetic Dextrose	SD
Target of Rapamycin	TOR
Ultra-Violet	UV
Yeast nitrogenous base	YNB
Zone of Inhibition	ZOI

Chapter 1 – Introduction

1.1. Fungi, fungal pathogens, and *Candida albicans* as an opportunistic pathogenic fungus on humans

The fungal kingdom is diverse with species ranging from unicellular to multicellular, saprophytes to parasites, and sessile to motile. Although having a wide range of features, fungi have common characteristics that classify them together such as the chitinous cell wall, true nucleus, and heterotrophic metabolism. These organisms are found everywhere, interact amongst themselves and with organisms from other kingdoms, and are key players in the biosphere [1]. The fungal kingdom encompasses nine phyla—Opisthosporidia, Chytridiomycota, Neocallimastigomycota, Blastocladiomycota, Zoopagomycota, Mucoromycota, Glomeromycota, Basidiomycota, and Ascomycota—which exhibit a vast spectrum of genomic and functional diversity, ranging from minimalistic parasites like Opisthosporidia to highly complex, ecologically versatile fungi like those of the Ascomycota and Basidiomycota, all shaped by extensive evolutionary changes [1]. Of these phyla, Ascomycota is the largest with about 66% of fungal species, including extensively studied organisms like *Saccharomyces cerevisiae, Neurospora crassa, Emericella nidulans, Schizosaccharomyces pombe* and *Candida albicans* [2, 3].

Fungi can be symbiotic with mutualistic relationships with other organisms like in mycorrhiza, a symbiotic association of fungi with plant roots, and in lichens, a relationship of a fungus with an algae or cyanobacteria. In plants, mutualistic relationships with fungi help in disease resistance, drought tolerance, and salt tolerance [4]. Fungi like *Penicillium*, *Saccharomyces*, *Aspergillus*, *Rhizopus*, as well as *Neurospora* can be involved in various industrial and research processes such as fermentation and dairy production. Many of the Italian cheese like Gorgonzola, Blu di Capra, Blu del Moncenisio etc. are produced by one of the *Penicillium* species named *Penicillium gravinivasei* [5]. Dramatically, the famous French cheese Camembert is in crisis because of decreases in biodiversity of the fungus *Penicillium biforme* due to cheesemakers' continuous use of the single strain *Pencicillium camemberti* [6].

Other important fungi include the yeast *Kluyveromyces marxianus* which is used by biotechnology companies to break down lignocellulosic biomass sources to produce ethanol [7], and is being engineered here at Concordia to produce fumaric acid from lactose-rich dairy waste. *Saccharomyces cerevisiae,* commonly called baker's or brewer's yeast is the primary producer of ethanol for human consumption and for renewable transportation fuels, as well as for CO_2 production in leavened bread. In addition, because it is easily cultured and genetically manipulated, it frequently serves as an important model organism to study the general molecular genetics of eukaryotes [8, 9]. Multiple times scientists have made use of yeast's advantages to gain insights into diseases by examining genes in *S. cerevisiae* similar to those implicated in human ailments. For example, mutations in the human *RAD51* gene are implicated in breast and ovarian cancers, while studies in yeast have identified that Rad51p plays a role in DNA repair. Thus understanding the molecular mechanisms of protein function in yeast can inform potential therapeutic strategies in humans [10]. While some fungi can cause pathogenesis and dramatically threaten human health, fungal benefits to humans as well as the ecosystem as a whole clearly outweigh their negative aspects [8]. These positive contributions demonstrate the critical roles fungi play in advancing technology and science, as well as maintaining ecological balance.

Still, despite their many beneficial characteristics, there has been considerable focus on their detrimental effects. Parasitic fungi associated with plants, for instance, can significantly impair growth and, in severe cases, lead to plant death, thus disrupting agricultural systems and ecosystems [4]. Up to 150 species of fungi have been documented to cause human infection [11]. Out of the 1.5 million estimated existence of different fungal species in nature [12], apart from *C. albicans***,** there are Aspergillus spp. like *niger*, *fumigatus,* and *flavus* which produce mycotoxins that can lead to death when ingested [13]. The severity such of fungal diseases can vary from superficial to life threatening. Most of these infections are opportunistic because the infecting agents are commonly found as commensal organisms residing as part of their microbial flora of the human host. For example *Candida albicans*, of same Ascomycota family as *S. cerevisiae,* is a member of the normal microflora of the human oral, gastrointestinal and urinogenital tracts, but can cause serious infections in immunocompromised individuals [14, 15].

This dual nature underscores the complexity of fungal roles in natural and applied settings. The phylogeny of fungi suggests that species have undergone evolution over a span of a billion years [16]. *C. albicans* belongs to the genus *Candida*, home to other clinically significant species such as *C. dubliniensis*, *C. auris*, *C. tropicalis*, and *C. krusei* which are well-recognized as opportunistic pathogens in humans. Recent studies estimate that *Candida* species alone are responsible for a significant proportion of fungal infections worldwide, underscoring their clinical importance [17]. The genus *Candida* is notable for its ecological and metabolic versatility, as members of this group can inhabit diverse environments, including the human microbiome, where they exist as commensals or pathogens [18].The pathogenicity of these fungi is attributed to their ability to adapt to different host niches, evade immune responses, and resist antifungal treatments, making them a focus of extensive research in medical mycology [19].

Candida infections, designated Candidiasis, now represents the third-to-fourth most frequent hospital acquired infections in the U.S. and worldwide [20-23]. The incidence of systemic candidiasis in the US is approximately 20 cases per 100,000 people (or about 60,000 cases per year) and in high-risk hospitalized patients this incidence increases by a factor of 50. Of note, these rates represent a 20-fold increase compared to just two decades ago, mostly as a result of an expanding population of immunocompromised patients [21, 24, 25]. Disseminated candidiasis carries unacceptably high mortality rates, about 40–60%, even with treatment using antifungal agents. This high mortality may be due to poor diagnosis, inappropriate disease management, associated septic shock, or the general critical condition of the patient.

Although *C. albicans* and *S. cerevisiae* have similar features that group them in the same family, they have distinguishing features that separate them such as their sexual cycle and morphogenesis. *C. albicans* appear to lack the complete meiotic sexual cycle present in *S. cerevisiae.* In addition, *C. albicans* is invasive and involved in pathogenicity in humans; these characteristics are absent in *S. cerevisiae*. Due to these differences, some genes specific to features of *C. albicans* such as hyphal genes like *ECE1*, *HWP1*, *ALS3* do not have homologs in *S. cerevisiae* [26]. *C. albicans* and *S. cerevisiae* diverged roughly 300 million years ago [27, 28]. Evolutionary conservation between the two is 40% based on in-silico studies, and they belong to different clusters in the evolutionary tree of life [29]. *C. albicans* is part of the CTG clade, a group of fungi characterized by their unique use of the CTG codon to encode serine instead of leucine [30]. This divergence in codon usage reflects a significant evolutionary adaptation, further distinguishing *C. albicans* from *S. cerevisiae*. Although some genes have the same biological function in both organisms, the duplication of the *S. cerevisiae* genome means that some genes have redundant or unrelated functions [26]. Some essential genes in *S. cerevisiae* do not have the same role in *C. albicans* and deletion of the same gene in the two organisms may not have the same effect. For example, deletion of *spt3* in *S. cerevisiae* causes defects in pseudohyphal formation but in *C. albicans*, cells remain hyper filamentous [31]. Similarly, multiple genes like *ALS1, ALS3, ECE1, SAP10*, and transcription factors (TFs) like Efg1, Tec1, Wor1, Flo8, Ppr1, Gal4, Tbf, Met28 have shown implications in such functional changes, creating a major difference between *C. albicans* and *S. cerevisiae*, with pathogenicity being the key differentiator [32-39].

S. cerevisiae is a budding yeast that can undergo morphogenesis to pseudohyphae while *C. albicans* can exist in each of yeast, pseudohyphal and true hyphal cells [26]. To be able to adapt to the host cell and its changing microenvironment, *C. albicans* has evolved to transform between many cellular phenotypic forms [40, 41]. These forms include yeast white [42], yeast opaque [43, 44], pseudohyphae [45, 46], and true hyphae [47]. The filamentous form of *C. albicans* is vital for host tissue invasion. This form is induced because of environmental cues that signal the fungus to start forming pseudohyphae and hyphae [48, 49]. The hyphal form allows the fungus's entry into the host's bloodstream, which can cause systemic infections [49]. The hyphal form of the fungus is important to invade epithelial cells and damage tissues, causing disease [50]. The yeast form on the other hand is considered important for dissemination through the bloodstream [26]. Morphological changes contribute to *C. albicans'* pathogenicity in host tissues and involve genetic networks of genes and TFs. For example, the switch from the yeast to pseudohyphal and hyphal forms essential in biofilm formation involves the Hyphae Wall Protein (HWP) family [51]. Such biofilm formation, a virulence factor implicated in many nosocomial and immunocompromised infections, has a genetic network comprising genes involved in biofilm formation at every stage together with their controlling TFs) including Bcr1, Efg1, Tec1, Ndt80, Brg1, and Rob1, which regulate adhesion, morphogenesis, biofilm maturation, and dispersion [51-56].

1.2. Treating *Candida albicans* infections

Recently, the development of drug resistance in *C. albicans* is contributing to the rising death rate caused by *Candida* infections. With the cost of treatments exceeding 2 billion USD per year 10 decades ago [57], studying the evolution of this opportunistic fungus has been a fundamental step in understanding its behavior and may help identify ways to prevent infection.

More effective treatments are needed to overcome tolerance to normal minimal inhibitory concentrations of drugs [58]. Most *C. albicans* infections are nosocomial and can be categorized into two categories: mucosal and systemic [18]. Mucosal infections affect the extremities and other regions with normal flora such as the skin, nails, vagina, oropharynx, and esophagus [18]. Of these, vulvovaginal candidiasis is the most common, occurring even in healthy individuals. On the other hand, onychomycosis (candidiasis of the nail) is not common, and cutaneous candidiasis is rare, occurring in patients with certain inborn immunity defects [18]. Systemic infections are disseminated infections that affect tissues and organs like the blood, central nervous system (CNS), liver, spleen, heart, and kidneys, causing sepsis with mortality exceeding 70% [59].

A common feature of antifungals is their ability to block or destroy key fungal machinery without damaging the host. There are three major classes of antifungals: azoles, polyenes, and echinocandins. Azoles are the most common class of antifungals and the first choice for treatment of invasive infections. Their mechanism of action is to block ergosterol biosynthesis in the fungal membrane by inhibiting the enzyme lanosterol 14α-demethylase encoded by *ERG11*. This causes accumulation of sterol intermediates in the ergosterol pathway including a toxic sterol 14α-methyl-3,6-diol produced by Δ5,6-desaturase (Erg3) [60]. This in turn disrupts the production of ergosterol and inhibits cell growth. There are three generations of azoles, the first generation such as clotrimazole, bifonazole, econazole, and ketoconazole contain imidazole in their ring system. The second-generation class, including itraconazole and the most common antifungal fluconazole, has a triazole moiety [61]. The third and most recent generation of azoles includes voriconazole, posaconazole, and isavuconazole, used for the treatment of aspergillosis and mucormycosis [60]. The mechanism of action of polyenes such as Amphotericin B is by intercalating between ergosterol molecules in the fungal cell membrane. This creates pores that destabilize the fungal membrane causing ion leakage and disrupting the proton gradient [62, 63]. Unfortunately, they can also act on cholesterol membranes leading to toxicity in human cells [62]. Apart from these major classes we also have pyrimidine analogs like 5-flucytosine (5-FC) are antimetabolites that imitate nucleotide bases during the synthesis of nucleosides, disturbing the synthesis of fungal

RNA, DNA, and protein leading to cell cycle arrest [61]. Cytosine deaminase converts 5-FC into its active form 5-fluorouracil which is the toxic compound that interrupts DNA and RNA synthesis. Flucytosine treatment is prone to generate antifungal resistance, so it is often used in combination with other antifungals [62]. Echinocandins are inhibitors of β-1,3 glucan synthase encoded by *FSK1* in *C. albicans* [64]. β-1,3 glucan synthase is involved in cell wall synthesis and inhibition of this enzyme disrupts cell wall formation resulting in osmotic instability and cell lysis [65]. Echinocandins include antifungals like Caspofungin, Micafungin, and Anidulafungin, with Caspofungin being the most common echinocandin. One of the specific uses of this antifungal is for the treatment of nosocomial candidemia and invasive candidiasis [66].

Antifungal resistance has become a global problem, with about 7% of blood samples from patients suffering from *Candida* showing resistance to fluconazole [61, 67]. In response to azoles, resistance can be created by disruptions in the *ERG* genes, which are major players in the ergosterol pathway. For example, mutations in *ERG3,* which encodes Δ5,6-desaturase involved in the conversion of 14α-demethylase (lanosterol) to a toxic sterol 14α-methyl-3,6-diol, results in the survival of the isolate under azole stress [68, 69]. Mutations in *ERG11,* the gene encoding 14α-demethylase, overexpression of its activator Upc2, and overexpression of ABC transporters due to point mutations in the transcriptional factor Tac1 and Mrr1's activation domain causing transport of fluconazole out of the cell can also lead to fluconazole resistance [61, 70, 71].

It is an evolutionary adaptation for fungal cells to survive against selective pressure exerted by antifungals over time [72]. Factors that govern the rate of developing mutations in these include population size, doubling time, pathways that confer resistance, and the fitness costs associated with each of them [72]. Resistance can arise within the host during fungal infections or from exposure to fungicides used on crops, which may also affect human fungal pathogens [72]. Due to the high mortality rate from fungal pathogens especially *C. albicans*, current efforts are being directed to identify new drug targets that bypass resistance.

Pathogenesis in *C. albicans* is controlled by different environmental signals interacting with fungal gene networks. These include signaling pathways that are part of regulatory circuits comprising a cascade of genes and transcription factors (TFs) that activate or repress various processes. In morphogenesis, for instance, the MAPK pathway with the Cph1 and Efg1 TFs regulate the yeast to hyphae switch, while TFs like Ndt80 [73], Bcr1 [74, 75], Brg1, Tec1, Efg1, and Rob1 regulate genes controlling the formation of biofilms [54, 76, 77].

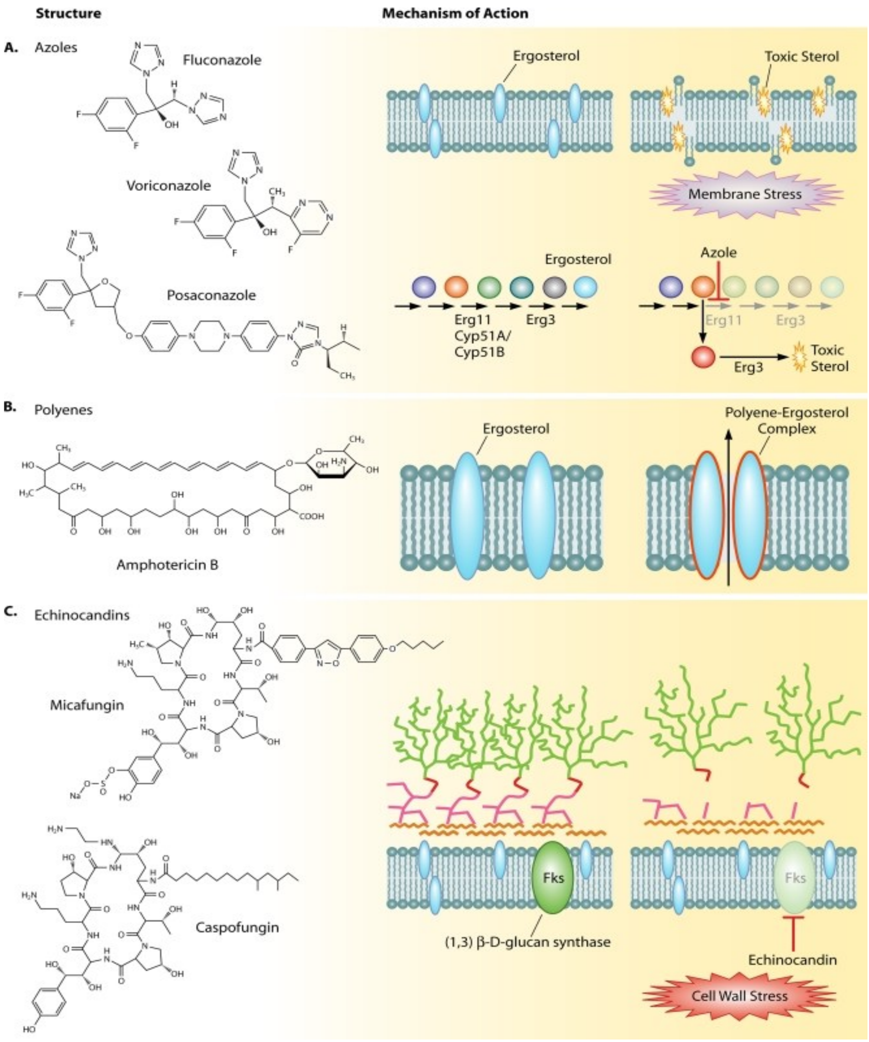

Figure 1.1. Diagram illustrating the mechanism of action and targets of C. albicans against the antifungal drugs A) azoles, B) polyenes, and C) echinocandins [78].

1.3. Importance of drug screening and identifying antifungal targets for drug development

Identifying essential genes is a powerful strategy for discovering potential drug targets because essential genes are critical for the survival of the pathogen. Drugs designed to inactivate the products of these genes can effectively kill the organism, making them attractive candidates for therapeutic intervention [79, 80]. In *Candida albicans*, essential genes have been systematically identified using tools like the GRACE (Gene Replacement and Conditional Expression) library, which allows researchers to construct mutant strains with conditional expression of essential genes and observe their phenotypes under non-permissive conditions [80]. The antifungal classes consist of both fungistatic (inhibits the growth of fungi) like azoles, and fungicidal (kill the fungi) like polyenes, allylamines and echinochandins [81]. Currently, two out of the four major classes of antifungals target the ergosterol pathway although through different mechanisms [82]. This, in addition to the alarming rate of antifungal resistance in patients with *C. albicans,* means that there is a need to explore alternative drug targets, especially the products of fungal genes absent in humans to reduce side effects in the human host [82]. Since *C. albicans* infection is overrepresented among immunocompromised and hospitalized patients, a shift towards more fungicidal drugs is needed especially in patients with candidiasis [17]. Even more drug targets can be explored by synthetic lethality, where the deletion of two genes that results in lethality, but single mutants are viable because individual genes are non-essential [83]. For example, targeting the chaperone protein Hsp90 in combination with other stress-response pathways has shown promise in combating antifungal resistance by destabilizing cellular homeostasis under drug pressure [84]. Therefore, multiple high-throughput screenings (HTS) have been a common method to identify the genes that are involved in virulence and drug resistance [85-87]. For example, the GRACE 1.0 collection in which the tetracycline promoters have been looped out contains 887 strains containing nonessential genes that was used to investigate drug response in the null mutants [85]. Recently, HT drug screening and advanced genomic tools have further accelerated the identification of antifungal targets [88, 89]. CRISPR-Cas9-based systems and RNA interference (RNAi) libraries allow precise manipulation of fungal genomes, enabling the discovery of essential genes and synthetic lethal pairs [89]. Coupling these methods with phenotypic assays ensures robust validation of novel drug targets.

Predicting how genetic variations influence phenotypic traits remains challenging despite advances in sequencing and large-scale experiments. Many traits result from complex interactions between multiple genes and environmental factors. Epistatic (gene-by-gene) interactions further complicate this process [90, 91]. To address this, researchers are integrating HTS with epistasis analysis. For example, one study genotyped ~200,000 diploid *S. cerevisiae* progenies and identified multiple genetic interactions [92]. Another study introduced natural gene variants into four yeast strains, revealing background-dependent effects on growth, particularly near key interaction hub genes [93, 94]. By combining HTS with epistasis analysis, researchers can uncover genetic interactions that drive traits like drug resistance and fungal pathogenicity, leading to better antifungal treatments.

Epistasis and fungal adaptation

Epistasis can occur when the function of one gene depends on or is modified by another gene, and this can create a complex network of interactions that influence traits [95]. Epistatic interactions can broadly be classified in three classes [96]:

- **Epistatic interactions when one gene modifies the phenotypic effect of a mutation in another gene (genetic–genetic interaction)**
  For example, *EFG1* and *CPH1* are TFs involved in regulating morphogenesis. Individually, the deletion of either gene results in mild defects in filamentation, however when both genes are deleted, the defect becomes severe. This shows how the combined genetic mutations can amplify the phenotypic outcome [97]. Similarly, in *A. nidulans,* the double mutant of genes encoding LaeA, a global regulator of secondary metabolism, and VeA, developmental regulator exhibits greater defects in secondary metabolite production than either single mutant [98].
- **Epistatic interactions when one gene's mutation indirectly affects pathways regulated by another gene through epigenetic modifications (genetic–epigenetic interaction)**
  For example, deletion of *HDA1*, a histone deacetylase, affects the expression of genes involved in biofilm formation in *C. albicans*. If a transcription factor like *BCR1* is deleted alongside *HDA1*, the interaction may further suppress biofilm formation due to the compounded effects of disrupted transcription and epigenetic regulation [56]. Similarly in *S. cerevisiae*, the deletion of *SET1*, a histone methyltransferase, shows genetic-epigenetic interaction with *HSP104*, leading to altered stress tolerance phenotypes [99].
- **Epistatic interactions when two genes simultaneously vary in expression (epigenetic–epigenetic interaction)**

GCN5 (encoding histone acetyltransferase) and *RPD3* (encoding histone deacetylase) affect morphogenesis and stress responses. If both genes are deleted, the effects on hyphal formation or oxidative stress tolerance may be non-linear, highlighting an epigenetic–epigenetic interaction [100]. Similarly, in *Neurospora crassa*, the variable expression of *DIM-5* (a histone methyltransferase) and *HP1* (a heterochromatin protein) epistatically affects heterochromatin formation and genome stability and shows epigenetic–epigenetic interplay [101].

In *C. albicans* such interactions play important roles for adaption and regulation of morphogenesis [102], stress responses [103], and drug resistance [104]. Transcription factors play a key role in these interactions by controlling the activity of multiple genes [35]. For example, Efg1, which drives the yeast-to-hyphal transition in *C. albicans*, interacts with other regulators like Cph1 and Nrg1. However if *EFG1* is deleted, these other TFs can compensate partially, maintaining the cell's ability to form hyphae in specific conditions [105, 106]. Similarly, the MAPK pathway activates Hog1 during osmotic stress that epistatically (genetic-epigenetic) interacts with a chromatin remodeller like the SNF complex. These relationships ensure that the transcription of genes required for survival is efficiently regulated [107-109].

This dynamic relationship between TFs and epistatic effects enables *C. albicans* to adapt to conditions like stress, nutrient limitation, and host environment, helping to make it a successful pathogen. Understanding these interactions could provide insights into potential antifungal targets, particularly by disrupting the compensatory mechanisms that underlie the pathogen's adaptability. Moreover, transcriptional rewiring can occur, where transcription factor orthologs of closely related species regulate different circuits to control gene expression patterns. This rewiring not only enables shifts between commensal and pathogenic states in response to host immune pressures but also allows *C. albicans* to fine-tune metabolic pathways, stress responses, and drug resistance mechanisms. By uncovering how transcriptional circuits are rewired, researchers may identify key regulatory nodes that can be targeted to disrupt fungal survival and virulence [35].

1.4. Transcription factor gene regulation and rewiring

Transcription factors consist of at least two functional domains: a DNA-binding domain, which recognizes and binds specific DNA sequences, and a transcriptional regulation domain, which activates or represses gene expression. TFs are classified into families based on their DNA-binding domains, and over 100 such domains have been identified [110]. In the Saccharomycotina, four major TF families—zinc cluster, leucine zipper (bZIP), C2H2 zinc finger, and homeodomain proteins—play central regulatory roles [111].

In *Candida albicans*, approximately 4% of the genome encodes transcription factors, making them the largest family of proteins [112]. These TFs are essential for processes such as biofilm formation [56], drug resistance [104], and the transition between commensal and pathogenic lifestyles [113]. Manipulation of these factors can provide critical information of cellular regulatory circuits. For example, zinc cluster proteins can be artificially activated by adding an activation domain with specific mutations, enabling researchers to create a library of hyperactive TF variants useful for a variety of functional studies [34, 114].

For organisms to adapt to environments and possibly develop new traits, TFs can be rewired to regulate different genes or even networks. This phenomenon of transcriptional rewiring is a powerful tool to study evolution across a wide range of organisms. For instance, in *C. albicans*, the zinc cluster TF Ppr1 regulates allantoin metabolism, while its ortholog in *S. cerevisiae* controls pyrimidine biosynthesis [34]. Similarly, extensive rewiring of genetic networks has been documented between sea urchin species, where cis-regulatory changes in genes like *ALX1* and Delta drive differences in skeletogenesis [33].

Transcriptional rewiring can occur through several mechanisms [35]:

- Cis-regulatory mutations: Gain or loss of DNA-binding sites alters TF binding and gene regulation.
- Protein-protein interactions: Mutations enable TFs to interact with new partners, creating new regulatory combinations.
- Effector domain changes: Mutations convert a TF from an activator to a repressor or vice versa.

S. cerevisiae and *C. albicans* have diverged significantly over 300 million years yet show similarities in their transcriptional networks. One computational study estimate that 16% of regulatory connections are conserved between these species [115, 116]. However, another analyses documented rewiring among fungal species, with less than 10% similarity among regulons over similar evolutionary timescales [117]. TFs often interact with other proteins to regulate gene expression. For instance, in *S. cerevisiae*, the TF Met4 partners with Met28, Met32, and Cbf1 to control methionine biosynthesis. However, in *C. albicans*, Met4 functions independently alongside Cbf1 to regulate the same pathway [38]. Similarly, Mcm1 in *S. cerevisiae* regulates mating type, the cell cycle, and arginine metabolism by partnering with different proteins, and this regulatory network has undergone significant turnover across yeast species [118].

In animal species, regulatory network evolution involves gains and losses of TF binding sites, leading to functional diversification. For example, studies on fly species have shown that even closely related organisms exhibit quantitative differences in TF binding, driven by changes in DNA recognition sequences [119]. Understanding how transcriptional networks evolve provides insights into cellular adaptation and complexity. Rewiring allows organisms to fine-tune gene expression, adapt to new environments, and evolve novel traits. While species divergence generally reduces network similarity, exceptions like *C. albicans* and *S. cerevisiae* reveal the potential for evolutionary constraints and selective pressures to maintain functional conservation in key processes. By examining transcriptional network evolution in diverse species, researchers can uncover general principles of gene regulation, which may have implications for fields like synthetic biology and disease management.

Thesis objectives

High-throughput screening has become an invaluable tool for studying *C. albicans*. This approach enables the simultaneous analysis of numerous genetic or phenotypic factors, allowing researchers to systematically explore the molecular mechanisms underlying its transition from a commensal organism to a pathogen. High-throughput methods are particularly suited for *C. albicans* due to its phenotypic variability, and its ability to adapt to diverse and challenging environments within the human host. I identified two objectives for my study. These involve two distinct strategies for the high-throughput investigation of *C. albicans* function.

Objective 1: Previously our lab derived a set of approximately 900 nonessential, expression-defective *C. albicans* strains called GRACE 1.0 derived from the tetracycline-regulated GRACE collection of strains [80, 85]. Utilizing high throughput methods, they screened the collection against antifungal drugs including two azoles fluconazole and posaconazole, two echinocandins caspofungin and anidulafungin, and a polyene, amphotericin B. They identified 119 genes showing resistance to fluconazole. While high-throughput and large-scale screenings are valuable for identifying genes involved in responses like drug resistance, more focused, gene-specific studies are essential to uncover the underlying mechanisms, paving the way for future synergistic therapies and targeted drug development. Therefore, the goal of my study is to investigate in greater detail three previously identified genes *RAP1*, *ERG3* and *HSC1* for their individual roles in fluconazole response, as well as their genetic interactions in this process. This approach focuses on functional studies involving loss-of-function manipulations.

Objective 2: High-throughput screening is a powerful tool for dissecting the rewiring of transcriptional networks in *C. albicans* compared to non-pathogenic relatives like *S. cerevisiae*. It provides a comprehensive framework for identifying novel targets for antifungal therapies and for understanding how *C. albicans* evolves and adapts to its niches, making it a cornerstone of modern fungal pathogenesis research. Thirty poorly characterized *C. albicans* transcription factors were selected based on in-silico data predicting them to be involved either in infection or drug resistance or to have been rewired compared to *S. cerevisiae*. Each transcription factor was activated, using VP64 DNA-binding activation domain, and used to test strains for phenotypes regarding morphogenesis, carbon source utilization, osmotic and cell wall stress, pH and temperature tolerance, genotoxicity, and drug resistance. Interestingly, our results reveal a rewired transcription factor Adr1 involved in drug resistance in C. *albicans*. In contrast to the previous objective, these investigations focus on manipulations that direct gain-of-function phenotypes.

Chapter 2 – Materials and Methods

2.1. Strains, Cultures, and Growth Conditions

All *C. albicans* and *Escherichia coli* strains used in this study were maintained at -80°C in 30-50% glycerol stocks. For this study, we used two libraries: GRACE 1.0 [85] and Activated Transcription Factor Using VPR [37], together with new six strains with gene deletions, namely *rap1Δ/Δ, erg3Δ/Δ, hsc1Δ/Δ,* and the double mutants *rap1Δ/Δ erg3Δ/Δ, erg3Δ/Δ hsc1Δ/Δ, and rap1Δ/Δ hsc1Δ/Δ*, along with their respective wildtypes, as detailed in Table 1 . Before each experiment, glycerol stocks were spread on solid media plates and incubated for 1-2 days at the specified temperature. Yeast peptone dextrose (YPD) with uridine [2% w/v bactopeptone, 1% w/v yeast extract, 2% w/v glucose, 50 µg/mL uridine, and 2% w/v agar] was used for *C. albicans* growth at 30°C for 1-2 days [120], or Luria-Bertani (LB) with ampicillin [1% w/v tryptone, 0.5% w/v yeast extract, 0.5% w/v NaCl, 100 µg/mL ampicillin, and 2% w/v agar] for *E. coli* growth at 37°C for 1 day (Son and Taylor, 2021). For all media preparation, the pH was adjusted to be neutral (6.5). Only individual unsectored colonies were inoculated to start overnight cultures in 5 mL liquid YPD at 30°C with shaking at 220 rpm, unless specified otherwise.

2.2. Construction of Single and Double Gene Deletions

2.2.1 CRISPR-Cas9 Transient System

C. albicans mutant strains with single and double gene deletions were constructed using the CRISPR-Cas9 transient system [121], adapted from the Vyas et al., CRISPR system [122] without requiring genomic integration. Two sgRNAs were used for constructing mutants with single and double gene deletions. The DNA sequences of *C. albicans* genes of interest (GOI), along with 1 kb of their upstream and downstream sequences, were retrieved from the Candida Genome Database (CGD) [123] and input into the Benchling (https://www.benchling.com/crispr/) software for annotation. The sgRNAs were either designed using Benchling's CRISPR program (20 bp with NGG, ON-target score >60%, GC >45%) or retrieved from [122]. The transient protocol was accomplished by Polymerase Chain Reactions (PCR) to amplify the *C. albicans* codon-optimized Cas9 nuclease gene (CaCAS9), the single-guide RNA (sgRNA) cassettes, and the mutagenic donor repair DNA fragments [121]. In this study, OneTaq 2X Master Mix with Standard Buffer was used as the DNA polymerase, and the protocol followed as per the manufacturer's instructions (NEB, M0482L). CaCAS9 was amplified from PV1093 using primers flanking the *ENO1* promoter and *CYC1* terminator regions. Three rounds of PCR were performed to amplify the sgRNA cassettes from plasmid pV1098: 1) The promoter SNR52 and the sgRNA scaffold + terminator *ENO1* components were separately amplified using primers flanking their regions and an overlapping 20 bp chimeric guide sequence, 2) the two constructed fragments were joined by primers from the internal chimeric sequence, 3) the fused product was amplified to form the cassette (Fig. S). Plasmids pFA-ARG4 and pFA-HIS1 were used to construct the repair DNA fragments for the first and second gene disruptions, respectively [124]. The primers were designed for integration via homology-directed repair (HDR) and consisted of an ~80 bp flanking region of the disrupted gene and an overlapping ~20 bp sequence of the donor DNA downstream of the flanking region (S1 and S2 sites from PFA plasmids). The amplified fragments were verified for their correct length using gel electrophoresis (R0611, Thermo Scientific). The primers are listed in Appendix, I) and the constructs are in Appendix II).

2.2.2. *Candida albicans* Transformation

The lithium acetate (LiAc) protocol was adapted from Noble and Johnson, 2015 to transform the *C. albicans* strains. The SN148 lab strains, auxotrophic for the amino acids arginine, leucine, histidine, and uracil [125], was used as a background to construct the six mutants. The culture was harvested at log phase (OD_{600} nm 0.7-0.8) from YPD to ensure cell competency, washed with nuclease-free water, and suspended in 600 µL LiAc-TE solution [100 mM lithium acetate, 10 mM Tris-HCl (pH 7.5), 1 mM EDTA (pH 8.0)] for membrane permeability disruption and to enhance DNA uptake. The transformation mixture contained 100 µL competent cells, PCR products (1 µg CaCas9, 1 µg sgRNA, and 3-6 µg repair DNA), 100 µg single-stranded salmon sperm (Thermo Fisher Cat. no. 15632011), and 600 µL 0.1M LiAc/40% PEG [50% polyethylene glycol 4000 in LiAc/TE solution]. The mixture was incubated to allow cell division and then subjected to heat shock at 44°C to introduce pores and improve transformation efficiency. The cells were then washed and incubated in rich YPD media for recovery, after which they were washed again and plated on synthetic dextrose media lacking arginine or histidine (SD -Arg, SD -His) for marker selection. The plates were left to grow for 3-5 days at 30°C.

2.2.3. Colony PCR: DNA extraction and restriction enzyme digestion

Candidate transformed colonies grown on the SD media were collected and verified for their corresponding gene deletion. A colony PCR was performed to confirm the gene deletion construct. After each transformation attempt, ~50 colonies were transferred to new solid SD plates, and at least 30 colonies were tested until 3 successfully transformed constructs for the desired gene deletions were found. DNA was extracted from each colony using the YeaStar Genomic DNA Kit (D2002, Cederlane Labs) and used as the template DNA with OneTaq 2X Master Mix. Three different reactions per construct were run on the gel to check for the presence of the gene of interest (GOI), the presence of the replaced marker (*ARG4* or *HIS1* from PFA plasmids), and their correct placement in the genome. Diagnostic primers were designed in Benchling, including a pair of primers for each of the following: external of the replaced GOI region, internal of the GOI, and internal of the marker sequences (Table S). The *EcoRI* restriction enzyme (RE) protocol from New England Biolabs was used for digestion as a double verification for the *HCS1* deletion. Verified constructs were stored in glycerol stocks in triplicates.

2.3. Phenotypic assay screenings for different phenotypic behaviors

Overnight cultures were stepwise diluted by a factor of 10 to reach cell concentrations between 1 million and 1 thousand cells per milliliter, either by hand or using automated liquid-handling machines such as Opentrons OT-2 robot and the Biomek FXP Workstation. The equipment was programmed to spot 3 µL of each dilution on various media plates for different phenotypic assays or add culture to a 96-well drug plate. All plates were incubated for 48 hours unless otherwise specified, at 30°C, with exceptions for temperature-specific plates (15°C, 30°C, and 45°C), Spider and serum media plates (37°C), and phloxine B plates (25°C). To examine white and opaque morphologies, the library was screened by incubating cells overnight in Yeast Nitrogenous Base-N-Acetylglucosamine (YNB-GlcNAc) medium supplemented with 5 µg/mL phloxine B [0.67% w/v yeast nitrogen base, 0.15% w/v amino acid mix, 50 µg/mL uridine, 1.25% w/v GlcNAc, and 2% w/v agar for solid medium]. Following incubation, cells were plated onto YNB-GlcNAc solid medium and incubated at room temperature for 1–5 days, depending on the strain, to allow colony differentiation. White and opaque cells were then selected periodically from single colonies and examined microscopically using Calcofluor white (CFW) staining to visualize cell wall structures. Cells then were suspended in water, the cell concentration was adjusted, and the suspensions plated on agar medium containing 5 µg/mL phloxine B and different carbon sources. Plates were incubated at room temperature. Data were collected and plates were scanned on the 7th day and the frequency of sectored colonies calculated by standard statistical methods.

For hyphal morphologies, screening was done in liquid Spider medium and in YPD supplemented with 10% fetal bovine serum (FBS). Transformants were also screened for their ability to utilize different carbon sources. Agar media for this experiment were prepared by replacing glucose with 2% of each of 4 different sugars: xylose, fructose, sucrose, and sorbitol.

2.4. Screening for environmental factors like pH and temperature, heavy metal tolerance, cell wall, genotoxic and osmotic stress

To test for heavy metal tolerance using solid YPD media plates with 1% of 1M arsenic, and 1% of 1M of cesium chloride was used. Furthermore, for response to genotoxic and osmotic stress using 30 mM of hydroxyurea (HU) and 1M of sodium chloride (NaCl) was infused in YPD agar media. For cell wall stress we used 150 µg/mL of Congo red and CFW (100, 200 and 600 µg/mL) in YPD. Cells were also tested for pH (range of 5 to 8) and temperature (15 to 43°C).

2.5. Adhesion assay

Overnight YPD cultures were washed with 1× PBS and diluted from an absorbance of OD_{600}=1. Aliquots of 5 µL of the adjusted sample dilutions were spotted on solid YPD plates and grown at 30°C for 5 days. The spots were equally subjected to a running stream of water until all nonadherent cells were washed off and adhesive cells remained on the agar. Images of the plates were captured using the spImager Canon EOS Rebel T7i camera before and after the wash.

2.6. Antifungal response tests

2.6.1. Liquid assay

To test the antifungal resistance in liquid media, cultures were grown in YPD or SC in the presence of drugs. From drug stock solutions, working solutions were prepared using DMSO for fluconazole (FLU) (5-120 μg/mL), posaconazole (10-50 μg/mL) and amphotericin B (1-4 μg/mL), water for Caspofungin (10 -50 μg/mL) and anidulafungin (5-50 μg/mL). All drugs were obtained from Sigma, except Caspofungin that was obtained from Merck. Once in solution, drugs were stored at −20°C. The 30 strains with activated TFs were screened against all 5 drugs. We made-up the volume of each well to 195 µl of YPD and combined it with 5 µl of overnight Candida cultures. MIC plates were incubated with a shaking TECAN infinite M200 PRO plate reader at 30°C with for overall 72 hours and optical densities were read at specific time points throughout 72 hours.

2.6.2. Spot assays

To test the antifungal resistance of the different strains in liquid media, cultures were grown in SC overnight. The overnight cultures were serially diluted in 10-fold stages to a density of 10^6 to 10^3 cells per mL and 3 µL of each dilution were spotted onto SC agar plates infused with the same 5 drugs fluconazole (5-120 μg/mL), posaconazole (10-50 μg/mL) and amphotericin B (1-4 μg/mL), water for Caspofungin (10 -50 μg/mL) and anidulafungin (5-50 μg/mL). For objective 1, strains which were created for this study (appendix III) were tested only for fluconazole with different concentrations as mentioned above. Plates were captured using the Epson Perfection v500 photo scanner or the spImager Canon EOS Rebel T7i camera.

2.6.3. Fluconazole disc diffusion

Disc diffusion was used to test the construct strains' level of susceptibility by measuring/observing their zone of inhibition (ZOI) formed on solid media compared to the background strain. Otherwise known as the Kirby-Baur method, this susceptibility test provides a visual representation of the growth of a starting culture as the drug diffuses into the solid media plate. The ability of cells to grow around this paper disc will determine the fitness of the strain under the effect of the drug diffused through the agar. The larger the zone of inhibition around the disc, the more susceptible the strain is at that dose of drug. The lack of growth caused around the paper filter caused by the drug diffusion is referred to as the ZOI, and its size is a direct correlation to the tested strain's susceptibility to the antifungal. The steps were followed as stated by [126] and Clinical &

Laboratory Standards Institute (CLSI) document M44-A2, with some modifications. The solid media used was YPD with a final concentration of ~1.3% agar concentration instead of 2%. The total depth of the solid media in the standard 100 mm diameter petri dish was considered, and an adequate volume (25 mL) was filled in the standard petri dish so as to get a depth of 4mm, and not get false results of susceptibility [126]. Overnight cultures were diluted from which 1 mL was seeded in approximately 6 mL media YPD (1.3% agar). The suspension of agar media and mutant cells was evenly poured over ~20 mL of 2% agar YPD. The fluconazole (drug) and control (no drug) discs were dispensed onto the cell-containing solid media using flamed forceps. The discs containing a concentration of fluconazole were either bought or prepared anew. Twenty-five µg fluconazole discs (Cat. No OXCT1806B) and sterile blank discs (Cat. No OXCT0998B) were obtained from Fisher Scientific. Following Kirkpatrick et al., 1998 [127], discs were prepared containing 25 µg of fluconazole added on sterile blank paper discs (Sigma-Aldrich Product No. F8929) with their corresponding negative control of blank discs containing the same amount of solvent DMSO only. The discs were dried in a desiccator and stored at 4°C to be used within 1 week. Plates were incubated according to standard conditions [120] and captured after growth using the spImager Canon EOS Rebel T7i camera.

2.7. Microscopy and imaging

2.7.1. Cell morphology

For white and opaque cell morphologies, overnight cultures grown in 5 mL YNB-GlcNAc medium at room temperature were subjected to phase differential interference contrast (DIC) microscopy. Cells were examined by DIC at 63X and fluorescent microscopy at 100X magnification using a Leica DMi6000 microscope mounted with a TIR camera. As for yeast and filamentous morphologies, overnight cultures were grown in YPD media at 30°C.

2.7.2. Calcofluor white staining and cell aspect ratio measurement

Single colonies of *C. albicans* strains were inoculated in 5 mL of liquid YPD and incubated overnight at 30°C with shaking at 220 rpm. The cells were washed twice with 1× phosphate-buffered saline (PBS) [0.8% w/v NaCl, 0.02% w/v potassium chloride, 0.144% w/v sodium phosphate dibasic, 0.0245% w/v potassium phosphate monobasic; pH=7.4] , and diluted to 10^7

colony forming unit (CFU) (or OD_{600}=1) from which 10 µL were mounted on a slide with 1 µg/mL CFW (M2R; Sigma, Cat.18909). Cells were then imaged using a Leica DMi6000 microscope with both DIC optics and a DAPI filter cube (377/50ex, 447/60em), using a 40x (N.A. 1.25) objective lens and a Hamamatsu Orca R2 camera. The cellular elongation was quantified after capture. CFW stain images were presented to a Region-based Convolutional Neural Net (R-CNN, [128]) trained to recognize yeast cells, resulting in a binary mask that represents the outline of most cells in the image; these masks were verified by a trained human observer, who could discard inappropriate masks that did not correlate well with merged DIC and CFW stain images. The remaining masks were measured in FIJI [129], using the Shape Descriptors option to extract the aspect ratio (AR) of each cell, being the ratio of the width of the cell to its height [130]. The AR of filament cells was calculated for each compartment of the filament.

2.8. Colony counting and measurement

From a growth plate of *C. albicans* strains (*rap1Δ/Δ, erg3Δ/Δ, hcs1Δ/Δ*, *erg3Δ/Δ hcs1Δ/Δ*, *rap1Δ/Δ hcs1Δ/Δ* and *rap1Δ/Δ and erg3Δ/Δ*), individual and segregated small and large colonies were picked and their area size (mm^2) was measured using FIJI [129], referred to as parental colonies. The samples were cultured in 5 mL of liquid YPD and incubated overnight at 30°C with shaking at 220 rpm. The next day they were washed twice and diluted with 1× PBS, spread on YPD plates and left to grow at 30°C for 2 days before capturing images. From the images of distributed colonies of the various strains, individual and segregated colonies were counted and assigned a measurement in FIJI; briefly, the growth area of the plate was outlined, the blue channel of the original RGB image was processed by performing white and black top-hat operations to enhance the colony structures and the zones between them (respectively), then marker-controlled watershed was performed using the morpholibj plugin [131] to separate individual colonies, and the label image was thresholded and the size of particles between 0.05 mm^2 and 10 mm^2 was measured. Any artifacts were manually removed after the run. The area measurements (mm^2) were divided into two groups, less than and greater than or equal to 1.4 mm^2, based on the size of their parental strain starting culture. These groups were identified as small and large colonies which resulted in a frequency (%) of small and large colonies distributed on the plates and captured after growth using the spImager Canon EOS Rebel T7i camera.

2.9. Colony susceptibility variation under different stressors

To investigate whether *RAP1* deletion results in variation of response between the smaller and larger colonies, we replica-plated a distribution of 50-100 CFU of WT, *rap1Δ/Δ* small and *rap1*Δ/Δ large onto different stressor solid plates and let them grow for 1 day. The solid plates were YPD-agar infused with the appropriate stressor reagent. The stress response tests were the following: oxidative stress using hydrogen peroxide (H_2O_2) (8-100 mM), osmotic stress using NaCl (0.5-1.5 M), DNA damage using ultra-violet (UV) light (45-100 seconds), temperature (40-48°C), cell wall stress using antifungal Caspofungin (0.25-1.25 µg/mL), and inhibition of the target of rapamycin (TOR) pathway which regulates cell growth and metabolism using rapamycin (1-3 ng/mL). To recognize variability between the two distinguish colonies in presence of stressor, we compared the colonies in presence of stress with the master plates. Images of the plates were taken using the spImager Canon EOS Rebel T7i camera.

2.10. Bioinformatic analyses

Sequences of genes of all transcription factors were obtained from the Candida Genome Database (CGD-http://www.candidagenome.org/) and the Saccharomyces Genome Database (SGD-https://www.yeastgenome.org/). Gene orthogroup assignments for all predicted protein-coding genes across 23 Ascomycete fungal genomes were obtained from the Fungal Orthogroups Repository [132] maintained by the Broad Institute (broadinstitute.org/regev/orthogroups). DNA sequence motifs were identified using the Web-based motif-detection algorithm MEME (Motif-based sequence analysis tools) Suite-FIMO (Find Individual Motif Occurrences) (https://meme-suite.org/meme/tools/fimo) [133] and CGD feature Go term finder [123]. For more stringent motif identification, we used MAST hits with an E-value of were detected from each individual TF protein sequence using INTERPROSCAN, PFAM and ELM motif definitions.

Chapter 3 – Investigating the genetic interplay of *RAP1, ERG3* and *HCS1 C. albicans*' resistance to fluconazole

3.1. Introduction

While High throughput screening (HTS) methods are effective for identifying candidate genes involved in various pathways due to their ease in scalability and availability of automation platforms, they should be followed up with further tailored screening of such strains to rule out false positives or negatives. Screening of the GRACE 1.0 library of 887 nonessential gene disruptions in *C. albicans* against a range of commercial drugs including fluconazole highlighted three mutant strains of gene, *RAP1*, *ERG3*, and *HCS1*, that exhibited growth discrepancies in presence of fluconazole compared to the WT [85]. *ERG3* is documented for its role in ergosterol biosynthesis and antifungal resistance [69, 134], *RAP1* and *HCS1* remain understudied in this context. These genes are intriguing candidates for further investigation due to their similar phenotype but potential involvement in diverse cellular processes. *ERG3* (ERGosterol biosynthesis 3) synthesizes the enzyme Erg3, a C-5 sterol desaturase that plays an important role in the conversion of sterol intermediates to ergosterol during ergosterol biosynthesis. Disruptions in ergosterol biosynthesis alter cell membrane composition and functions [135]. It is also involved in other biological responses such as biofilm formation where its expression is upregulated, contributing to infections and drug resistance [135]. *ERG3* gene's role in drug resistance and cell membrane formation within the ergosterol genes has been extensively studied so our research focuses on its relationship with other genes involved in resistance to fluconazole.

RAP1 (Repressor Activator Protein 1) is a transcriptional factor (TF) present in a variety of other species from human to budding yeast *S. cerevisiae,* whose regulatory network has been well studied in yeast. Comparative genomics approaches pointed out evolutionary changes in Rap1 domains. For example, *C. albicans* Rap1 lacks a C-terminal domain and is non-essential [32, 39], while the *S. cerevisiae* ortholog is essential. In *C. albicans* It is known that Rap1 plays a role in telomere maintenance and biogenesis [136], and recent research shows that it is multifunctional and also regulates other cellular functions such as cell wall composition and response to cell wall-disrupting agents, biofilm formation and virulence [137].

HCS1 has orthologs in many fungal species but has not been directly studied in *C. albicans*, where it is an uncharacterized gene encoding a putative ATP dependent 5'-3' DNA helicase with a role in post-replication repair [85, 123]. The role of helicases, particularly *HCS1,* in drug resistance has not been well explored.

This study aims to investigate potential roles of *RAP1*, *ERG3*, and *HCS1* in mediating fluconazole response by integrating phenotypic assays, genetic constructs, and morphological analyses. The findings will not only provide insight on transcriptional regulation and cellular adaptation but also lay a foundation for potentially developing synergistic therapeutic strategies against drug-resistant *C. albicans*.

3.2. Results

The slower response of *rap1*, *erg3* and *hcs1* GRACE 1.0 strains to fluconazole suggests that these mutants may alter known resistance mechanisms such as ergosterol biosynthesis pathway or fitness characteristics (Fig. 6B, [85]). With this preliminary information, we designed new constructs using CRISPR to have complete knockouts of these genes and characterized each knockout strain (*rap1Δ/Δ*, *erg3Δ/Δ* and *hsc1Δ/Δ*) separately and in combination (Fig. 3.2.1).

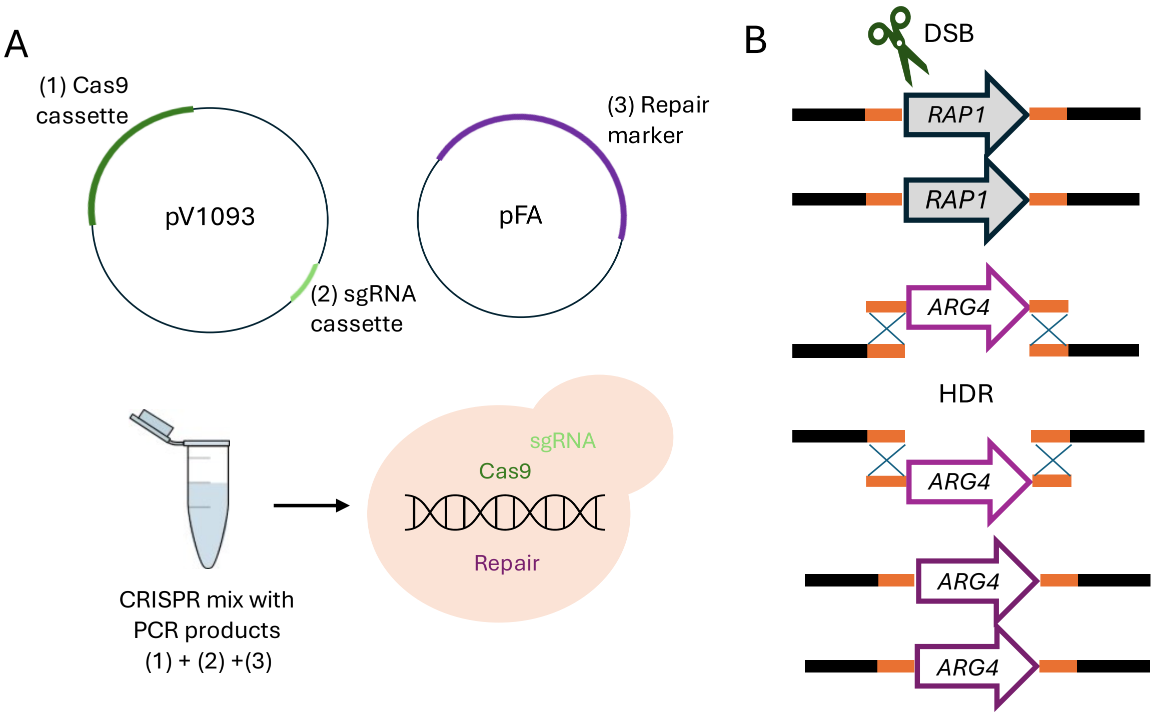

Figure 3.2.1. Transient CRISPR schematic design. A. PCR-amplified Cas9 and sgRNA expression cassettes from plasmid pV1093 and repair marker from plasmid pFA were transformed into competent C. albicans yeast cells. B. Cas9-sgRNA complex guides the double-stranded break (DSB) at GOI RAP1 and ARG4 selectable marker replaces GOI by HDR.

3.2.1. Variability of *rap1Δ/Δ* strains in response to fluconazole

To observe variation in response behaviour of the respective gene deletion strains and identify possible limitations of spot assays such as uneven distribution of antifungals across the agar, temperature control and antifungal evaporation, we sought out to test the new CRISPR-generated mutant strains using a disc diffusion assay (Fig 3.2.2). The *hcs1Δ/Δ* strain behaves as wild type in its response to fluconazole having a similar clear ZOI around the 25 µg fluconazole disc in contrast to previous studies using GRACE 1.0 strains. The *erg3Δ/Δ* strain was resistant, showing no ZOI (Fig. 3.2.2). The *rap1Δ/Δ* strain showed variation in fluconazole response; 17% of culture replicates (n=12) showing clear zones identical to the WT, 33.3% showing mild ZOI and 50% showing no ZOI at all (Fig. 3.2.2).

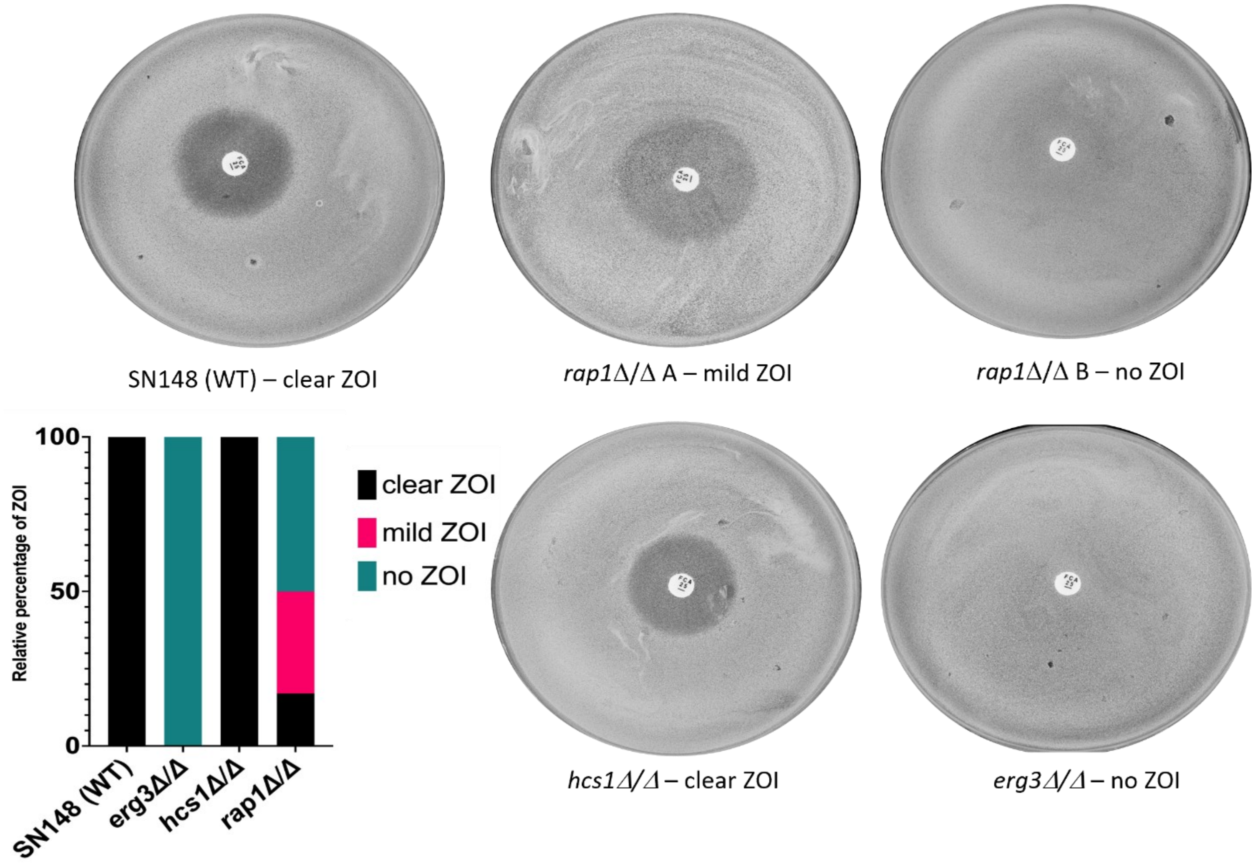

Figure 3.2.2. Response of rap1, erg3 and hcs1 strains respectively to the fluconazole disc diffusion. A. Disc diffusion assay results showed that rap1Δ/Δ strain exhibits inconsistent behavior in presence of fluconazole, hcs1Δ/Δ strain showed no variation in fluconazole response compared to WT and erg3Δ/Δ strain showed resistance in response to fluconazole. B. Analysis of all disc diffusion tests performed revealed that 17% of the time it showed a clear zone of inhibition (ZOI), 33.3% of the time it exhibited a mild ZOI, and 50% of the time it showed no ZOI compared to WT.

3.2.2. Colony size variation for the *rap1Δ/Δ* strain

Another phenotype affected by the *rap1Δ/Δ* mutation is colony size distribution, where colonies grown on a YPD solid plate exhibit a predominant phenotype of small colony sizes with the occasional emergence of larger colonies in the growth of offspring colonies (Fig. 3.2.3 A). To test whether colony sizes (area in mm²) are heritable from parent to offspring, we spread colony forming units (CFU) coming from small *rap1Δ/Δ* colonies (area <1.4 mm²) as well as from large *rap1Δ/Δ* strain colonies (area ≥1.4 mm²). The distribution of offspring colonies shows that small *rap1Δ/Δ* strain parental colonies gave rise to 84% small and 16 % larger offspring colonies whereas larger *rap1Δ/Δ* colonies gave rise to 76% small and 24% larger offspring colonies (Fig. 3.2.3 B). While both small and large *rap1Δ/Δ* colonies predominantly give rise to small colonies, large colonies generate a lower frequency of small colonies compared to small colonies themselves. This suggests that colony size is not strictly inherited in a simple manner but rather follows a biased distribution, where larger colonies have a slightly increased likelihood of producing larger progeny.

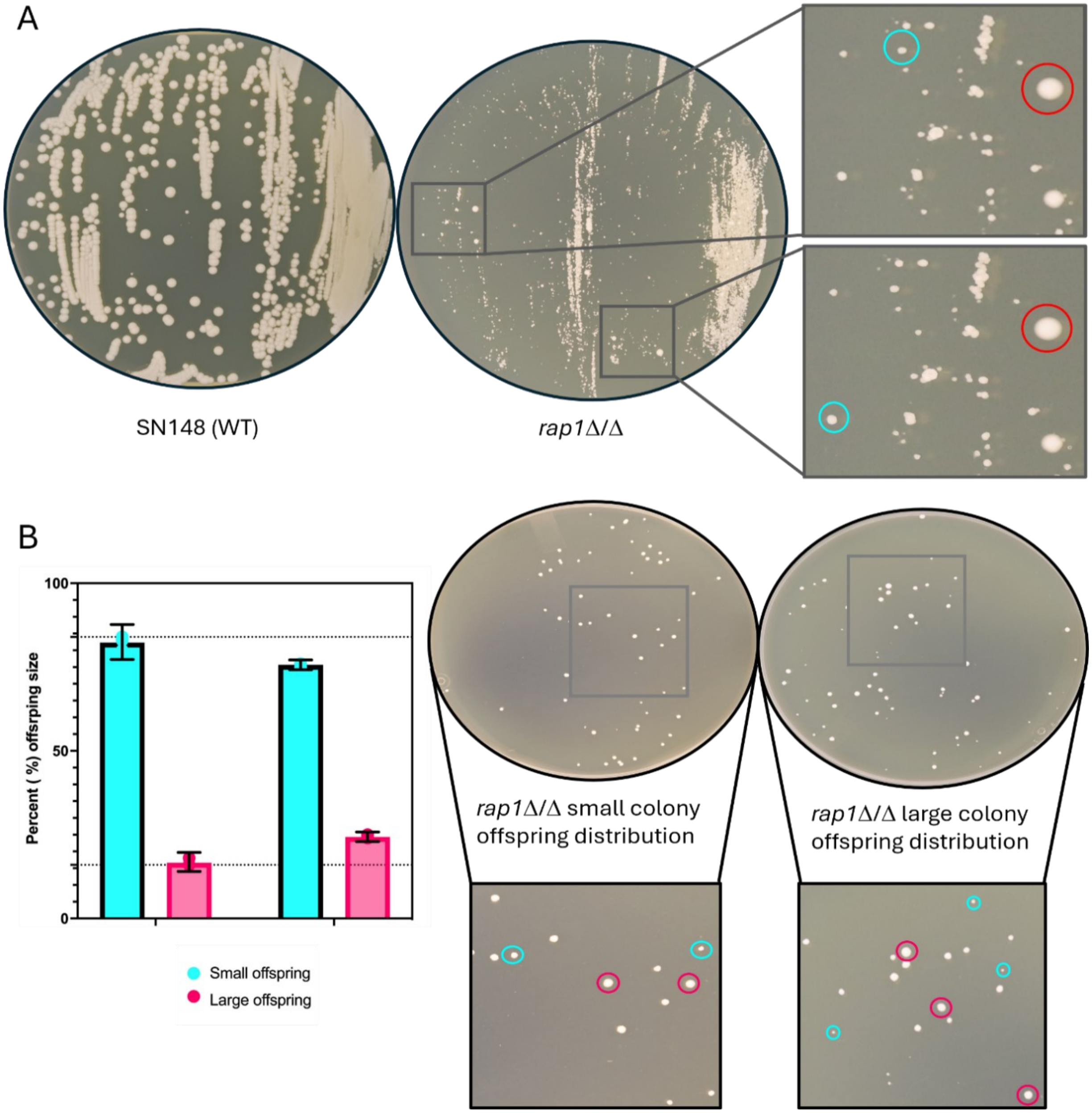

Figure 3.2.3. Impact of RAP1 deletion on colony size distribution on YPD. A. rap1Δ/Δ strain colonies predominantly exhibit a small colony size phenotype, with occasional emergence of larger colonies. Small parental colonies gave rise to 84% small and 16% larger offspring colonies, whereas large parental colonies produced 76% small and 24% larger offspring colonies. B. Offspring colony size distribution based on WT minimum size, classified as small (<1.4 mm²) and large (≥1.4 mm²) parental colony size of rap1Δ/Δ strains. Example of experimental plate with approximately 50 CFU plated from a small and a large parental colony from part A; small and large offspring colonies are circled in turquoise and pink respectively. The small rap1Δ/Δ parental strain gives rise to 79% small and 21% large offspring colonies, and the large parental strain gives rise to 76% small and 24% large ones.

3.2.3. Search for genetic interactions among *RAP1*, *ERG3* and *HSC1* for fluconazole resistance

To investigate potential interactions among these genes, we systematically created double knockout strains. The double mutants were then tested for fluconazole response to assess possible functional relationships.

Our spot assay results revealed intriguing patterns of resistance and susceptibility in the presence of fluconazole (Fig. 3.2.4). The *erg3Δ/Δ* strain exhibited strong resistance, as evidenced by prominent growth compared to the wild type (WT) whereas strain *rap1Δ/Δ* displayed only mild resistance. Interestingly, the double mutant strain of *rap1Δ/Δ erg3Δ/Δ* showed decreased resistance compared to *erg3Δ/Δ* strain, reversing the phenotype observed in *erg3Δ/Δ* alone. This suppression phenotype reveals a positive interaction between RAP1 and ERG3. If *ERG3* was solely dominant in the pathway, we would expect the double mutant to behave like *erg3Δ/Δ* strain, but instead, resistance is reduced, indicating an epistatic interaction.

The phenotype of the *erg3Δ/Δ hcs1Δ/Δ* strain was almost as robust as that of single deleted *erg3Δ/Δ* strain, while the *hcs1Δ/Δ* strain showed growth like WT. This suggests that *HCS1* does not significantly influence the resistance mechanism driven by *ERG3*. Furthermore, deletion of *HCS1* in combination with RAP1, *rap1Δ/Δ hcs1Δ/Δ*, resulted in a response similar to that of *rap1Δ/Δ*, further supporting the idea that *HCS1* might have no detectable role in fluconazole resistance.

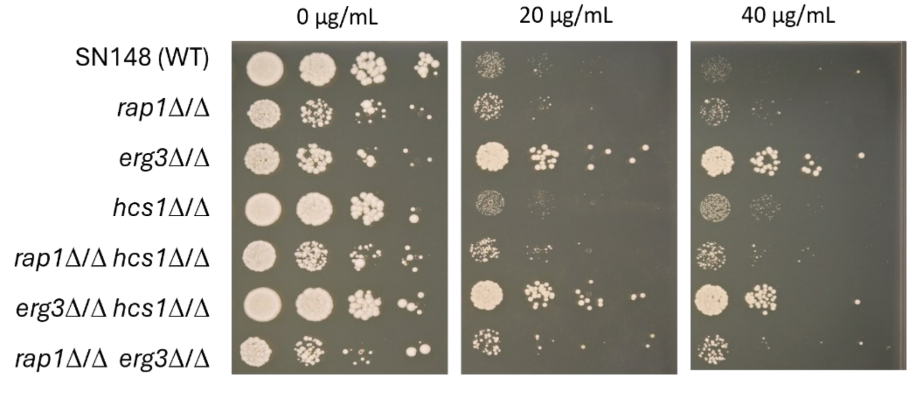

Figure 3.2.4. Fluconazole spot assay response of double mutants. The erg3Δ/Δ strain showed strong resistance, while rap1Δ/Δ was mildly resistant. rap1Δ/Δ erg3Δ/Δ double mutant had reduced resistance, suggesting RAP1-ERG3 interaction. erg3Δ/Δ hcs1Δ/Δ double KO remained resistant, and rap1Δ/Δ hcs1Δ/Δ double KO behaved like rap1Δ/Δ strain, indicating HCS1 plays a minor role.

3.2.4. Colony area size in *rap1Δ/Δ erg3Δ/Δ and rap1Δ/Δ hsc1Δ/Δ*

Consistent with having different colony size phenotype within a genetically identical *rap1Δ/Δ* strain, we observed the same epigenetic behavior of *rap1Δ/Δ erg3Δ/Δ* and *rap1Δ/Δ hcs1Δ/Δ* (Fig. 3.2.5). We similarly checked the progeny distribution of these two-size category colonies and found a positive correlation between small parental colonies and small offspring even for the *rap*1 double mutants. The distribution of small double mutant *rap1Δ/Δ erg3Δ/Δ* parental colonies gave rise to 90% small offspring and 10% larger ones, and larger parental colonies gave rise to 71% small offspring and 29% larger ones (Fig. 3.2.5). For the double mutant *rap1Δ/Δ hcs1Δ/Δ*, small parental colonies produced a dramatic 98% small CFU and 2% large colonies, while the large parental colonies produced 77% small CFU and 23% large colonies (Fig. 3.2.5). Interestingly, this also suggests that larger progeny have a greater likelihood of emerging from a larger colony.

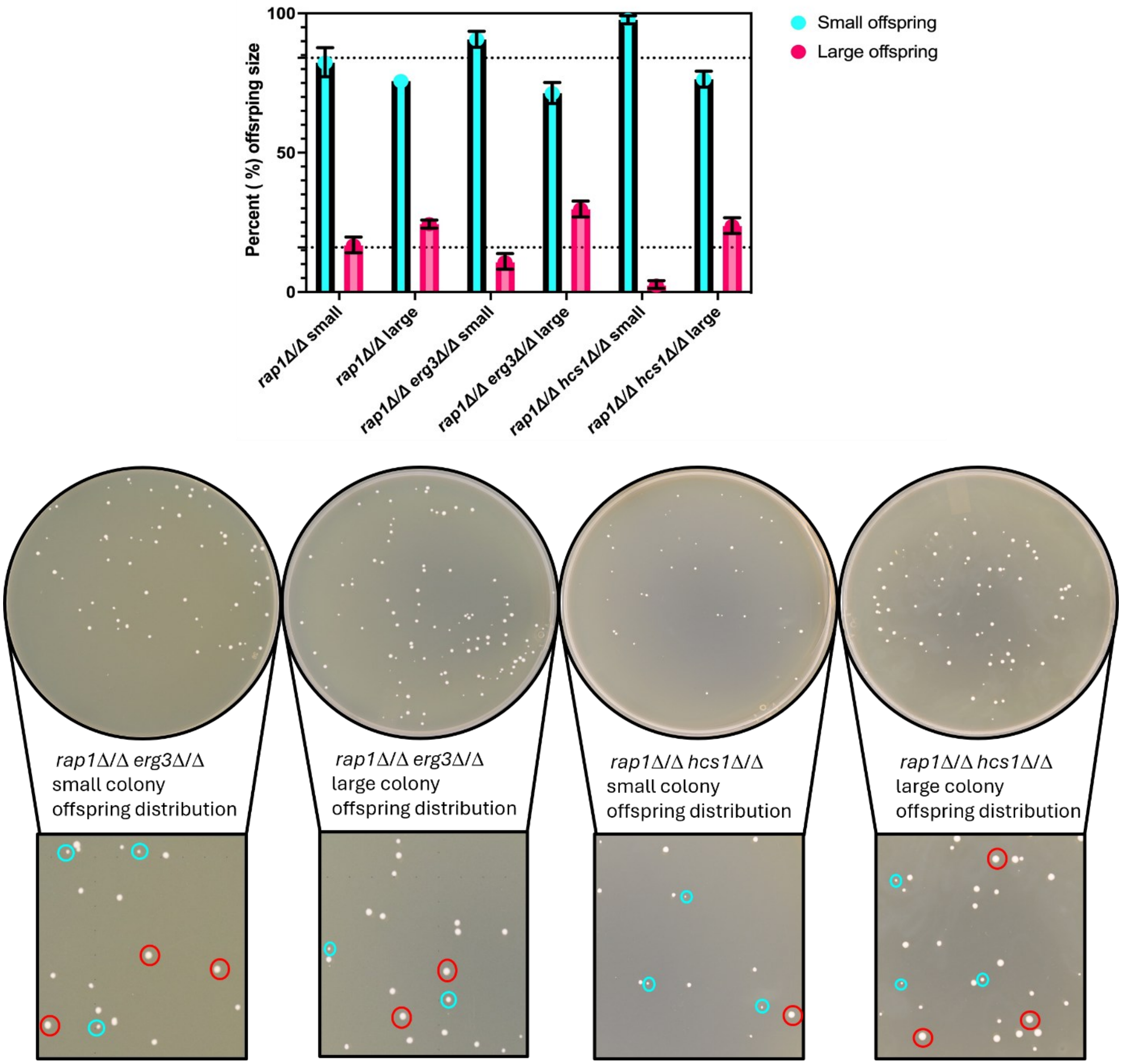

Figure 3.2.5. Colony size distribution in rap1Δ/Δ erg3Δ/Δ, and *rap1Δ/Δ hcs1Δ/Δ strains. Bar graph representing the relative percentages offspring size in different strains. The double mutants show different size variation as rap1Δ/Δ single mutant. Small parental colonies of rap1Δ/Δ erg3Δ/Δ* and *rap1Δ/Δ hcs1Δ/Δ produced 90% and 98% small offspring, respectively, while large parental colonies produced 71% and 77% small offspring. Example of experimental plate with approximately 50 CFU plated from a small and a large parental colony from part A; small and large offspring colonies are circled in turquoise and pink respectively.*

3.2.5. Colony morphology between sizes of *rap1* strains

Cell morphology is also different in *rap1Δ/Δ* strains. Colonies contain predominantly pseudohyphal cells as opposed to the yeast type cells seen with the wild type, *erg3Δ/Δ* and *hcs1Δ/Δ* strains (Fig. 3.6). To assess morphological differences, we measured the AR (width-to-height) of cells from WT and both small and large *rap1Δ/Δ* colonies. Among the 2 phenotypic colony sizes of *rap1Δ/Δ* single mutants, the variation in AR is minor with *p*-value significance < 0.05 (Fig. 3.2.6).

As for the *rap1* double mutants, the AR of the cells were assessed, and they maintained their pseudohyphal form compared to the yeast cell morphologies of *erg3Δ/Δ* and *hcs1Δ/Δ* single mutants (Fig 3.2.7). This indicates that *rap1Δ/Δ* is epistatic to both *erg3Δ/Δ* and *hcs1Δ/Δ* for cell shape.

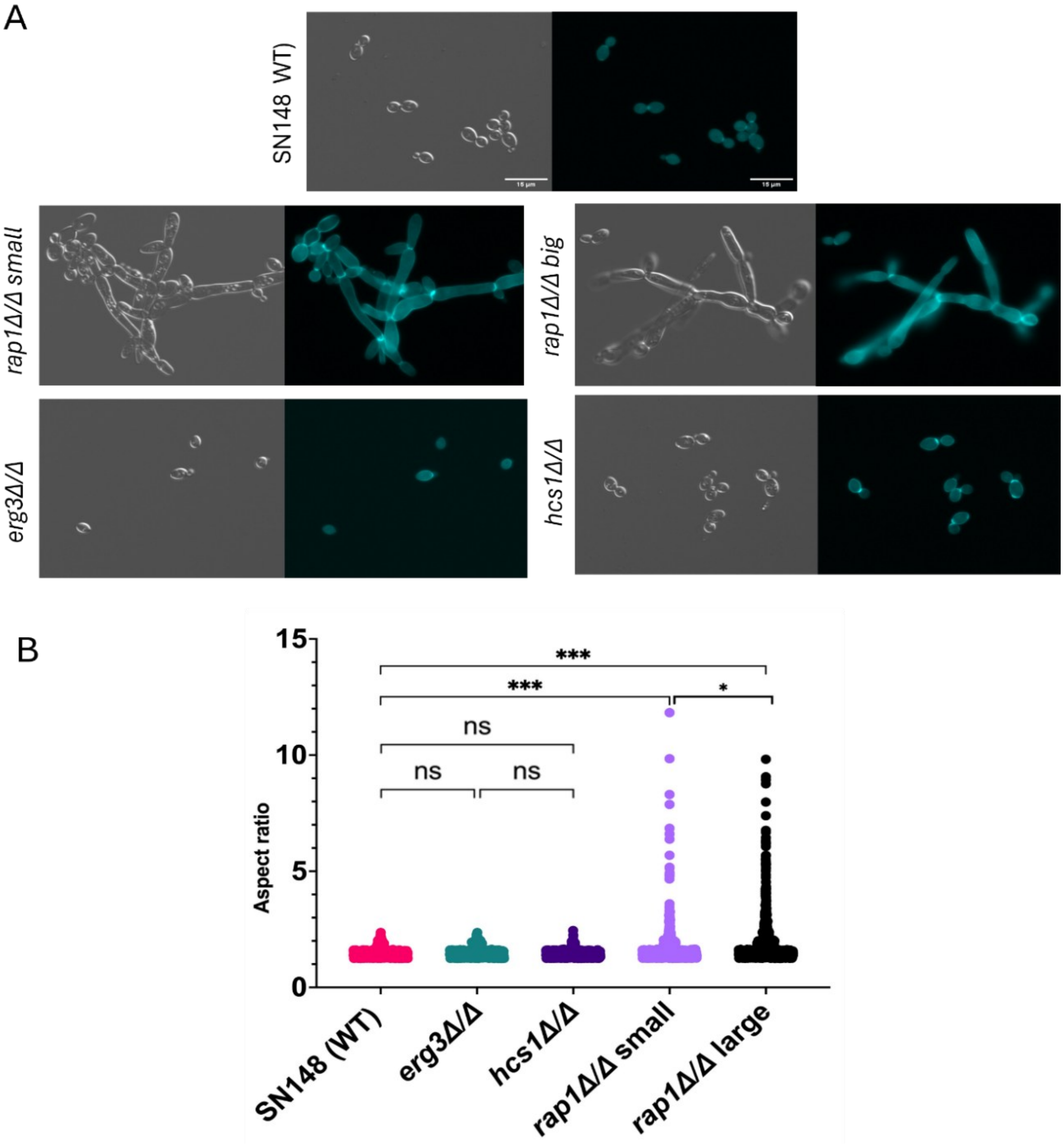

Figure 3.2.6. Cell morphology and aspect ratio analysis of rap1Δ/Δ strains. A. Samples were stained with CFW and imaged with Leica DMi600 microscope. DIC and fluorescence images represent the cellular morphology of strains grown in liquid YPD. Strains of rap1Δ/Δ colonies predominantly exhibit pseudohyphal cell morphology, unlike the yeast cells observed in wild type (WT), erg3Δ/Δ, and hcs1Δ/Δ strains. Cells are shown under 100x magnification with scale bar at 15 µm. B. Cell AR show significant differences between WT and strains of rap1Δ/Δ small and large colonies. We used ordinary one-way ANOVA test comparison with Turkey multiple comparison test with single pooled variance where mean difference was calculated and significance is calculated based on the F-statistic and its corresponding p-value ($P < 0.05$), which determine whether the group means are significantly different while accounting for unequal variances.

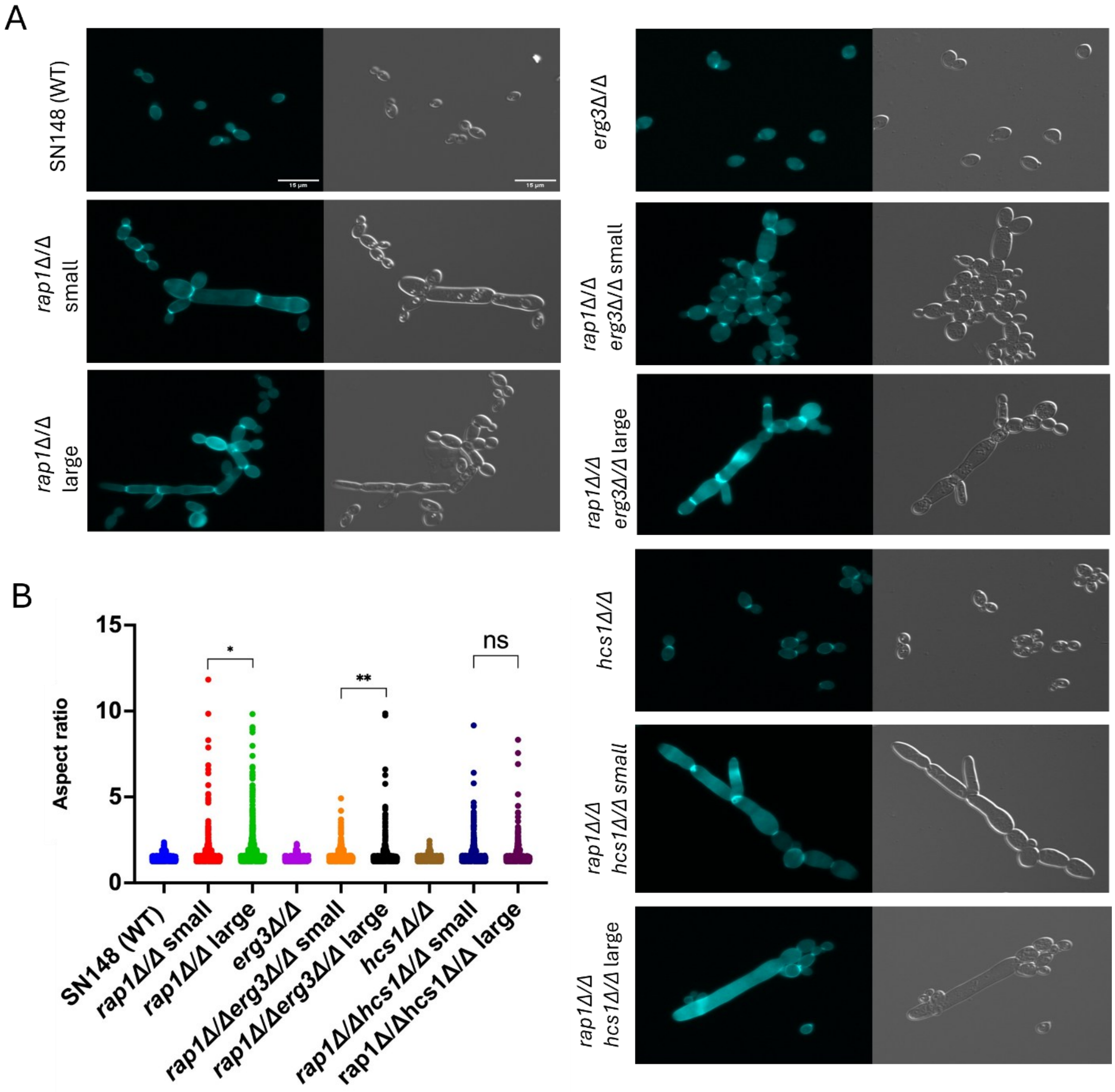

Figure 3.2.7. Cell morphology and size in the CRISPR mutants. A. DIC and CFW-fluorescence images represent the cellular morphology of strains grown in liquid YPD. Strains of rap1Δ/Δ colonies predominantly exhibit pseudohyphal cell morphology, unlike the yeast cells observed in wild type (WT), erg3Δ/Δ, and hcs1Δ/Δ strains. Cells are shown under 100x magnification with scale bar at 15 µm. rap1Δ/Δ erg3Δ/Δ keeps a pseudohyphal shape, unlike erg3Δ/Δ. B. The cell AR show significant differences between erg3 Δ/Δ strain and rap1Δ/Δ small and large colonies. The AR variation between small and large rap1Δ/Δ colonies is not significant We used ordinary one-way ANOVA test comparison with Turkey multiple comparison test with single pooled variance where mean difference was calculated, and significance is calculated based on the F-statistic and its corresponding p-value ($P < 0.05$), which determine whether the group means are significantly different while accounting for unequal variances.

3.2.6. Correlation of fluconazole response in fast and slow growing *rap1* colonies

To test if the variation in fluconazole resistance is due to the different colony sizes observed in the *rap1Δ/Δ* strain, we performed the disc diffusion assay with 25 µg fluconazole with both sizes of the *RAP1* deletion construct (Fig. 3.2.8). From this experiment, 33% of both cultures from small and large colony sizes of *rap1Δ/Δ* had no ZOI, indication full resistance, whereas 67% had a mild ZOI, indication slight susceptibility (Fig. 3.2.8). This suggests that there is no correlation between the *rap1Δ/Δ* colony sizes and fluconazole response. Because of the different colony sizes and variability of *rap1Δ/Δ* mutants in response to fluconazole, we further investigated using the disc diffusion assays with double mutants and assessed colony size-specific phenotypes. Intriguingly, the variability in fluconazole response observed in *rap1Δ/Δ* persisted in these combinations, although the extent differed. For instance, half of *rap1Δ/Δ erg3Δ/Δ* mutants of small colonies revealed similar proportions of mild and no ZOI. In the case of *rap1Δ/Δ erg3Δ/Δ* large colonies, 33% exhibited a mild ZOI and 67% had none (Fig. 3.2.8). As for the *rap1Δ/Δ hcs1Δ/Δ* small mutants, their culture displayed 33% no ZOI and 67% a mild zone (Fig. 3.2.8). However, *rap1Δ/Δ hcs1Δ/Δ* mutants of the larger colonies were more distributed, showing 17% clear ZOI, 33% mild and 50% no ZOI. In contrast, the double deletion of *hcs1Δ/Δ* and *erg3Δ/Δ* consistently resulted in a mild ZOI across all replicates respective of sizes (Fig. 3.2.8).

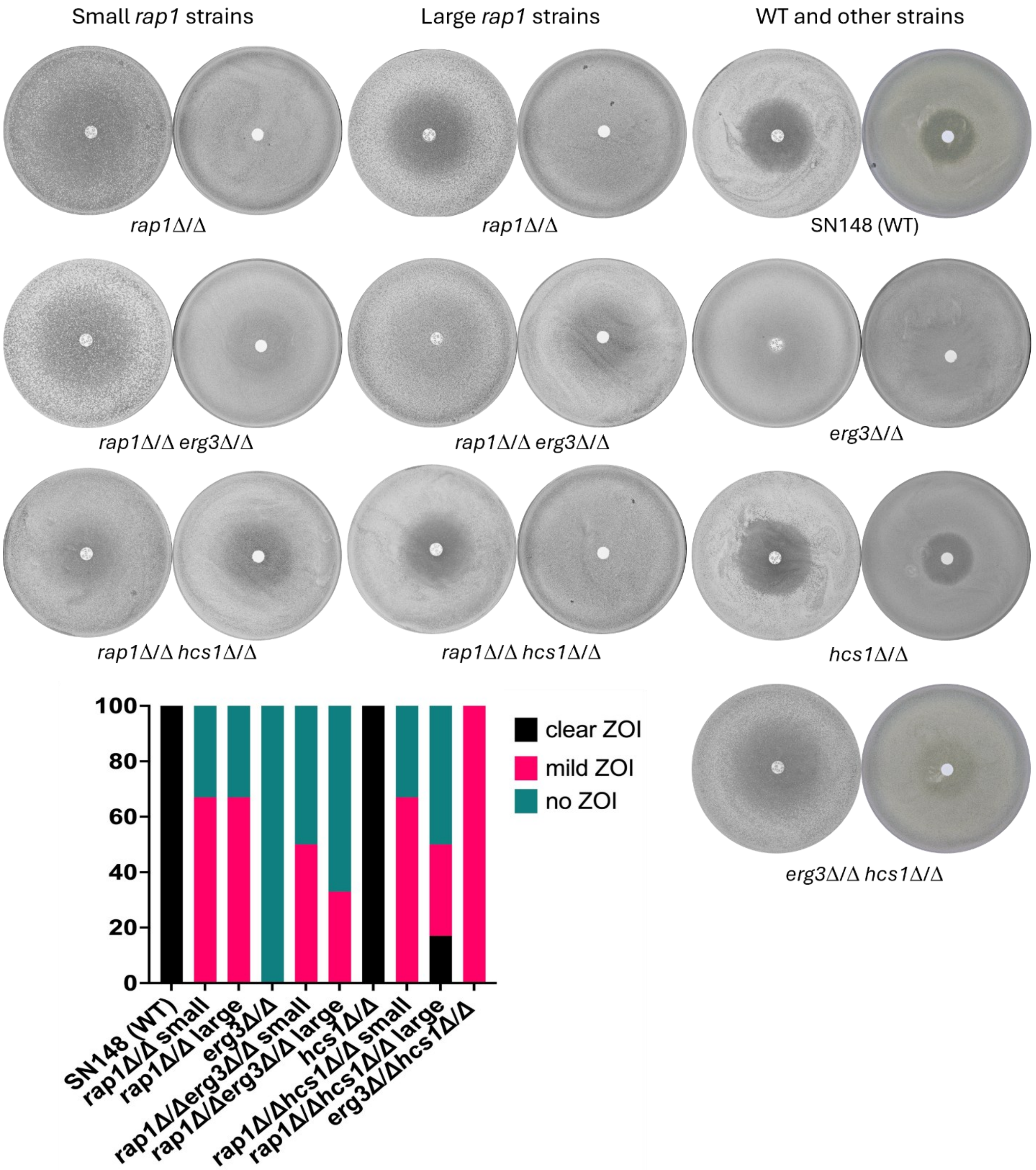

Figure 3.2.8: *Fluconazole resistance in the CRISPR mutants and small and large colonies of rap1* single and double *mutants using the disc diffusion assay. Bar graph represents fluconazole responses across replicates of the constructs. It shows consistent response of no ZOI for the erg3Δ/Δ single mutant, clear for hcs1Δ/Δ single mutant and mild for erg3Δ/Δ hcs1Δ/Δ while rap1 mutants from both colony sizes have differing resistance levels in ZOI.*

3.2.7. Adhesion of *RAP1, ERG3 and HCS1* deleted strains

Often filamentation is linked to invasiveness in *C. albicans*. Because of the pseudohyphal nature of the *rap1Δ/Δ* strain*,* and because one of the key elements for it is a cell's ability to adhere to a surface, we used an assay testing adhesion to a solid surface after washing. Colonies were grown for 4 days prior to washing. We found that for the *rap1Δ/Δ* strains both smaller and larger colonies showed an increased adhesiveness with little difference between them (Fig. 3.2.9A-B). However, the *erg3Δ/Δ* strain showed no evidence for adhesion, with cells completely washed off from the surface, suggesting that *ERG3* plays an important role in adhesion (Fig. 3.2.9A). Strain *hcs1Δ/Δ*, like WT, has a partial adhesive nature as some cells remained adhered to the surface (Fig. 3.9B).

We were curious to expand the assay to the double mutants to investigate the persistence of the *erg3* non-adhesive behavior. We saw the adhesiveness in the *rap1*Δ/Δ *erg3*Δ/Δ double KOs similar to the *rap1Δ/Δ* strains after wash, and the colonies showed a wrinkly morphology before wash (Fig. 3.2.9A-B). Both smaller and larger colonies of the *rap1*Δ/Δ *hcs1*Δ/Δ double mutant have a fuzzier and less wrinkly morphology (Fig. 3.2.9B). As mentioned earlier, when *hsc1Δ/Δ* mutant behaves similarly to WT when it comes to adhesiveness while *erg3Δ/Δ strain* is completely non-adhesive. However, strains of erg*3Δ/Δ hcs1Δ/Δ* becomes slightly more adherent than that of *erg3Δ/Δ* yet still maintains the wrinkly morphology before wash (Fig. 3.2.9C).

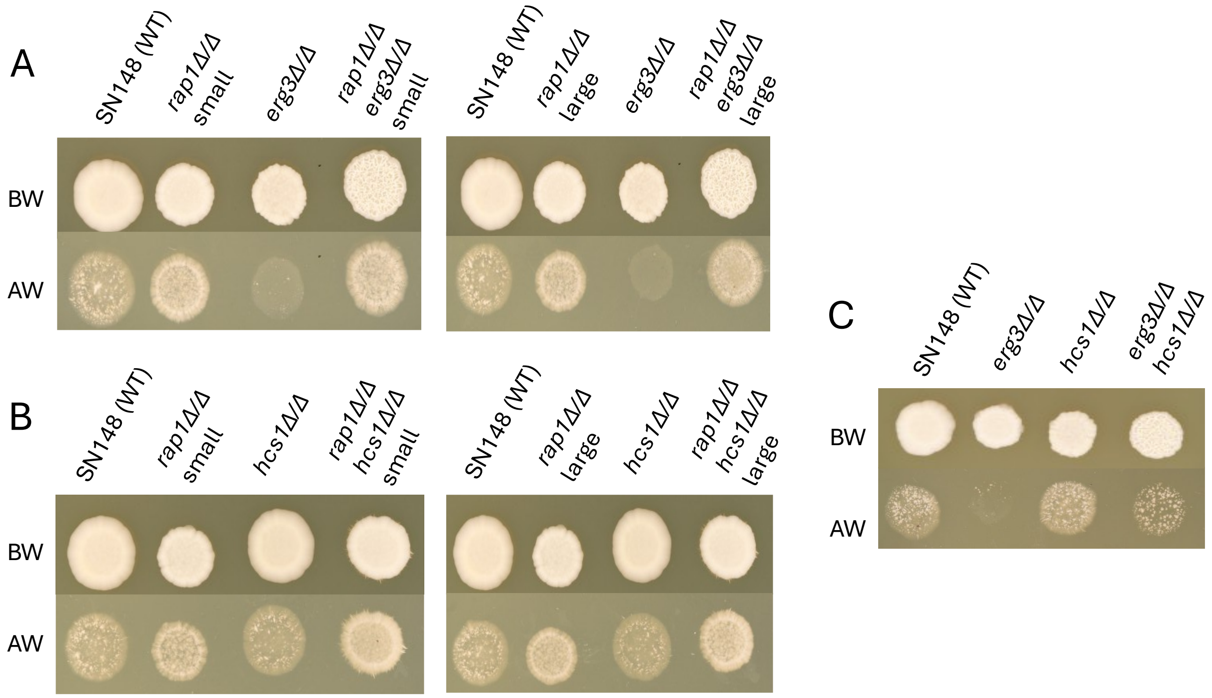

Figure 3.2.9. Adhesion in the constructed mutants. A. Both small and large rap1Δ/Δ colonies show high adhesion after wash (AW), while strain rap1Δ/Δ erg3Δ/Δ small and large colonies are winkled before wash (BW), and small colonies adhere similarly to that of rap1Δ/Δ, large colonies are less adhesive. B. Strain rap1Δ/Δ hcs1Δ/Δ colonies show fuzziness BW, with small colonies being more adhesive than rap1Δ/Δ AW. C. Strains erg3Δ/Δ hcs1Δ/Δ shows increased adherence compared to non-adhesive erg3Δ/Δ strain, indicating an interaction between these genes in adhesion regulation.

3.2.8. Switchable response of *rap1Δ/Δ*, *rap1Δ/Δ erg3Δ/Δ and rap1Δ/Δ hcs1Δ/Δ* large resistant strains

With large *rap1Δ/Δ* strain colonies having a greater chance in having a resistance response, we tested if this phenotype remains consistent. We streaked the larger resistant *rap1Δ/Δ* strain colonies on a fresh YPD plate and selected larger offspring colonies to perform disc assays. The offspring also had variability and did not all result in complete resistance; half had a clear and mild ZOI, and the other half had no ZOI (Fig. 3.2.10).

We picked the larger *rap1* double mutants to test whether we observe a switchable response. We found that resistant candidates of larger *rap1Δ/Δ erg3Δ/Δ* strain colonies can indeed switch to a more susceptible response; 75% of replicates showed no ZOI and 25% had a mild ZOI

(Fig. 3.2.10). As for larger *rap1Δ/Δ hcs1Δ/Δ* strains, they resulted in a similar proportion of mild to no ZOI (Fig. 3.2.10).

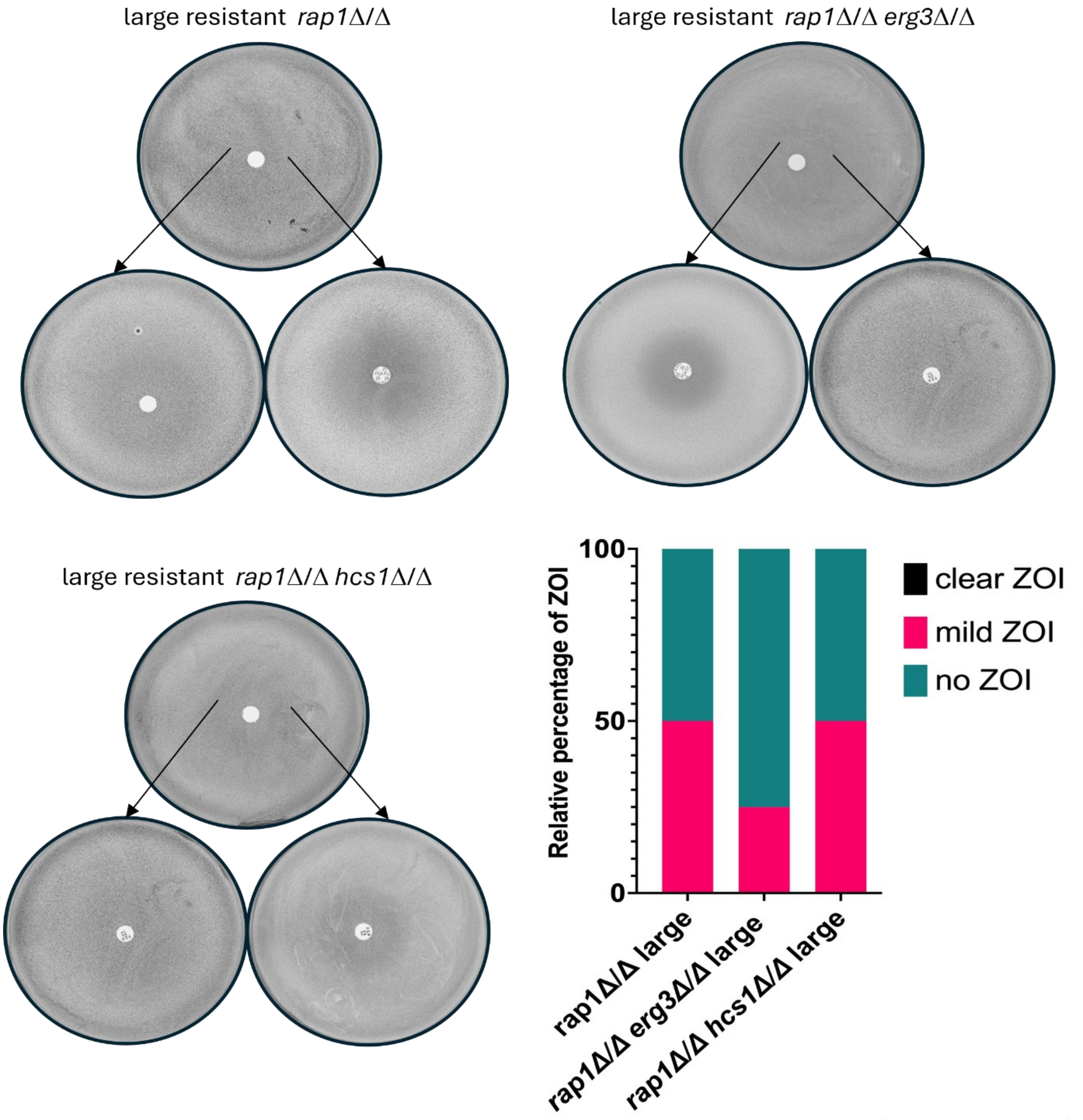

Figure 3.2.10. Fluconazole resistance stability in single and double mutants of rap1Δ/Δ. Disc diffusion assay of large rap1 resistant mutants in presence of 25 µg fluconazole shows their switch to susceptibility. Bar graph presenting variability in resistance: Single mutant rap1Δ/Δ and double mutant rap1Δ/Δ hcs1Δ/Δ with 50% mild ZOI whereas double mutant rap1Δ/Δ erg3Δ/Δ showed 25% mild ZOI.

3.2.9. Response of *rap1Δ/Δ* in different stressors

After testing with the fluconazole response of *rap1Δ/Δ* strains and observing the colony sizes, we tested if this phenotype remains consistent with other stressors, specifically DNA damaging UV, high temperature, osmotic stressor (NaCl), target of rapamycin (TOR) inhibitor (rapamycin) and echinocandin antifungal (Caspofungin). To do this we used the replica method as described in 2.9.

We did not find any variability in both small and large parental type *rap1Δ/Δ* strains compared to the different stressor except oxidative and genotoxic stress. In presence of H_2O_2 and UV, both small and large parental type *rap1Δ/Δ* strains showed sensitivity (Figure 3.2.12, Fig. S1). Under most stress conditions, both small and large parental *rap1Δ/Δ* strains behaved similarly to the wild type (WT). The variability between small and large replica colonies did not show a consistent correlation with the distribution of affected *rap1Δ/Δ* parental strains. However, under oxidative stress, both *rap1Δ/Δ* strains exhibited sensitivity to H_2O_2 at 12.5 mM. At lower concentrations, a shift in the distribution of colony sizes was observed and larger colonies had a higher survival rate.

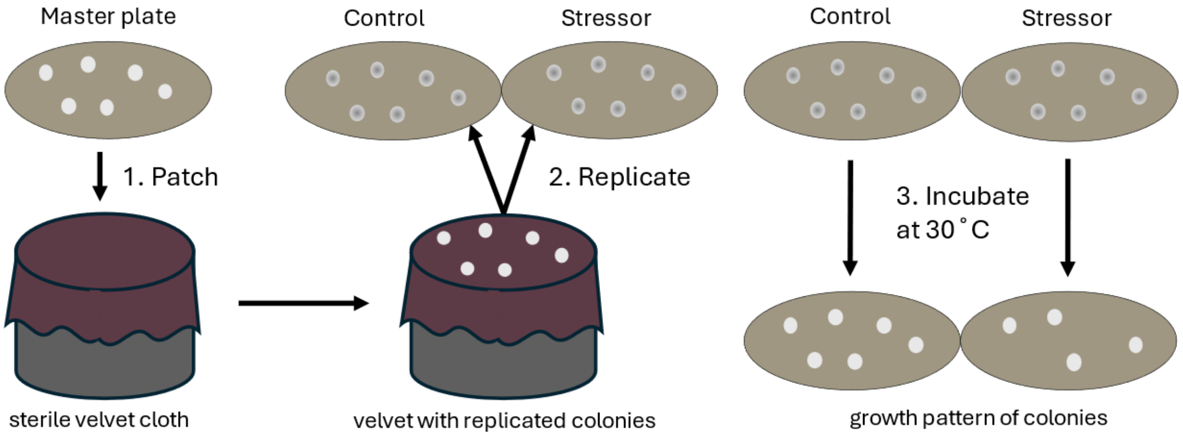

Figure 3.2.11. Diagram representing the replica-plating system used for stress-testing. Firstly, a previously grown plate with colonies is pressed on a velveteen to transfer to colonies. Secondly, new plates with stress-inducing agent (H_2O_2,Caspofungin, UV, high temperature, Rapamycin, NaCl) are pressed onto the velveteen for colony transfer. Lastly, the new plates were incubated, and the growth pattern of the stressor-plates was compared to the WT and analyzed against the control plate.

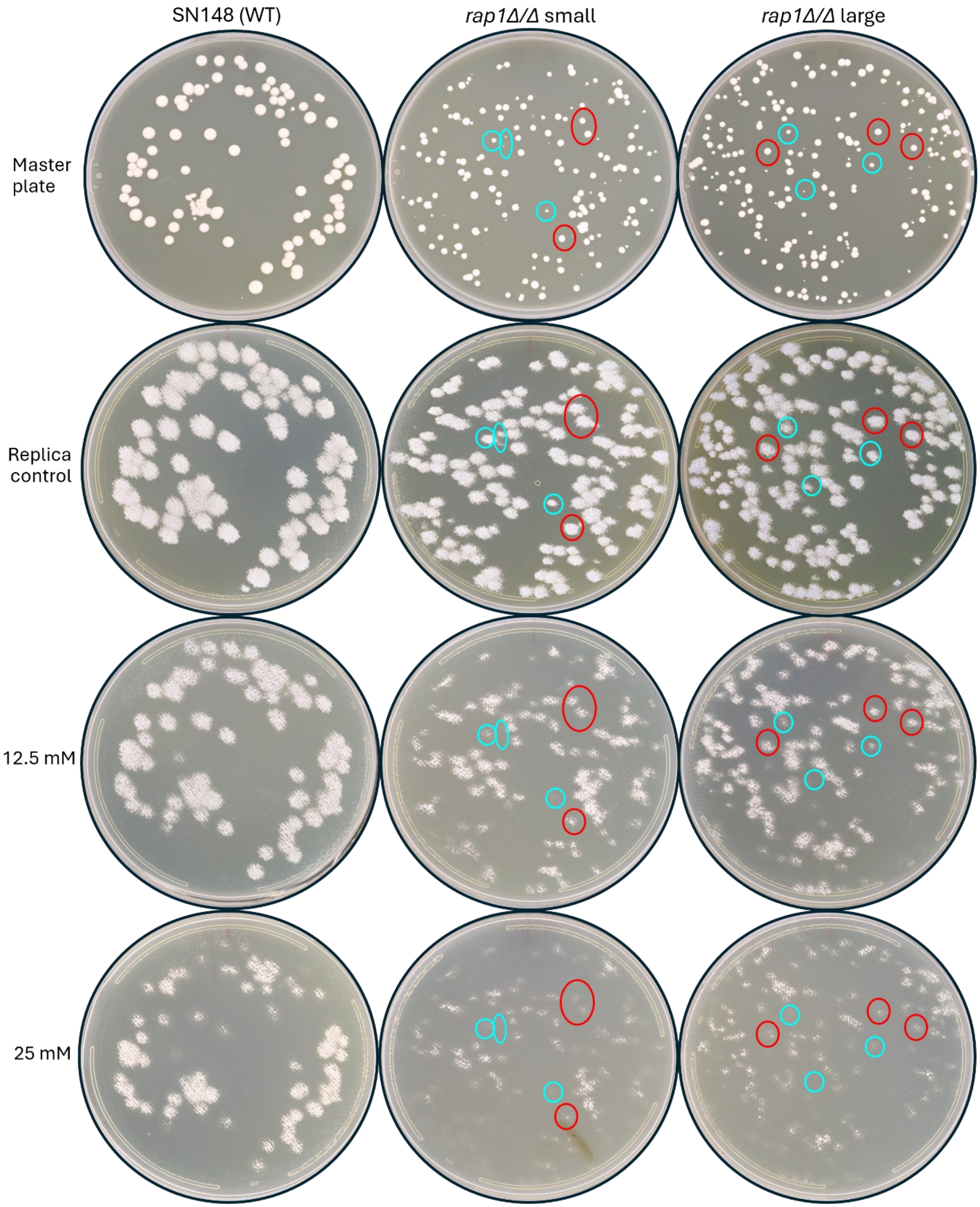

Figure 3.2.12. Sensitivity of rap1Δ/Δ small and large strains in presence of oxidative stress. Colonies were exposed to 12.5 and 25 mM H_2O_2 *using the replica-plating method. Smaller slower-growing and larger faster-growing isolated replica colonies are circled in turquoise and pink respectively.*

3.2.10. Possible function and motif search of Rap1

The transcription factor Rap1 is well characterized in *S. cerevisiae* and binds to a 5'-CACCCATACA-3’ motif. In *C. albicans* it binds to the same sequence however, it is already known to be rewired [39]. Therefore, to understand the global role of Rap1, we searched its motif 5'-CACCCATACA-3' in the promoter regions of the *Candida* genome and identified more than 1400 possible candidate binding sites. These includes genes responsible for stress response, hyphal growth as well as many transcription factors including Cap1, Adr1 and Upc2 all well known for influencing fluconazole resistance (Fig 3.2.13). We also find *CDR1* and *CDR4* transporter genes and a few Ergosterol biosynthesis genes like *ERG5, ERG9, ERG25* etc., but not Erg3, indicating that Rap1 may not bind to the *ERG3* promoter.

3.3. Discussion

The dynamics of drug resistance in *C. albicans* have been a prime focus of research for many years, yet much remains to be uncovered. Drug resistance in *C. albicans* is a complex process and involves multiple mechanisms [138]. Multiple genes from different pathways including the ergosterol pathway (such as *ERG11*[139], *UPC2* [140], *ERG3*, *ERG6* [141],) transporter proteins (like *CDR1, CDR2* [142] and *MDR1* [143, 144]), TFs (like *TAC1, MRR1, ADR1, MRR2, CAP1*) [145, 146], cell wall synthesis protein encoding *FSK1* as well as heat shock proteins *HSP90* [147] are all known to be involved in drug resistance. In a previous large-scale screening, our lab found that *RAP1*, encoding a transcription factor, *ERG3*, encoding a C-5 sterol desaturase of the ergosterol biosynthesis pathway, and *HCS1*, encoding a helicase, played a role in fluconazole response. More specifically, the study identified candidate GRACE 1.0 mutants *rap1*, *erg3* and *hcs1*, showing a resistance response to fluconazole [85]. In this study, we constructed independent disruption mutants of these genes and retested the fluconazole resistance with various concentrations of the drug. While our spot assays showed that the *rap1Δ/Δ* and *erg3Δ/Δ* mutants conferred resistance to the drug, the *hcs1Δ/Δ* mutant strain remained unaffected. It is common to see the variation between partial loss of function mutants and complete knockout [148]. Rap1 in *S. cerevisiae* binds the sequence 5'-CACCCATACA-3', and regulates telomere function, silencing, and the activation of glycolytic and ribosomal protein genes [149]. However in *C. albicans*, Rap1 is rewired and together with the TF Cbf1 is involved in ribosomal gene regulation [39]. Also, *C. albicans* Rap1 lacks a C-terminal domain found in ScRap1, making it interesting to investigate further. ScRap1 has been found to target ~5% of yeast genes and contribute to activation of ~37% of RNA polymerase II-mediated transcription [137, 150]. CaRap1 is observed to be implicated in oxidative stress response, cell wall integrity, biofilm formation and virulence [137, 151]. In this study, we confirmed the large-scale screening of Chen et al., 2018 that the deletion of *RAP1* can confer resistance to fluconazole, deletion of *ERG3* confers resistance but that of *HCS1* does not [85]. Previously, TFs such as Upc2, Mrr1, and Tac1 have been associated with fluconazole resistance, but their deletion typically results in increased susceptibility [140, 145, 152]. In contrast, our findings show that *RAP1* deletion enhances fluconazole resistance, but this resistance level is variable. The deletion of *RAP1* results in a delay in lag phase resulting in slower growth rate in *C. albicans.* Often in slow growing cells metabolism is supressed and altered pathways are in use resulting in resistance [106, 153-156]. This might be simply because in such altered pathways the drug target is not needed. Similar observations were made in other studies exploring the role of Rap1 in *C. albicans* [137]. Strikingly, we observed a variation in colony size distribution within the *rap1Δ/Δ* strain, with a predominance of small colonies and occasional emergence of larger colonies. Previously a similar epigenetic pattern in the case of the *SIR2* deletion resulting in two morphologically distinct colonies, wrinkled and smooth has been observed [157]. This suggests a genetic component influencing colony morphology in *rap1* mutants, potentially linked to *RAP1*'s regulatory role in gene expression. Therefore, we checked the morphology of *rap1Δ/Δ* strains under the microscope of both large and small colonies. We observed a pseudohyphal morphology in *rap1Δ/Δ* irrespective of colony sizes, in contrast to the wild-type yeast morphology. Other TFs are known to be involved in formation of pseudohyphae are Efg1, Bcr1, Hwp1 [158, 159]. Further, we tested *rap1Δ/Δ* for chemical and environmental stressors including H_2O_2, Rapamycin, NaCl, Caspofungin, temperature and UV. We did not observe any change in the response to these stressors except under oxidative and genotoxic stress where *rap1Δ/Δ* strain is sensitive compared to WT. Moreover, in lower concertation, only the large colonies were resistant and able to survive, aligning with the expected correlation between fitness and colony size, where smaller colonies exhibit reduced growth under stress while larger colonies persist.

Overall, our findings suggest that Rap1 plays a role in regulating morphogenesis, and disrupting this gene can lead to changes in cell shape, possibly affecting fungal invasion. To test this, we performed an adhesion assay and confirmed that Rap1 is indeed involved. Our results indicate a strong link between the Rap1 transcription factor and adhesion leading potentially to virulence though further transcriptomic analysis and *in vivo* virulence studies are needed to understand the underlying genetic mechanisms.

In *S. cerevisiae,* small colonies can appear because of a block in the aerobic respiratory chain pathway involved in ATP generation. These small colonies are thus unable to grow on nonfermentable carbon sources (such as glycerol or ethanol), and form small anaerobic-sized colonies when grown in the presence of fermentable carbon sources (such as glucose)[160]. Therefore, we grew the small colonies of strains *rap1Δ/Δ*, *rap1Δ/Δ erg3Δ/Δ* and *rap1Δ/Δ hcs1Δ/Δ* on a glycerol source instead of glucose and they all had the ability to grow, indicating healthy mitochondria.

HCS1 is a DNA helicase in *C. albicans*, and its ortholog in *S. cerevisiae* is well characterized with a known 5'-3', double-stranded DNA helicase and ATP hydrolysis function [161]. In yeast, Hcs1 potentially interacts with Rad5 and that it likely acts upstream of Rad5 [162]. It is also known absence of *HSC1* shows resistance to cycloheximide in *S. cerevisiae* [163]. However, in *C. albicans* very little is known about *HCS1.* Unlike the previous large-scale screening we found that *hcs1* deletion does not have any impact on growth in presence of fluconazole, morphology, or invasiveness. Further investigation will be needed to understand its role in *C. albicans*. Intriguingly, we did find that introducing the *hcs1Δ/Δ* disruption in the *erg3Δ/Δ* background reverses the drug resistance of *erg3Δ/Δ*. Moreover, during adhesion test, the *hcs1Δ/Δ erg3Δ/Δ* double mutant was able to rescue the loss of adhesion created by the *erg3Δ/Δ* mutation*,* indicating they might have epistatic interactions for some phenotypes - however this needs to be studied further to determine the exact mechanisms.

Mutation of Erg3 is known for altering the metabolic pathway resulting in resistance to azoles. If Erg3 remains active, it converts 14a-methylated sterols into a toxic 3,6-diol derivative resulting in azole hyposensitivity to azole [164], and studies have reported *erg3* mutation results in azole resistance by altering the pathway and decrease the role of Erg11 the target of azole [165-168]. We found a similar result when we deleted *ERG3* using our CRISPR construction. However, it was suggested that loss of ergosterol biosynthesis will have a negative impact on the switch between the yeast and hyphal morphology and thus could potentially contribute to decreased fitness *in vivo* [169]. Therefore, we tested if *erg3* mutant impacts *Candida albicans* morphology and adhesiveness. Intriguingly, we found that *ERG3* is critical for adhesiveness of *C. albicans.* Previously, *erg3* mutants *in vivo* studies have also shown lower adhesion [156, 170]. Often cell membrane plays a critical role in adhesion [171] and since *erg3* disturbs cell membrane integrity, this might be the reason for such low adhesiveness. Due to similar behavior between *rap1* and *erg3* for showing resistance to fluconazole, we decided to further investigate it and decipher whether they have genetic interactions.

We have observed genetic interactions of the deletions of *RAP1* and *ERG3* in *C. albicans*. The *rap1Δ/Δ erg3Δ/Δ* strain showed a similar pattern of drug resistance response like the *rap1Δ/Δ* strain with variability in resistance, even though the *erg3* mutant strain showed clear drug resistance. Moreover, the cellular morphology of *rap1Δ/Δ erg3Δ/Δ* strain demonstrated pseudohyphae like the *rap1Δ/Δ* small strain, while *erg3Δ/Δ* was in yeast form. These all indicate a connection between Rap1 and Erg3. Previously, it has been reported that Rap1 can impact the cell membrane composition [137], and Erg3, being part ergosterol biosynthesis, also does the same. Therefore, we checked the DNA binding motif of Rap1 in the upstream promoter region of the *ERG3* gene but were not able to find the motif. This indicates that Rap1 does not directly regulate *ERG3* however we did find other genes associated with the ERG-pathway like *ERG6, ERG25* that are potential targets of Rap1 binding. We found approximately 1400 genes with potential to be Rap1 targets indicating possibility of multiple roles of Rap1 in *C. albicans*. Strikingly, among them were transcription factors Cap1 and Adr1. We found roughly 60 TFs which might be downstream of Rap1 including Bcr1 which is known for its role in the adhesion. Previously, our lab has created a library of 30 activated TFs using VPR which includes Adr1 [37]. Therefore, we performed high throughput screening for these TFs for different phenotypes and were able to find the role of Adr1 in drug resistance as described in next objective of my studies (chapter 4).

Previously many genetic interactions of different genes have been reported in other *Candida* species, highlighting the broader relevance of our findings. For example, the *ERG11* gene, encoding the fluconazole target enzyme lanosterol demethylase, interacts with *UPC2*, a transcription factor that regulates sterol biosynthesis. Deletion of *UPC2* leads to impaired fluconazole resistance, even in strains overexpressing *ERG11*, illustrating how mutations in one gene can affect another's function in antifungal resistance [156, 172]. Additionally, interactions between the stress-response kinase *HOG1* and *FKS1*, a glucan synthase gene, also reveal complex genetic networks. The *hog1Δ/Δ fks1Δ/Δ* double mutant shows altered cell wall integrity and stress responses, which mirrors the effects observed when *RAP1* is deleted in *C. albicans*, reinforcing the idea that signal transduction pathways and cellular stress responses are tightly connected [173-175].

Our findings also point to possible feedback loops in sterol biosynthesis as a critical aspect of *RAP1*-*ERG3* interaction. The *ERG3* gene is part of a complex biosynthetic pathway, and its expression might be regulated in response to changes in sterol composition, which is influenced by *RAP1*. The loss of *RAP1* might interfere with ergosterol production, leading to changes in membrane composition, and, in turn, stress tolerance, and antifungal susceptibility. Based on our observation in *Candida albicans*, the relationship between *RAP1* and *ERG3* in our study adds to this body of knowledge, suggesting that the functional loss of *RAP1* could potentially alter sterol biosynthesis or resistance to environmental stressors. Moreover, the presence of two distinct inheritable phenotypes emphasizes the complexity of genotype-phenotype interactions and epistasis, highlighting the intricate relationship between genetic variations and phenotypic outcomes

Chapter 4 – Identifying transcription factors that influence the response of *C. albicans* strains upon activation

4.1 Introduction

Transcription factors (TFs) represent the largest functional protein family in *C. albicans,* comprising approximately 4% of its genome (about 240 TFs) [114]. These master regulators coordinate multiple virulence mechanisms that enable fungal pathogenicity and drug resistance. Notably, TFs control biofilm formation - a key virulence trait that can increase antifungal resistance up to 1000-fold by creating physical and physiological barriers to drug penetration [54]. Additionally, they regulate the critical yeast-to-hypha morphological switch [113], stress response pathways [176], metabolic flexibility, and cell wall remodeling - all processes that contribute to antifungal resistance.

The central position of TFs in regulatory hierarchies means even minor alterations can dramatically impact drug susceptibility. For instance, gain-of-function mutations in Tac1 lead to constitutive overexpression of CDR-drug efflux pumps, resulting in azole resistance [177]. Similarly, mutations in MRR1 upregulate MDR1 gene expression, conferring resistance to multiple drug classes [178]. These examples underscore why TFs represent high-value targets for antifungal development.

Comparative genomic analyses reveal extensive evolutionary divergence between *C. albicans* and *S. cerevisiae* TF networks. While approximately 60% of TF genes are conserved between these species, only about 16% of regulatory connections (TF-target gene relationships) remain unchanged [115]. This transcriptional rewiring over 300 million years of evolution has resulted in several key differences:

- 42% of *C. albicans* TFs lack direct *S. cerevisiae* orthologs
- 25% of orthologous TFs regulate divergent sets of target genes
- *C. albicans*-specific expansions in TF families involved in stress response and virulence

To systematically investigate these differences, our lab generated a library of 30 chimeric TFs by fusing DNA-binding domains to the potent VPR activation domain, focusing on factors either lacking *S. cerevisiae* orthologs or bioinformatically predicted to have divergent functions. We further employed high-throughput functional screening using the VPR-strains in order to examine the roles of the TFs across the strains' phenotypes such as morphology, pH and temperature tolerance, carbon source metabolism, antifungal resistance, and metal ion response. Interestingly, among these TFs, activation of pC4_02500C_A conferred resistance to cell membrane-targeting drugs. Further analysis revealed that pC4_02500C_A is the ortholog of *S. cerevisiae* Adr1 but has evolved distinct functions. This finding highlights the extent of transcriptional rewiring between these two species and underscores the importance of understanding species-specific transcription factors networks to identify new therapeutic targets. We were able to extensively study this strain *C4_02500C_A* alias *ADR1* in *C. albicans* which recently was published [37] as attached in Appendix 1. 4.2.

4.2 Results

4.3.1. Screening the VPR activated transcription factor library for differential growth, metabolism, and morphology of strains

We first investigated carbon source utilization. We checked growth on various sugars as a sole source of carbon; these sugars included glucose, xylose, fructose, sucrose, and sorbitol. We did not find any variation in the growth of any strain containing an activated transcription factor. Next, we inspected morphological variation at standard conditions using microscopic analysis for all the TFs strains. We found that the strains containing activated *C2_05640W_A, C4_07150W_A, NRG2*, and *GLN3* showed a mixture of both yeast and filamentous cells (Fig. 4.1A). Subsequently, we screened all the strains for their morphology in spider media and during serum stimulation in both liquid and solid media. During the solid media assay all the strains transitioned to a filamentous form just like the wild type. However, in the liquid media we could see differences in the timing of the filamentous transition. We found that the strains containing activated *C2_05640W_A, C4_07150W_A, NRG2,* and *GLN3* showed a faster transition compared to the wild type. We also observed other strains containing activated TFs like *ADR1, MET32, UGA33, C1_11690W_A* and

PHO4 switched to the hyphal morphology faster than the wild type (Fig. 4.1B). Another morphological change *in C. albicans* is the transition from white cells to opaque cells. This can be identified by growing the *C. albicans* strains in the liquid media containing GlcNAc at room temperature and shaking at 110 rpm, followed by microscopy to check the cells where the opaque cells are more elongated [87]. One strain with activated OFI1 showed a pronounced switch from white to opaque cells during the screening (data not shown).

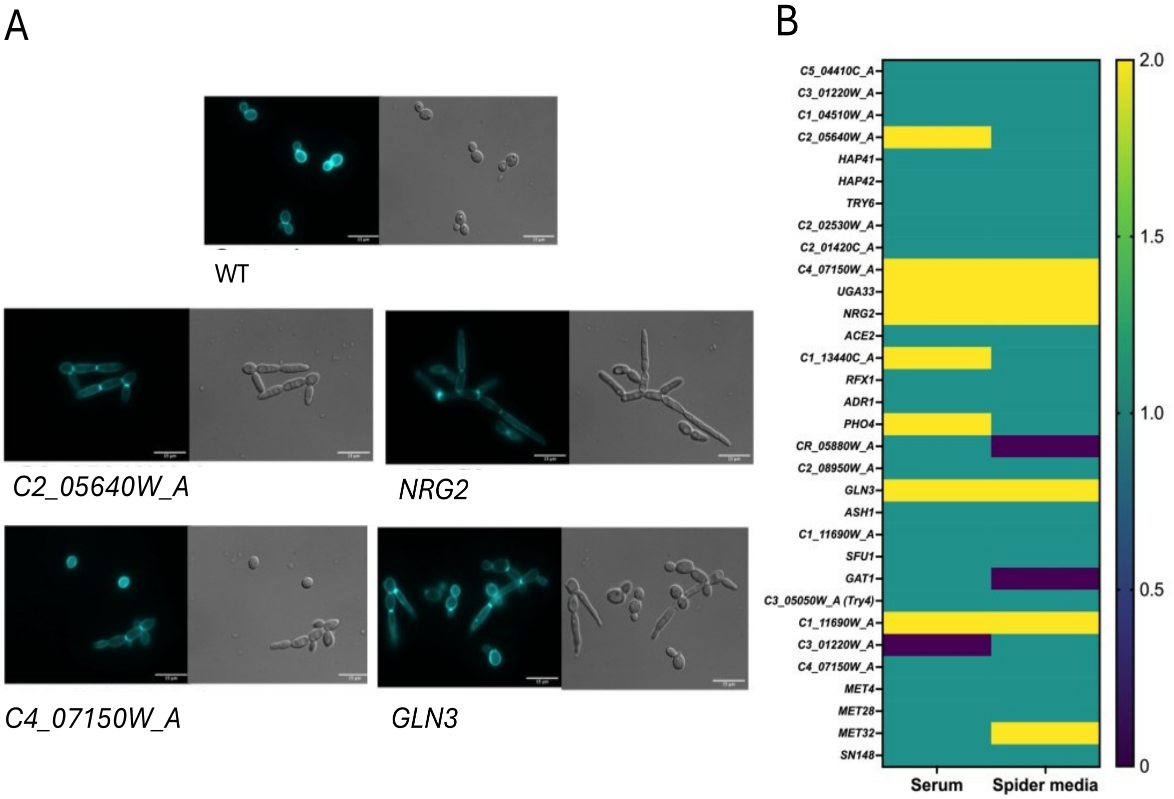

Fig. 4.1. Screening of activated TF library for morphological variation. A. DIC and CFW-fluorescence images represent the cellular morphology of strains grown in liquid YPD. Strains with activated C2_05640W_A, NRG2, C4_07150W_A and GLN2 enhanced filamentous morphology. Cells are shown under 100x magnification with scale bar at 15 µm. B. A heat map of morphological switch from yeast to hyphae was monitored in two different media every half an hour, Spider and serum until 2 hours. We found that 9 strains showing faster switch than the wild type, although only 5 strains showed a faster switch in both media. Adapted from [116].

4.2.2. Screening of the constructed library for response to cell wall stress and heavy metals

The cell wall is the first line of defense for most of the unicellular organisms including bacteria and fungi, and, in C. *albicans*, it contains 10% chitin for rigidity [179]. We investigated the impact of transcription factor activation on the cell wall by using Congo red and Caspofungin as they cause cell wall stress by targeting glucan and chitin synthase [155, 180]. We checked the growth of each strain with an activated transcription factor in the presence of Congo red and Caspofungin. We did not find any changes in the any of the strains. This suggests that none of the transcription factors are involved in cell wall integrity.

Heavy metals coupled with sulfate ions, for example $CdSO_4$ create signalling via the Rad53 pathway leading to cell cycle arrest and therefore have an important role in metal toxicity. We screened the library for tolerance to arsenic and cesium. We found the strain with activated *MET4,* which acts as a methionine biosynthesis regulator in *S. cerevisiae,* showed impressive tolerance to both heavy metals. Often an increased level of methionine (sulfur containing amino acid) biosynthesis has been observed to give tolerance to heavy metals as yeast uptakes the sulfate ion from the environment [181, 182]. This tolerance to the heavy metal might be due to the upregulation of methionine biosynthesis (sulfur utilization). Met28 and Met32 are also known transcription factors which are involved in methionine biosynthesis in *S. cerevisiae* [116, 183]. Unlike transcription factor Met4, activation of Met28 and Met32 did not create much resistance to heavy metals (Fig 4.2A), although Met32 activation permits slight growth in the presence of arsenic~~.~~

4.2.3. Screening activated transcription factor library for temperature and pH response

We know that pH 7 is ideal for *Candida albicans* growth under in vitro conditions; however, in certain host niches, such as the vagina, the pH can drop below 6.5, while in others, it can be higher[184]. Therefore, it is important to assess the adaptability of *Candida* to different pH environments. In addition to pH, temperature is another critical factor influencing *C. albicans* physiology and pathogenicity. *Candida* can encounter a wide range of temperatures in different host environments. For example, surface tissues may be slightly cooler, while febrile conditions can raise the temperature above 37°C. Therefore, it is essential to evaluate how *Candida* adapts to and survives across various temperature ranges to better understand its fitness and virulence in diverse host conditions. Therefore, we screened the activated transcription factor for response to pH (5 to 8) and temperature (15 to 43˚C). We found 1 transcription factor, pC5_04410C_A which, when activated, improved growth at low temperature, although it did not generate any modulation in behaviour at the higher temperatures. Most of the strains behaved similarly to WT at the high temperature, although strains with activated *ADR1, MET32, UGA33, pC1_11690W_A* and *HAP42* grew slightly better in higher temperature compared to wildtype but that might be due to fact that they generically grow faster than the wild type and the rest of the strains. We found the strains with activated *ASH1, HAP41, MET28, MET32* significantly, and *C3_01220W_A* to a minor extent, gave a tolerance to low pH 5, while *C1_04510W_A, PHO4* and *TRY4* activation allowed better growth at a pH above 7.

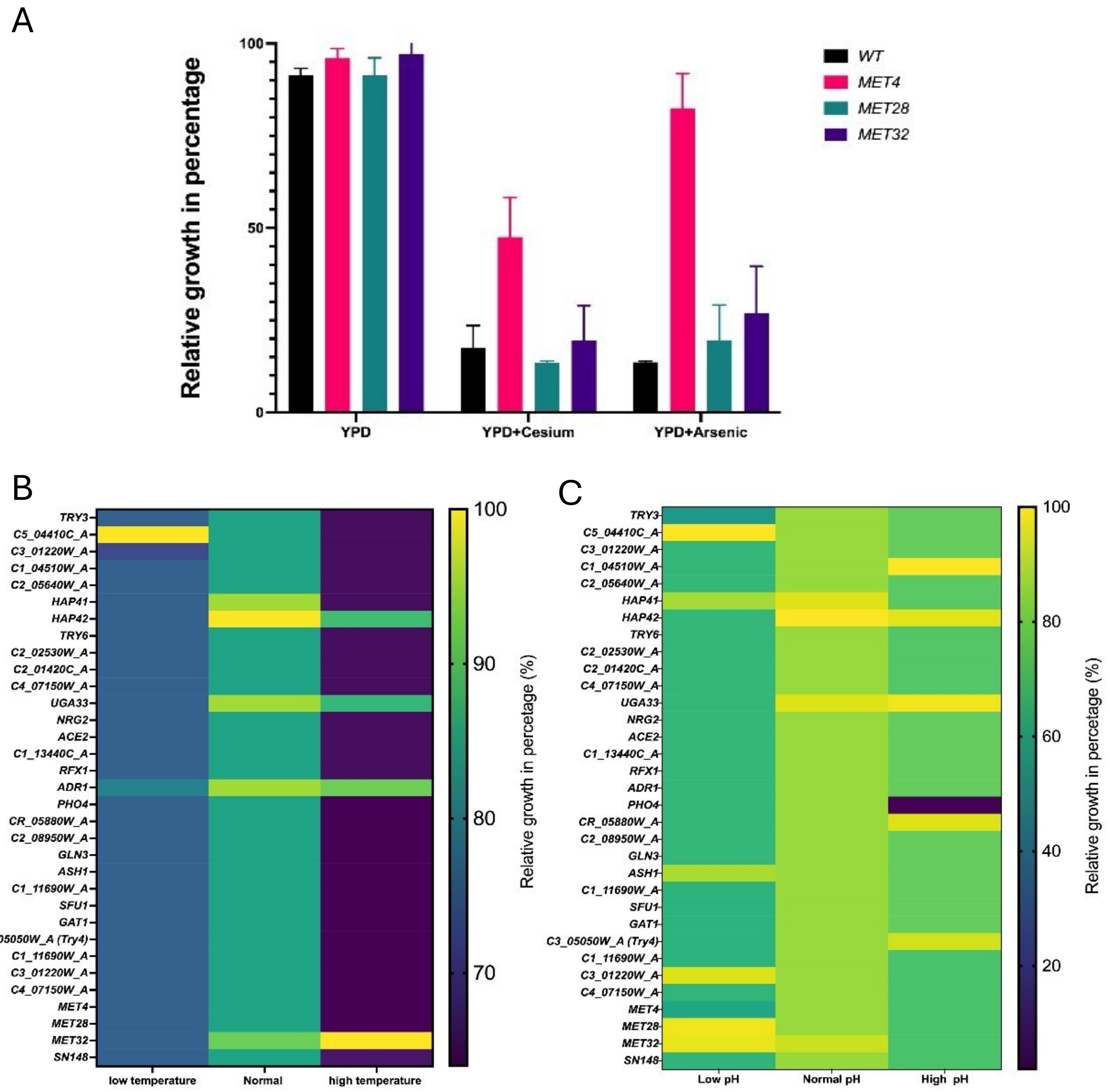

Fig. 4.2. Screening of activated TF library for environmental stress. A. A relative growth assay in liquid media results showing tolerance to heavy metals like cesium and arsenic through activation of Met4. B. Heat map of relative growth assay performed in solid media of temperature tolerance in different strains. Strains HAP42, UGA33, MET32 and ADR1 generated tolerance to the high temperature whereas C5_04410C_A strain results in low temperature tolerance. C. Heat map of relative growth assay in solid media of pH (from 5-8 pH) tolerance in various strains containing activated transcription factors. ASH1, HAP41, MET28, MET32 and C3_01220W_A strains created tolerance to acidic pH whereas strains C1_04519W_A, PHO4 and TRY4 allowed alkaline tolerance [116].

4.2.4. Screening the activated transcription factor library for genotoxicity

Genotoxic (Genome-Toxic) stress compromises the genome integrity of an organism. There are several mutagens that can induce genotoxic stress, such as the DNA replication stalling chemical HU and the thymine-dimer-causing UV light. We screened the library of activated transcription factor strains for DNA damage using HU. Interestingly, the 4 strains with activation of TF pC2_08950W_, Rfx1, Hap42 and Hap41 showed resistance (Fig 4.3.A).

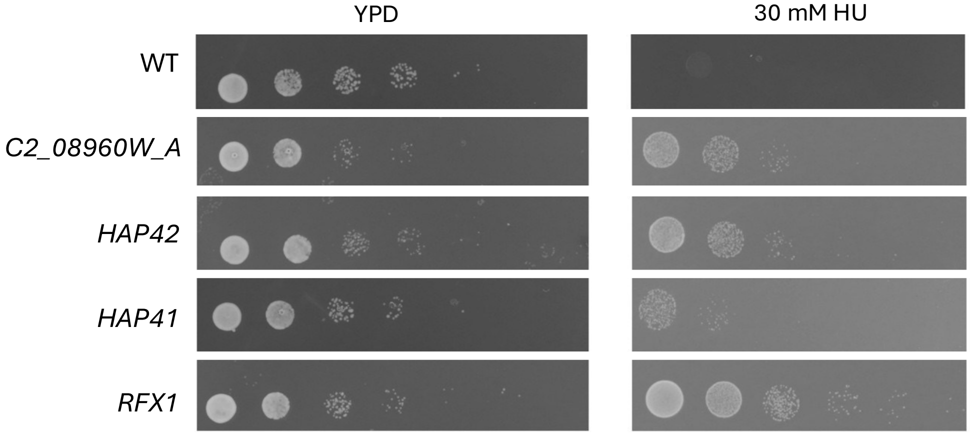

Fig.4.3. Spot assay of activated TF strains under genotoxic stress. Strains of activated C2_08950W_A, HAP42, HAP41 and RFX1 showing growth in the presence of 30 mM DNA damaging agent HU.

4.3. Discussion

As discussed in chapter 1, TFs are crucial regulators across all organisms, coordinating various cellular processes. In *Candida albicans*, TFs play pivotal roles in infection, morphogenesis, biofilm formation, and mating [185-187]. Using comprehensive processes such as phenotypic screening approaches, we examined the impact of respective activated transcription factors on pH tolerance, temperature response, morphology, carbon source utilization, cell wall stress, adhesion, hydroxyurea resistance, drug resistance and salt tolerance, in order to elucidate their possible roles in *C. albicans*. Additionally, cellular morphology was analyzed in media such as serum, Phloxine B, and Spider, which are crucial for studying hyphal formation and white-opaque switching.

Our results identified at least 9 *C. albicans* TFs that generated different phenotypes, specifically Met4, Ofi1, Met28, Met32, Adr1, Try3, Try4, Hap41, and Hap42. For instance, Met4 activation promoted heavy metal tolerance and strong adhesion, while Met28 provided resistance to acidic conditions and low temperatures. These findings contrast with the known roles of Met4, Met28, and Met32 in *S. cerevisiae*, where they primarily regulate methionine biosynthesis [38, 188-190] This divergence highlights potential "rewiring" of regulatory networks in *C. albicans*, likely driven by the need to adapt to its pathogenic lifestyle [38].

The transcription factor Ofi1 *(C1_13440C_A*) was particularly intriguing, as its activation induced opaque colony formation during white-opaque switching, a process typically governed by Wor1 [191]. Despite previous findings that *OFI1* gene deletion has minimal effects on switching [191], our results point to a potential important role in white-opaque switching. Very recently it was uncovered that activated Ofi1 bypasses the rule of only having switching in aa and αα cells, and permits switching even in aα cells [192]. It will be interesting to see the coordination between Ofi1 and other white-opaque related transcription factors.

Hap41 and Hap42, members of the CCAAT-binding family, presented another example of *Candida*-specific divergence. While Hap4 in *S. cerevisiae* is well-known for its role in iron homeostasis and respiration [193, 194], Hap41 and Hap42 in *C. albicans* generated enhanced adhesion, high pH and temperature tolerance, and resistance to HU, suggesting possible roles in DNA repair or adhesion mechanisms. This divergence from canonical Hap4 function emphasizes the specialized adaptations of *C. albicans* to its pathogenic niche.

Microbial adherence, a critical factor in pathogenesis, also revealed differences among TFs. While canonical regulators of hyphal formation and biofilm growth, such as Gal4, Rfx2, Flo8, and others, showed expected adhesion enhancements [195, 196], *Candida*-specific TFs like Try3 and Try4 presented unique contributions. These TFs, already implicated in adhesion by large-scale studies, merit further characterization to clarify their roles in establishing pathogenic biofilms.

Compared to other fungi, *C. albicans* displays significant regulatory innovation, likely driven by its dual lifestyle as a commensal and pathogen. For example, while transcription factors in *Aspergillus* species often regulate secondary metabolism in response to environmental stress [197], *C. albicans* TFs like Hap41 and Hap42 appear to specialize in stress resistance and adhesion, reflecting its adaptation to host environments. Similarly, regulators of white-opaque switching in

C. albicans (e.g., Wor1, Ofi1) have no direct counterparts in *S. cerevisiae*, illustrating the unique transcriptional network in *C. albicans* to support pathogenicity.

Adr1 presented another case of functional divergence. While its activation resulted in only slight growth enhancement under standard conditions, transcriptional profiling suggested a role in lipid biosynthesis [37]. This was confirmed by the observed azole resistance conferred by Adr1 activation, as azoles target the ergosterol biosynthesis pathway. Such a connection underscores Adr1's potential involvement in drug resistance mechanisms, which are less prominent in its *S. cerevisiae* ortholog [37].

This work indeed highlights the multifaceted roles of *C. albicans* transcription factor and their divergence from model fungi, emphasizing the importance of phenotypic screening and comparative analysis in understanding fungal adaptation and pathogenesis.

Chapter 5 – Conclusion and future perspectives

The insights gained from this research not only enhance our understanding of *C. albicans* biology but also underscore the importance of studying genetic interactions in pathogenic fungi. Our prime focus was to investigate the roles of *RAP1*, *ERG3*, *HCS1 and ADR1* in *C. albicans* fluconazole resistance, colony morphology, and cellular adaptation which were identified in the HTS.

Large drug screens using resources such as the GRACE 1.0 library are useful to find candidate genes that are linked to drug response, but more studies are needed to confirm this involvement and to understand how these genes function. In objective 1, we studied the genes *ERG3, RAP1*, and *HCS1* from the GRACE 1.0 library in order to learn if they are functionally associated with fluconazole resistance. We carried out targeted deletions and phenotypic analysis of these genes. *Erg3*, a key component of the ergosterol biosynthesis pathway, exhibited a crucial role in drug resistance. *ERG3* deletion conferred strong fluconazole resistance by altering sterol accumulation, consistent with its known function. Notably, interactions between *Erg3* and *Rap1* revealed an epistatic relationship, where their combined deletion resulted in phenotypes distinct from individual knockouts like lower fluconazole resistance, suggesting a complex genetic network behind drug resistance. Rap1, primarily recognized for its role in telomere maintenance, generated a variable response to fluconazole. The absence of a Rap1 DNA-binding motif upstream of *ERG3* suggests that *Rap1* does not directly regulate *ERG3*. Instead, their epistatic interaction indicates a more intricate regulatory mechanism requiring further investigation. Our bioinformatics analysis pointed out that Rap1 may have a bigger role as it has the potential to regulate more than 60 TFs including Bcr1, Tac1, Cap1, Cbf1, Ume6, Adr1. To understand the exact role of Rap1, in future we need a deep transcriptomics study.

In the second part of our study, we conducted a large-scale screening of activated transcription factors in *C. albicans*, leading to the identification of Adr1 as a key regulator of antifungal resistance. While its orthologs in *S. cerevisiae* regulate metabolic pathways, in *C. albicans*, Adr1 plays a distinct role in mediating resistance to antifungal agents, particularly those targeting the fungal cell membrane. Adr1 was shown to modulate ergosterol biosynthesis pathways, a critical component of the fungal cell membrane, resulting in resistance to cell membrane targeting drugs like fluconazole. This finding highlight how species-specific adaptations in transcription factors can drive unique drug resistance mechanisms, emphasizing the need for targeted therapeutic strategies that consider the evolutionary divergence between fungal species. We were able to successfully publish this [37]. Apart from Adr1, we were also able to identify TFs like Met4, Met28, Ofi1, Try3 etc. potentially to have distinct role in *C. albicans* compared to *S. cerevisiae*. These TFs indeed are interesting and need further detailed investigation.

Future studies could focus on dissecting the regulatory networks involving Adr1, Erg3, and Rap1, as well as exploring their interactions with other components of the ergosterol biosynthesis pathway. The use of advanced genomic and proteomic tools could further elucidate the molecular mechanisms driving these interactions. Additionally, investigating the environmental and host-specific factors influencing gene regulation in *C. albicans* could reveal new targets for intervention.

References

1. Naranjo-Ortiz MA, Gabaldón T. Fungal evolution: diversity, taxonomy and phylogeny of the Fungi. Biological Reviews. 2019;94(6):2101-37.
2. Lutzoni F, Kauff F, Cox CJ, McLaughlin D, Celio G, Dentinger B, et al. Assembling the fungal tree of life: progress, classification, and evolution of subcellular traits. American journal of botany. 2004;91(10):1446-80.
3. Schoch CL, Sung G-H, López-Giráldez F, Townsend JP, Miadlikowska J, Hofstetter V, et al. The Ascomycota tree of life: a phylum-wide phylogeny clarifies the origin and evolution of fundamental reproductive and ecological traits. Systematic biology. 2009;58(2):224-39.
4. Redman RS, Dunigan DD, Rodriguez RJ. Fungal symbiosis from mutualism to parasitism: who controls the outcome, host or invader? New Phytologist. 2001;151(3):705-16.
5. Kure CF, Skaar I. The fungal problem in cheese industry. Current Opinion in Food Science. 2019;29:14-9.
6. Ropars J, Didiot E, de La Vega RCR, Bennetot B, Coton M, Poirier E, et al. Domestication of the emblematic white cheese-making fungus Penicillium camemberti and its diversification into two varieties. Current Biology. 2020;30(22):4441-53. e4.
7. Leonel L, Arruda P, Chandel A, Felipe M, Sene L. Kluyveromyces marxianus: A potential biocatalyst of renewable chemicals and lignocellulosic ethanol production. Critical Reviews in Biotechnology. 2021;41(8):1131-52.
8. Reece JB, Urry LA, Cain ML, Wasserman SA, Minorsky PV, Jackson RB. Cellular respiration and fermentation. Campbell biology. 2011:163-83.
9. Botstein D, Fink GR. Yeast: an experimental organism for 21st century biology. Genetics. 2011;189(3):695-704.
10. Grundy MK, Buckanovich RJ, Bernstein KA. Regulation and pharmacological targeting of RAD51 in cancer. NAR cancer. 2020;2(3):zcaa024.
11. Marichal P, Bossche H. Mechanisms of resistance to azole antifungals. Acta Biochimica Polonica. 1995;42(4):509-16.
12. Stajich JE, Berbee ML, Blackwell M, Hibbett DS, James TY, Spatafora JW, et al. The fungi. Current Biology. 2009;19(18):R840-R5.
13. Seyedmousavi S, Lionakis M, Parta M, Peterson S, Kwon-Chung K, editors. Emerging Aspergillus species almost exclusively associated with primary immunodeficiencies. Open forum infectious diseases; 2018: Oxford University Press US.
14. Zeise KD, Woods RJ, Huffnagle GB. Interplay between Candida albicans and lactic acid bacteria in the gastrointestinal tract: impact on colonization resistance, microbial carriage, opportunistic infection, and host immunity. Clinical microbiology reviews. 2021;34(4):e00323-20.
15. Denning DW. Antifungal drug resistance: an update. European Journal of Hospital Pharmacy. 2022;29(2):109-12.
16. Hedges SB, Blair JE, Venturi ML, Shoe JL. A molecular timescale of eukaryote evolution and the rise of complex multicellular life. BMC evolutionary biology. 2004;4:1-9.
17. Denning DW. Renaming Candida glabrata—A case of taxonomic purity over clinical and public health pragmatism. Plos Pathogens. 2024;20(3):e1012055.
18. Lopes JP, Lionakis MS. Pathogenesis and virulence of Candida albicans. Virulence. 2022;13(1):89-121.
19. Khalaf AS, Ahmed LT. Enteritis Distribution among People Infected with Candidiasis.
20. Beck-Sague CM, Jarvis WR, System NNIS. Secular trends in the epidemiology of nosocomial fungal infections in the United States, 1980-1990. The Journal of infectious diseases. 1993:1247-51.

21. Edmond MB, Wallace SE, McClish DK, Pfaller MA, Jones RN, Wenzel RP. Nosocomial bloodstream infections in United States hospitals: a three-year analysis. Clinical infectious diseases. 1999;29(2):239-44.
22. Wisplinghoff H, Ebbers J, Geurtz L, Stefanik D, Major Y, Edmond MB, et al. Nosocomial bloodstream infections due to Candida spp. in the USA: species distribution, clinical features and antifungal susceptibilities. International journal of antimicrobial agents. 2014;43(1):78-81.
23. Assessment M-S, Chukwu D, Ikporukpo API, Anam B, Adebayo E, Traynor T, et al. 10TH GLOBAL RESEARCH CONFERENCE. 2024.
24. Hajjeh RA, Sofair AN, Harrison LH, Lyon GM, Arthington-Skaggs BA, Mirza SA, et al. Incidence of bloodstream infections due to Candida species and in vitro susceptibilities of isolates collected from 1998 to 2000 in a population-based active surveillance program. Journal of clinical microbiology. 2004;42(4):1519-27.
25. Viudes A, Peman J, Canton E, Ubeda P, López-Ribot J, Gobernado M. Candidemia at a tertiary-care hospital: epidemiology, treatment, clinical outcome and risk factors for death. European Journal of Clinical Microbiology and Infectious Diseases. 2002;21:767-74.
26. Berman J, Sudbery PE. Candida albicans: a molecular revolution built on lessons from budding yeast. Nature Reviews Genetics. 2002;3(12):918-31.
27. Berbee ML, Taylor JW. Fungal molecular evolution: gene trees and geologic time. Systematics and evolution: Springer; 2001. p. 229-45.
28. Heitman J. Microbial pathogens in the fungal kingdom. Fungal biology reviews. 2011;25(1):48-60.
29. Dujon B. Yeast evolutionary genomics. Nature Reviews Genetics. 2010;11(7):512-24.
30. Santos MA, Tuite MF. The CUG codon is decoded in vivo as serine and not leucine in Candida albicans. Nucleic acids research. 1995;23(9):1481-6.
31. LaPrade RF, Muench C, Wentorf F, Lewis JL. The effect of injury to the posterolateral structures of the knee on force in a posterior cruciate ligament graft: a biomechanical study. The American Journal of Sports Medicine. 2002;30(2):233-8.
32. Mallick J, Whiteway M. The evolutionary rewiring of the ribosomal protein transcription pathway modifies the interaction of transcription factor heteromer Ifh1-Fhl1 (interacts with forkhead 1-forkhead-like 1) with the DNA-binding specificity element. Journal of Biological Chemistry. 2013;288(24):17508-19.
33. Erkenbrack EM, Davidson EH. Evolutionary rewiring of gene regulatory network linkages at divergence of the echinoid subclasses. Proceedings of the National Academy of Sciences. 2015;112(30):E4075-E84.
34. Tebung WA, Choudhury BI, Tebbji F, Morschhäuser J, Whiteway M. Rewiring of the Ppr1 zinc cluster transcription factor from purine catabolism to pyrimidine biogenesis in the Saccharomycetaceae. Current Biology. 2016;26(13):1677-87.
35. Johnson AD. The rewiring of transcription circuits in evolution. Current opinion in genetics & development. 2017;47:121-7.
36. Parvizi Omran R, Ramírez-Zavala B, Aji Tebung W, Yao S, Feng J, Law C, et al. The zinc cluster transcription factor Rha1 is a positive filamentation regulator in Candida albicans. Genetics. 2022;220(1):iyab155.
37. Shrivastava M, Kouyoumdjian GS, Kirbizakis E, Ruiz D, Henry M, Vincent AT, et al. The Adr1 transcription factor directs regulation of the ergosterol pathway and azole resistance in Candida albicans. MBio. 2023;14(5):e01807-23.
38. Shrivastava M, Feng J, Coles M, Clark B, Islam A, Dumeaux V, et al. Modulation of the complex regulatory network for methionine biosynthesis in fungi. Genetics. 2021;217(2):iyaa049.
39. Lavoie H, Hogues H, Mallick J, Sellam A, Nantel A, Whiteway M. Evolutionary tinkering with conserved components of a transcriptional regulatory network. PLoS biology. 2010;8(3):e1000329.

40. Gasch AP. Comparative genomics of the environmental stress response in ascomycete fungi. Yeast. 2007;24(11):961-76.
41. Staib F, Arasteh K. Chlamydospore formation on Staib agar. Observations made before Candida dubliniensis was described. Mycoses. 2001;44(1-2):23-7.
42. Slutsky B, Staebell M, Anderson J, Risen L, Pfaller Mt, Soll D. " White-opaque transition": a second high-frequency switching system in Candida albicans. Journal of bacteriology. 1987;169(1):189-97.
43. Pande K, Chen C, Noble SM. Passage through the mammalian gut triggers a phenotypic switch that promotes Candida albicans commensalism. Nature genetics. 2013;45(9):1088-91.
44. Bommanavar SB, Gugwad S, Malik N. Phenotypic switch: The enigmatic white-gray-opaque transition system of: Candida albicans. Journal of Oral and Maxillofacial Pathology. 2017;21(1):82-6.
45. Tao L, Cao C, Liang W, Guan G, Zhang Q, Nobile CJ, et al. White cells facilitate opposite-and same-sex mating of opaque cells in Candida albicans. PLoS Genetics. 2014;10(10):e1004737.
46. Prasad P, Tippana M. Morphogenic plasticity: The pathogenic attribute of Candida albicans. Current Genetics. 2023;69(2-3):77-89.
47. Calderone RA, Fonzi WA. Virulence factors of Candida albicans. Trends in microbiology. 2001;9(7):327-35.
48. Csank C, Schröppel K, Leberer E, Harcus D, Mohamed O, Meloche S, et al. Roles of the Candida albicans mitogen-activated protein kinase homolog, Cek1p, in hyphal development and systemic candidiasis. Infection and immunity. 1998;66(6):2713-21.
49. Felk A, Kretschmar M, Albrecht A, Schaller M, Beinhauer S, Nichterlein T, et al. Candida albicans hyphal formation and the expression of the Efg1-regulated proteinases Sap4 to Sap6 are required for the invasion of parenchymal organs. Infection and immunity. 2002;70(7):3689-700.
50. Sudbery PE. Growth of Candida albicans hyphae. Nature Reviews Microbiology. 2011;9(10):737-48.
51. Blankenship JR, Mitchell AP. How to build a biofilm: a fungal perspective. Current opinion in microbiology. 2006;9(6):588-94.
52. Richard ML, Nobile CJ, Bruno VM, Mitchell AP. Candida albicans biofilm-defective mutants. Eukaryotic Cell. 2005;4(8):1493-502.
53. Nobile CJ, Andes DR, Nett JE, Smith Jr FJ, Yue F, Phan Q-T, et al. Critical role of Bcr1-dependent adhesins in C. albicans biofilm formation in vitro and in vivo. PLoS pathogens. 2006;2(7):e63.
54. Nobile CJ, Fox EP, Nett JE, Sorrells TR, Mitrovich QM, Hernday AD, et al. A recently evolved transcriptional network controls biofilm development in Candida albicans. Cell. 2012;148(1):126-38.
55. Nobile CJ, Mitchell AP. Regulation of cell-surface genes and biofilm formation by the C. albicans transcription factor Bcr1p. Current Biology. 2005;15(12):1150-5.
56. Nobile CJ, Nett JE, Hernday AD, Homann OR, Deneault J-S, Nantel A, et al. Biofilm matrix regulation by Candida albicans Zap1. PLoS biology. 2009;7(6):e1000133.
57. Stevens I, Silao FG, Huch S, Liu H, Ryman K, Carvajal-Jimenez A, et al. The early transcriptional and post-transcriptional responses to fluconazole in sensitive and resistant Candida albicans. Scientific Reports. 2024;14(1):1-10.
58. Ksiezopolska E, Gabaldón T. Evolutionary emergence of drug resistance in Candida opportunistic pathogens. Genes. 2018;9(9):461.
59. Pappas PG, Lionakis MS, Arendrup MC, Ostrosky-Zeichner L, Kullberg BJ. Invasive candidiasis. Nature Reviews Disease Primers. 2018;4(1):1-20.
60. Perfect JR. The antifungal pipeline: a reality check. Nature reviews Drug discovery. 2017;16(9):603-16.
61. Atriwal T, Azeem K, Husain FM, Hussain A, Khan MN, Alajmi MF, et al. Mechanistic understanding of Candida albicans biofilm formation and approaches for its inhibition. Frontiers in Microbiology. 2021;12:638609.

62. Perfect JR, Krysan DJ, Del Poeta M, Selmecki AM, Brown JC, Cowen LE. antifungal pipeline: build it strong; build it better! : Frontiers Media SA; 2022. p. 881272.
63. Taff HT, Mitchell KF, Edward JA, Andes DR. Mechanisms of Candida biofilm drug resistance. Future microbiology. 2013;8(10):1325-37.
64. Denning DW. Echinocandin antifungal drugs. The Lancet. 2003;362(9390):1142-51.
65. Chaffin WL, Lopez-Ribot JL, Casanova M, Gozalbo D, Martinez JP. Cell wall and secreted proteins of Candida albicans: identification, function, and expression. Microbiology and molecular biology reviews. 1998;62(1):130-80.
66. Pappas PG, Kauffman CA, Andes DR, Clancy CJ, Marr KA, Ostrosky-Zeichner L, et al. Clinical practice guideline for the management of candidiasis: 2016 update by the Infectious Diseases Society of America. Clinical infectious diseases. 2016;62(4):e1-e50.
67. Sanglard D. Emerging threats in antifungal-resistant fungal pathogens. Frontiers in medicine. 2016;3:11.
68. Kelly SL, Lamb DC, Baldwin BC, Corran AJ, Kelly DE. Characterization of Saccharomyces cerevisiae CYP61, sterol Δ22-desaturase, and inhibition by azole antifungal agents. Journal of Biological Chemistry. 1997;272(15):9986-8.
69. Morschhäuser J. The development of fluconazole resistance in Candida albicans–an example of microevolution of a fungal pathogen. Journal of microbiology. 2016;54:192-201.
70. Whaley SG, Berkow EL, Rybak JM, Nishimoto AT, Barker KS, Rogers PD. Azole antifungal resistance in Candida albicans and emerging non-albicans Candida species. Frontiers in microbiology. 2017;7:2173.
71. Ben-Ami R, Kontoyiannis DP. Resistance to antifungal drugs. Infectious Disease Clinics. 2021;35(2):279-311.
72. Robbins N, Caplan T, Cowen LE. Molecular evolution of antifungal drug resistance. Annual review of microbiology. 2017;71(1):753-75.
73. Sellam A, Tebbji F, Nantel A. Role of Ndt80p in sterol metabolism regulation and azole resistance in Candida albicans. Eukaryotic cell. 2009;8(8):1174-83.
74. Srikantha T, Daniels KJ, Pujol C, Kim E, Soll DR. Identification of genes upregulated by the transcription factor Bcr1 that are involved in impermeability, impenetrability, and drug resistance of Candida albicans a/α biofilms. Eukaryotic cell. 2013;12(6):875-88.
75. Ding C, Vidanes GM, Maguire SL, Guida A, Synnott JM, Andes DR, et al. Conserved and divergent roles of Bcr1 and CFEM proteins in Candida parapsilosis and Candida albicans. PloS one. 2011;6(12):e28151.
76. Brown AJ, Gow NA. Regulatory networks controlling Candida albicans morphogenesis. Trends in microbiology. 1999;7(8):333-8.
77. Kumar D, Kumar A. Molecular determinants involved in Candida albicans biofilm formation and regulation. Molecular Biotechnology. 2024;66(7):1640-59.
78. Shapiro RS, Robbins N, Cowen LE. Regulatory circuitry governing fungal development, drug resistance, and disease. Microbiology and molecular biology reviews. 2011;75(2):213-67.
79. Segal ES, Gritsenko V, Levitan A, Yadav B, Dror N, Steenwyk JL, et al. Gene essentiality analyzed by in vivo transposon mutagenesis and machine learning in a stable haploid isolate of Candida albicans. MBio. 2018;9(5):10.1128/mbio. 02048-18.
80. Roemer T, Jiang B, Davison J, Ketela T, Veillette K, Breton A, et al. Large-scale essential gene identification in Candida albicans and applications to antifungal drug discovery. Molecular microbiology. 2003;50(1):167-81.
81. Odds FC, Brown AJ, Gow NA. Antifungal agents: mechanisms of action. Trends in microbiology. 2003;11(6):272-9.
82. Lee Y, Puumala E, Robbins N, Cowen LE. Antifungal drug resistance: molecular mechanisms in Candida albicans and beyond. Chemical reviews. 2020;121(6):3390-411.

83. Scherens B, Goffeau A. The uses of genome-wide yeast mutant collections. Genome biology. 2004;5:1-8.
84. Cowen LE, Lindquist S. Hsp90 potentiates the rapid evolution of new traits: drug resistance in diverse fungi. Science. 2005;309(5744):2185-9.
85. Chen Y, Mallick J, Maqnas A, Sun Y, Choudhury BI, Côte P, et al. Chemogenomic profiling of the fungal pathogen Candida albicans. Antimicrobial agents and chemotherapy. 2018;62(2):10.1128/aac. 02365-17.
86. Islam A, Tebbji F, Mallick J, Regan H, Dumeaux V, Omran RP, et al. Mms21: a putative SUMO E3 ligase in Candida albicans that negatively regulates invasiveness and filamentation, and is required for the genotoxic and cellular stress response. Genetics. 2019;211(2):579-95.
87. Costa ACBP, Omran RP, Correia-Mesquita TO, Dumeaux V, Whiteway M. Screening of Candida albicans GRACE library revealed a unique pattern of biofilm formation under repression of the essential gene ILS1. Scientific Reports. 2019;9(1):9187.
88. Uthayakumar D, Sharma J, Wensing L, Shapiro RS. CRISPR-based genetic manipulation of Candida species: historical perspectives and current approaches. Frontiers in Genome Editing. 2021;2:606281.
89. Shapiro RS, Chavez A, Collins JJ. CRISPR-based genomic tools for the manipulation of genetically intractable microorganisms. Nature Reviews Microbiology. 2018;16(6):333-9.
90. Bloom JS, Kotenko I, Sadhu MJ, Treusch S, Albert FW, Kruglyak L. Genetic interactions contribute less than additive effects to quantitative trait variation in yeast. Nature communications. 2015;6(1):8712.
91. Tsouris A, Fournier T, Friedrich A, Hou J, Dunham MJ, Schacherer J. Species-wide survey of the expressivity and complexity spectrum of traits in yeast. PLoS Genetics. 2024;20(1):e1011119.
92. Matsui T, Mullis MN, Roy KR, Hale JJ, Schell R, Levy SF, et al. The interplay of additivity, dominance, and epistasis on fitness in a diploid yeast cross. Nature Communications. 2022;13(1):1463.
93. Costanzo M, VanderSluis B, Koch EN, Baryshnikova A, Pons C, Tan G, et al. A global genetic interaction network maps a wiring diagram of cellular function. Science. 2016;353(6306):aaf1420.
94. Ang RML, Chen S-AA, Kern AF, Xie Y, Fraser HB. Widespread epistasis among beneficial genetic variants revealed by high-throughput genome editing. Cell Genomics. 2023;3(4).
95. Phillips PC. Epistasis—the essential role of gene interactions in the structure and evolution of genetic systems. Nature Reviews Genetics. 2008;9(11):855-67.
96. Park S, Lehner B. Epigenetic epistatic interactions constrain the evolution of gene expression. Molecular systems biology. 2013;9(1):645.
97. Lo H-J, Köhler JR, DiDomenico B, Loebenberg D, Cacciapuoti A, Fink GR. Nonfilamentous C. albicans mutants are avirulent. Cell. 1997;90(5):939-49.
98. Bayram O, Krappmann S, Ni M, Bok JW, Helmstaedt K, Valerius O, et al. VelB/VeA/LaeA complex coordinates light signal with fungal development and secondary metabolism. Science. 2008;320(5882):1504-6.
99. Queitsch C, Sangster TA, Lindquist S. Hsp90 as a capacitor of phenotypic variation. Nature. 2002;417(6889):618-24.
100. Rashki A. Transcriptional analysis of the histone acetylation and deacetylation in Candida albicans. 2009.
101. Freitag M, Hickey PC, Khlafallah TK, Read ND, Selker EU. HP1 is essential for DNA methylation in Neurospora. Molecular cell. 2004;13(3):427-34.
102. Ernst JF. Transcription factors in Candida albicans–environmental control of morphogenesis. Microbiology. 2000;146(8):1763-74.
103. Islam A. Characterizing Genes of Candida albicans Involved in Invasiveness, Mating and Genotoxic Stress Response: Concordia University; 2018.

104. Cowen LE, Anderson JB, Kohn LM. Evolution of drug resistance in Candida albicans. Annual Reviews in Microbiology. 2002;56(1):139-65.
105. Glazier VE. EFG1, everyone's favorite gene in Candida albicans: A comprehensive literature review. Frontiers in Cellular and Infection Microbiology. 2022;12:855229.
106. Lu H, Shrivastava M, Whiteway M, Jiang Y. Candida albicans targets that potentially synergize with fluconazole. Critical reviews in microbiology. 2021;47(3):323-37.
107. Brown AJ, Haynes K, Gow NA, Quinn J. Stress responses in Candida. Candida and Candidiasis. 2011:225-42.
108. Sellam A, Chaillot J, Mallick J, Tebbji F, Richard Albert J, Cook MA, et al. The p38/HOG stress-activated protein kinase network couples growth to division in Candida albicans. PLoS genetics. 2019;15(3):e1008052.
109. Brewster JL, Gustin MC. Hog1: 20 years of discovery and impact. Science signaling. 2014;7(343):re7-re.
110. Kummerfeld SK, Teichmann SA. DBD: a transcription factor prediction database. Nucleic acids research. 2006;34(suppl_1):D74-D81.
111. Shelest E. Transcription factors in fungi: TFome dynamics, three major families, and dual-specificity TFs. Frontiers in genetics. 2017;8:53.
112. Homann OR, Dea J, Noble SM, Johnson AD. A phenotypic profile of the Candida albicans regulatory network. PLoS genetics. 2009;5(12):e1000783.
113. Liu H. Transcriptional control of dimorphism in Candida albicans. Current opinion in microbiology. 2001;4(6):728-35.
114. Schillig R, Morschhäuser J. Analysis of a fungus-specific transcription factor family, the C andida albicans zinc cluster proteins, by artificial activation. Molecular microbiology. 2013;89(5):1003-17.
115. Habib N, Wapinski I, Margalit H, Regev A, Friedman N. A functional selection model explains evolutionary robustness despite plasticity in regulatory networks. Molecular systems biology. 2012;8(1):619.
116. Shrivastava M. Activation of non-zinc cluster transcription factors in Candida albicans: Concordia University; 2021.
117. Sarda S, Hannenhalli S. High-throughput identification of cis-regulatory rewiring events in yeast. Molecular biology and evolution. 2015;32(12):3047-63.
118. Tuch BB, Li H, Johnson AD. Evolution of eukaryotic transcription circuits. Science. 2008;319(5871):1797-9.
119. Bradley RK, Li X-Y, Trapnell C, Davidson S, Pachter L, Chu HC, et al. Binding site turnover produces pervasive quantitative changes in transcription factor binding between closely related Drosophila species. PLoS biology. 2010;8(3):e1000343.
120. Chauhan N, Kruppa MD. Standard growth media and common techniques for use with Candida albicans. Candida albicans: Methods and Protocols. 2009:197-201.
121. Min K, Ichikawa Y, Woolford CA, Mitchell AP. Candida albicans gene deletion with a transient CRISPR-Cas9 system. MSphere. 2016;1(3):10.1128/msphere. 00130-16.
122. Vyas VK, Barrasa MI, Fink GR. A Candida albicans CRISPR system permits genetic engineering of essential genes and gene families. Science advances. 2015;1(3):e1500248.
123. d'Enfert C, Goyard S, Rodriguez-Arnaveilhe S, Frangeul L, Jones L, Tekaia F, et al. CandidaDB: a genome database for Candida albicans pathogenomics. Nucleic Acids Research. 2005;33(suppl_1):D353-D7.
124. Gola S, Martin R, Walther A, Dünkler A, Wendland J. New modules for PCR-based gene targeting in Candida albicans: rapid and efficient gene targeting using 100 bp of flanking homology region. Yeast. 2003;20(16):1339-47.

125. Noble SM, Johnson AD. Strains and strategies for large-scale gene deletion studies of the diploid human fungal pathogen Candida albicans. Eukaryotic cell. 2005;4(2):298-309.
126. Hudzicki J. Kirby-Bauer disk diffusion susceptibility test protocol. American society for microbiology. 2009;15(1):1-23.
127. Kirkpatrick B, Xu L, Cascella N, Ozeki Y, Sawa A, Roberts RC. DISC1 immunoreactivity at the light and ultrastructural level in the human neocortex. Journal of Comparative Neurology. 2006;497(3):436-50.
128. He K, Gkioxari G, Dollár P, Girshick R, editors. Mask r-cnn. Proceedings of the IEEE international conference on computer vision; 2017.
129. Schindelin J, Arganda-Carreras I, Frise E, Kaynig V, Longair M, Pietzsch T, et al. Fiji: an open-source platform for biological-image analysis. Nature methods. 2012;9(7):676-82.
130. Lu AX, Kraus OZ, Cooper S, Moses AM. Learning unsupervised feature representations for single cell microscopy images with paired cell inpainting. PLoS computational biology. 2019;15(9):e1007348.
131. Legland D, Arganda-Carreras I, Andrey P. MorphoLibJ: integrated library and plugins for mathematical morphology with ImageJ. Bioinformatics. 2016;32(22):3532-4.
132. Wapinski I, Pfeffer A, Friedman N, Regev A. Automatic genome-wide reconstruction of phylogenetic gene trees. Bioinformatics. 2007;23(13):i549-i58.
133. Bailey TL, Johnson J, Grant CE, Noble WS. The MEME suite. Nucleic acids research. 2015;43(W1):W39-W49.
134. Kelly SL, Lamb DC, Corran AJ, Baldwin BC, Parks LW, Kelly DE. Purification and reconstitution of activity of Saccharomyces cerevisiae P450 61, a sterol Δ 22-desaturase. FEBS letters. 1995;377(2):217-20.
135. Borecká-Melkusová S, Moran GP, Sullivan DJ, Kucharíková S, Chorvát Jr D, Bujdakova H. The expression of genes involved in the ergosterol biosynthesis pathway in Candida albicans and Candida dubliniensis biofilms exposed to fluconazole. Mycoses. 2009;52(2):118-28.
136. Yu EY, Yen W-F, Steinberg-Neifach O, Lue NF. Rap1 in Candida albicans: an unusual structural organization and a critical function in suppressing telomere recombination. Molecular and cellular biology. 2010;30(5):1254-68.
137. Wang W-H, Lai T-X, Wu Y-C, Chen Z-T, Tseng K-Y, Lan C-Y. Associations of rap1 with cell wall integrity, biofilm formation, and virulence in candida albicans. Microbiology Spectrum. 2022;10(6):e03285-22.
138. Prasad R. Candida albicans: Springer; 2017.
139. Xiang M-J, Liu J-Y, Ni P-H, Wang S, Shi C, Wei B, et al. Erg11 mutations associated with azole resistance in clinical isolates of Candida albicans. FEMS yeast research. 2013;13(4):386-93.
140. Vasicek EM, Berkow EL, Flowers SA, Barker KS, Rogers PD. UPC2 is universally essential for azole antifungal resistance in Candida albicans. Eukaryotic cell. 2014;13(7):933-46.
141. Henry KW, Nickels JT, Edlind TD. Upregulation of ERG genes in Candida species by azoles and other sterol biosynthesis inhibitors. Antimicrobial agents and chemotherapy. 2000;44(10):2693-700.
142. Tsao S, Rahkhoodaee F, Raymond M. Relative contributions of the Candida albicans ABC transporters Cdr1p and Cdr2p to clinical azole resistance. Antimicrobial agents and chemotherapy. 2009;53(4):1344-52.
143. White TC. Increased mRNA levels of ERG16, CDR, and MDR1 correlate with increases in azole resistance in Candida albicans isolates from a patient infected with human immunodeficiency virus. Antimicrobial agents and chemotherapy. 1997;41(7):1482-7.
144. Wirsching S, Moran GP, Sullivan DJ, Coleman DC, Morschhäuser J. MDR1-mediated drug resistance in Candida dubliniensis. Antimicrobial agents and chemotherapy. 2001;45(12):3416-21.

145. Schubert S, Rogers PD, Morschhäuser J. Gain-of-function mutations in the transcription factor MRR1 are responsible for overexpression of the MDR1 efflux pump in fluconazole-resistant Candida dubliniensis strains. Antimicrobial agents and chemotherapy. 2008;52(12):4274-80.
146. Prasad R, Kapoor K. Multidrug resistance in yeast Candida. International review of cytology. 2005;242:215-48.
147. Singh SD, Robbins N, Zaas AK, Schell WA, Perfect JR, Cowen LE. Hsp90 governs echinocandin resistance in the pathogenic yeast Candida albicans via calcineurin. PLoS pathogens. 2009;5(7):e1000532.
148. Wang R, Liu J, Liu Y, Lv Q, Yan L. Application of the mutant libraries for Candida albicans functional genomics. International Journal of Molecular Sciences. 2022;23(20):12307.
149. Chen Y, Rai R, Zhou Z-R, Kanoh J, Ribeyre C, Yang Y, et al. A conserved motif within RAP1 has diversified roles in telomere protection and regulation in different organisms. Nature structural & molecular biology. 2011;18(2):213-21.
150. Lieb JD, Liu X, Botstein D, Brown PO. Promoter-specific binding of Rap1 revealed by genome-wide maps of protein–DNA association. Nature genetics. 2001;28(4):327-34.
151. Wang W-H, Chen H-Y, Chen S-Y, Lan C-Y. Transcriptional profiling reveals the role of Candida albicans Rap1 in oxidative stress response. Bioscience Reports. 2024:BSR20240689.
152. Flowers SA, Barker KS, Berkow EL, Toner G, Chadwick SG, Gygax SE, et al. Gain-of-function mutations in UPC2 are a frequent cause of ERG11 upregulation in azole-resistant clinical isolates of Candida albicans. Eukaryotic cell. 2012;11(10):1289-99.
153. Cowen LE, Carpenter AE, Matangkasombut O, Fink GR, Lindquist S. Genetic architecture of Hsp90-dependent drug resistance. Eukaryotic cell. 2006;5(12):2184-8.
154. Georgiev VS. Membrane transporters and antifungal drug resistance. Current Drug Targets. 2000;1(3):261-84.
155. Ghannoum MA, Rice LB. Antifungal agents: mode of action, mechanisms of resistance, and correlation of these mechanisms with bacterial resistance. Clinical microbiology reviews. 1999;12(4):501-17.
156. Hirayama T, Miyazaki T, Sumiyoshi M, Ashizawa N, Takazono T, Yamamoto K, et al. ERG3-Encoding Sterol C5, 6-DESATURASE in Candida albicans is required for virulence in an enterically infected invasive candidiasis mouse model. Pathogens. 2020;10(1):23.
157. Pérez-Martín J, Uría JA, Johnson AD. Phenotypic switching in Candida albicans is controlled by a SIR2 gene. The EMBO Journal. 1999.
158. Basso V, d'Enfert C, Znaidi S, Bachellier-Bassi S. From genes to networks: the regulatory circuitry controlling Candida albicans morphogenesis. Fungal Physiology and Immunopathogenesis. 2019:61-99.
159. Chong PP, Chin VK, Wong WF, Madhavan P, Yong VC, Looi CY. Transcriptomic and genomic approaches for unravelling Candida albicans biofilm formation and drug resistance—an update. Genes. 2018;9(11):540.
160. Day M. Yeast petites and small colony variants: for everything there is a season. Advances in applied microbiology. 85: Elsevier; 2013. p. 1-41.
161. Jež M. of Thesis: Regulation of Recombination during Replication. Current topics in developmental biology. 52:1-53.
162. Putnam CD, Hayes TK, Kolodner RD. Post-replication repair suppresses duplication-mediated genome instability. PLoS genetics. 2010;6(5):e1000933.
163. Rzepnikowska W, Kaminska J, Kochański A. Validation of the pathogenic effect of ighmbp2 gene mutations based on yeast s. cerevisiae model. International Journal of Molecular Sciences. 2022;23(17):9913.
164. Kelly SL, Arnoldi A, Kelly DE. Molecular genetic analysis of azole antifungal mode of action. Biochemical Society Transactions. 1993;21(4):1034-8.

165. Chau AS, Gurnani M, Hawkinson R, Laverdiere M, Cacciapuoti A, McNicholas PM. Inactivation of sterol Δ5, 6-desaturase attenuates virulence in Candida albicans. Antimicrobial agents and chemotherapy. 2005;49(9):3646-51.
166. Prasad R, Nair R, Banerjee A. Emerging mechanisms of drug resistance in Candida albicans. Yeasts in Biotechnology and Human Health: Physiological Genomic Approaches. 2019:135-53.
167. Morio F, Pagniez F, Lacroix C, Miegeville M, Le Pape P. Amino acid substitutions in the Candida albicans sterol Δ5, 6-desaturase (Erg3p) confer azole resistance: characterization of two novel mutants with impaired virulence. Journal of antimicrobial chemotherapy. 2012;67(9):2131-8.
168. Eddouzi J, Parker JE, Vale-Silva LA, Coste A, Ischer F, Kelly S, et al. Molecular mechanisms of drug resistance in clinical Candida species isolated from Tunisian hospitals. Antimicrobial agents and chemotherapy. 2013;57(7):3182-93.
169. O'Meara TR, Veri AO, Ketela T, Jiang B, Roemer T, Cowen LE. Global analysis of fungal morphology exposes mechanisms of host cell escape. Nature communications. 2015;6(1):6741.
170. Keighley C, Gall M, Halliday CL, Chaw K, Newton P, Sintchenko V, et al. Breakthrough Candida albicans bloodstream infection associated with in vivo development of pan-azole resistance related to ERG3 gene deletion. Pathology. 2024;56(4):578-9.
171. Kennedy MJ. Adhesion and association mechanisms of Candida albicans. Current topics in medical mycology. 1988:73-169.
172. Heilmann CJ, Schneider S, Barker KS, Rogers PD, Morschhäuser J. An A643T mutation in the transcription factor Upc2p causes constitutive ERG11 upregulation and increased fluconazole resistance in Candida albicans. Antimicrobial agents and chemotherapy. 2010;54(1):353-9.
173. Víglaš J, Olejníková P. Signalling mechanisms involved in stress response to antifungal drugs. Research in microbiology. 2021;172(1):103786.
174. García R, Botet J, Rodríguez-Peña JM, Bermejo C, Ribas JC, Revuelta JL, et al. Genomic profiling of fungal cell wall-interfering compounds: identification of a common gene signature. BMC genomics. 2015;16:1-20.
175. Heilmann CJ, Sorgo AG, Mohammadi S, Sosinska GJ, de Koster CG, Brul S, et al. Surface stress induces a conserved cell wall stress response in the pathogenic fungus Candida albicans. Eukaryotic cell. 2013;12(2):254-64.
176. Cowen LE, Nantel A, Whiteway MS, Thomas DY, Tessier DC, Kohn LM, et al. Population genomics of drug resistance in Candida albicans. Proceedings of the National Academy of Sciences. 2002;99(14):9284-9.
177. Coste AT, Karababa M, Ischer F, Bille J, Sanglard D. TAC1, transcriptional activator of CDR genes, is a new transcription factor involved in the regulation of Candida albicans ABC transporters CDR1 and CDR2. Eukaryotic cell. 2004;3(6):1639-52.
178. Dunkel N, Liu TT, Barker KS, Homayouni R, Morschhäuser J, Rogers PD. A gain-of-function mutation in the transcription factor Upc2p causes upregulation of ergosterol biosynthesis genes and increased fluconazole resistance in a clinical Candida albicans isolate. Eukaryotic cell. 2008;7(7):1180-90.
179. Ruiz-Herrera J, Victoria Elorza M, Valentín E, Sentandreu R. Molecular organization of the cell wall of Candida albicans and its relation to pathogenicity. FEMS yeast research. 2006;6(1):14-29.
180. Roncero C, Duran A. Effect of Calcofluor white and Congo red on fungal cell wall morphogenesis: in vivo activation of chitin polymerization. Journal of bacteriology. 1985;163(3):1180-5.
181. Barbey P, Ayalew D, Yirgu G. Insight into the origin of gabbro-dioritic cumulophyric aggregates from silicic ignimbrites: Sr and Ba zoning profiles of plagioclase phenocrysts from Oligocene Ethiopian Plateau rhyolites. Contributions to Mineralogy and Petrology. 2005;149:233-45.
182. Yen JL, Su N-Y, Kaiser P. The yeast ubiquitin ligase SCFMet30 regulates heavy metal response. Molecular biology of the cell. 2005;16(4):1872-82.

183. Petti AA, McIsaac RS, Ho-Shing O, Bussemaker HJ, Botstein D. Combinatorial control of diverse metabolic and physiological functions by transcriptional regulators of the yeast sulfur assimilation pathway. Molecular biology of the cell. 2012;23(15):3008-24.

184. Buffo J, Herman MA, Soll DR. A characterization of pH-regulated dimorphism in Candida albicans. Mycopathologia. 1984;85(1):21-30.

185. Stoldt VR, Sonneborn A, Leuker CE, Ernst JF. Efg1p, an essential regulator of morphogenesis of the human pathogen Candida albicans, is a member of a conserved class of bHLH proteins regulating morphogenetic processes in fungi. The EMBO journal. 1997.

186. Whiteway M, Bachewich C. Morphogenesis in Candida albicans. Annu Rev Microbiol. 2007;61(1):529-53.

187. Pereira R, Dos Santos Fontenelle R, De Brito E, De Morais S. Biofilm of Candida albicans: formation, regulation and resistance. Journal of Applied Microbiology. 2021;131(1):11-22.

188. Carrillo E, Ben-Ari G, Wildenhain J, Tyers M, Grammentz D, Lee TA. Characterizing the roles of Met31 and Met32 in coordinating Met4-activated transcription in the absence of Met30. Molecular Biology of the Cell. 2012;23(10):1928-42.

189. Su N-Y, Ouni I, Papagiannis CV, Kaiser P. A dominant suppressor mutation of the met30 cell cycle defect suggests regulation of the Saccharomyces cerevisiae Met4-Cbf1 transcription complex by Met32. Journal of Biological Chemistry. 2008;283(17):11615-24.

190. Blaiseau P-L, Isnard A-D, Surdin-Kerjan Y, Thomas D. Met31p and Met32p, two related zinc finger proteins, are involved in transcriptional regulation of yeast sulfur amino acid metabolism. Molecular and cellular biology. 1997.

191. Du H, Li X, Huang G, Kang Y, Zhu L. The zinc-finger transcription factor, Ofi1, regulates white–opaque switching and filamentation in the yeast Candida albicans. Acta biochimica et biophysica Sinica. 2015;47(5):335-41.

192. Cui H, Yang D, Gong S, Zhang Y, Dong B, Su C, et al. The transcription factor Ofi1 is critical for white-opaque switching in natural MTL a/α isolates of Candida albicans. Molecular Microbiology. 2024;121(2):275-90.

193. Forsburg SL, Guarente L. Identification and characterization of HAP4: a third component of the CCAAT-bound HAP2/HAP3 heteromer. Genes & development. 1989;3(8):1166-78.

194. Mantovani R. The molecular biology of the CCAAT-binding factor NF-Y. Gene. 1999;239(1):15-27.

195. Bonhomme J, Chauvel M, Goyard S, Roux P, Rossignol T, d'Enfert C. Contribution of the glycolytic flux and hypoxia adaptation to efficient biofilm formation by Candida albicans. Molecular microbiology. 2011;80(4):995-1013.

196. Fox EP, Bui CK, Nett JE, Hartooni N, Mui MC, Andes DR, et al. An expanded regulatory network temporally controls C andida albicans biofilm formation. Molecular microbiology. 2015;96(6):1226-39.

197. Brakhage AA. Regulation of fungal secondary metabolism. Nature Reviews Microbiology. 2013;11(1):21-32.

Supplementary Figures

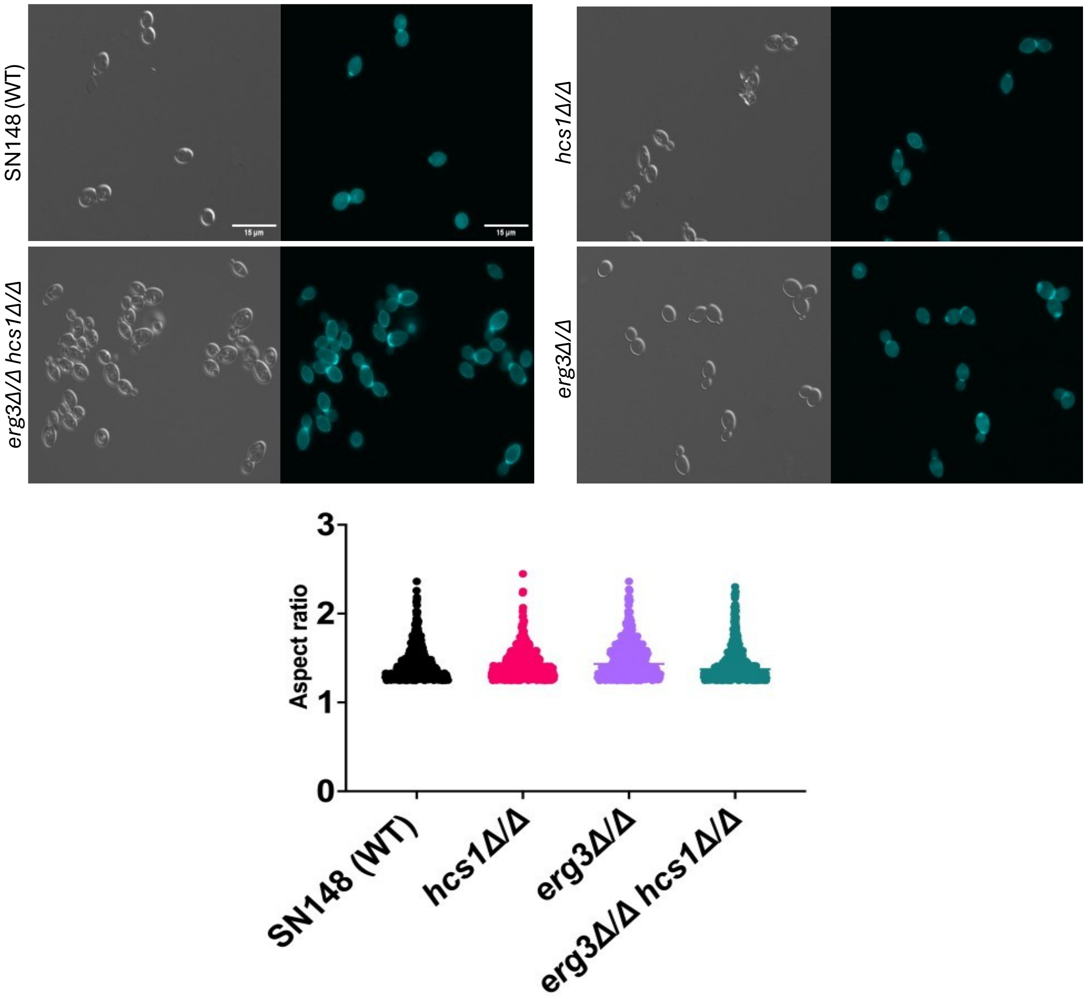

Fig. S1. Cell morphology and aspect ratio of erg3Δ/Δ and hcs1Δ/Δ single and double mutant strains.

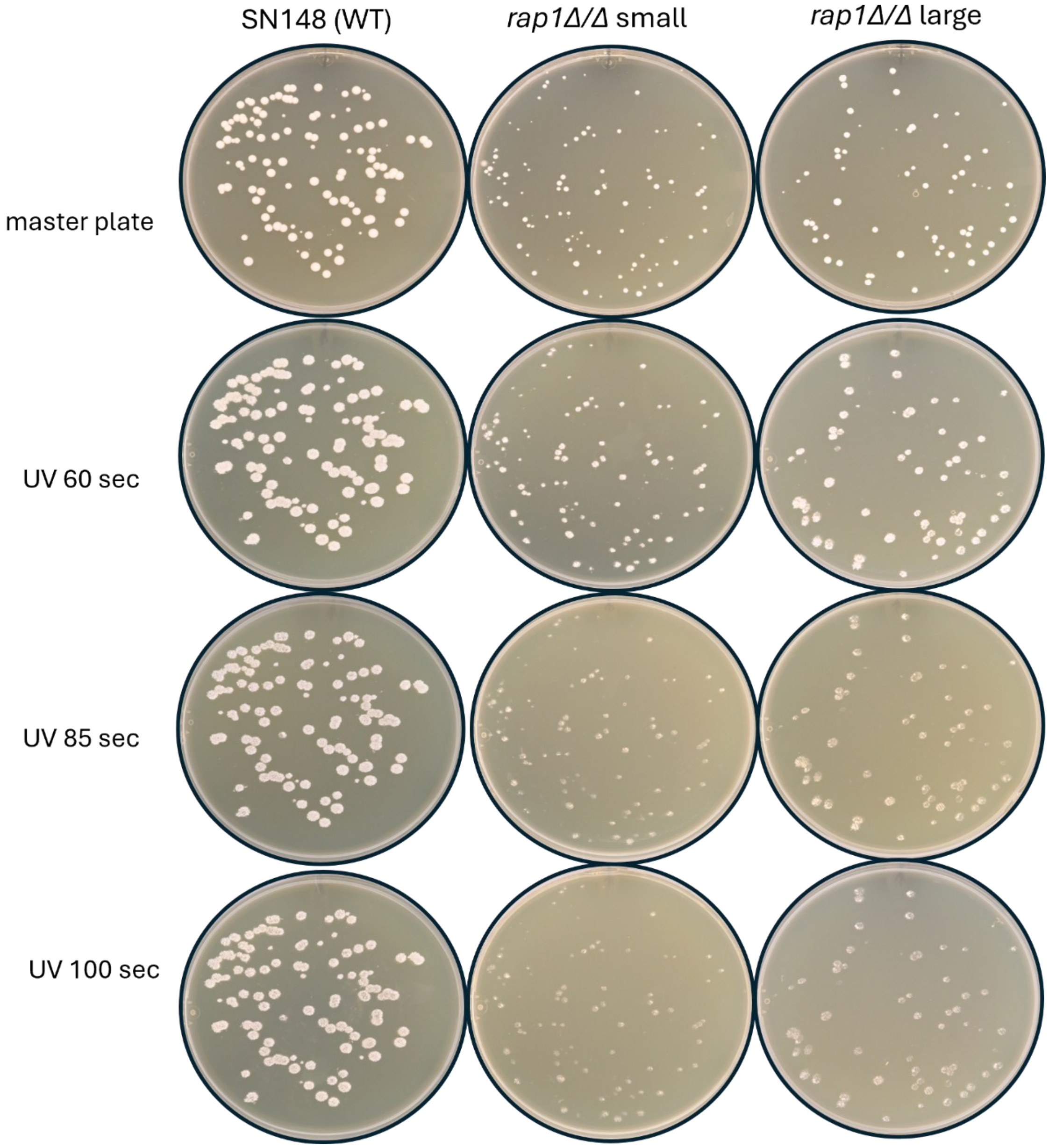

Fig. S2. UV exposure on rap1Δ/Δ single mutant strains. Genotoxicity induced by UV stress has an inhibitory effect on rap1Δ/Δ.

List of Supplementary Tables

Table S1: Oligonucleotides used in this study.

Oligo	Sequence (5'-3')	Description
P1_SNR52 promoter	aagaaagaaagaaaaccaggagtgaa	5' sgRNA
P2	caaattaaaaatagtttacgcaagtc	3' sgRNA
P3	gttttagagctagaaatagcaagttaaa	5' sgRNA
P4_ENO1 terminator	acaaatatttaaactcgggacctgg	3' sgRNA
P5_SNR52	gcggccgcaagtgattagact	5' sgRNA
P6_sgRNA	gcagctcagtgattaagagtaaagatgg	3' sgRNA
P7_CaCas9_F	atctcattagatttggaacttgtgggtt	5' CAS9
P8_CaCas9_R	ttcgagcgtcccaaaaccttct	3' CAS9
*ARG4*_internal-F	tatgtatgatgctgatttaactggtac	5' *ARG4* internal checking
*ARG4*_internal-R	ggaatagatttgatcgacattaaaaatcc	3' *ARG4* internal checking
HIS1 _internal-F	ttgctgttcctaaaaagggc	5' *HIS1* internal checking
*HIS1*_internal-R	cggttgcaccagctttcttcaattcgtcc	3' *HIS1* internal diagnostic
*ERG3*_guide-F1	GTGGTTATAAAGCTATCTTGgttttagagctagaaatagcaagttaaa	5' *ERG3* guide
*ERG3*_guide-R1	CAAGATAGCTTTATAACCACcaaattaaaaatagtttacgcaagtc	3' *ERG3* guide
*ERG3*_guide-F2	CTTGTCACACTGTCCATCACgttttagagctagaaatagcaagttaaa	5' *ERG3* guide
*ERG3*_guide-R2	GTGATGGACAGTGTGACAAGcaaattaaaaatagtttacgcaagtc	3' *ERG3* guide
*ERG3*_repair-F	CCATTTCTTTCCCTATTGTGCATATAAGTTCAATCTTTTTTTCTTTCTTTCGGATTCGGTTTAGCTAATTTTACTACCgaagcttcgtacgctgcaggtc	5' *ERG3* repair
*ERG3*_repair-R	AAAATAAAATAAAATAAAATATCTATATCATCAAAATTGGAAAAATAGTCAATGGTCCAAAACAAAGATGTACCAAtctgatatcatcgatgaattcgag	3' *ERG3* repair
*ERG3*_internal-F	GCCAGATCAAACATTTTCAG	5' *ERG3* internal checking
*ERG3*_internal-R	ATCATGAATCATGACAGTCC	3' *ERG3* internal checking
*ERG3*_external-F	CCTTCCCATCACATTACTGC	5' *ERG3* external checking
*ERG3*_external-R	TCATCATCACGACCGGGACC	3' *ERG3* external checking

*HCS1*_guide-F1	AAGAATTACGACAACGGGAAgttttagagctagaaatagcaagttaaa	5’ *HCS1* guide
*HCS1*_guide-R1	TTCCCGTTGTCGTAATTCTTcaaattaaaaatagtttacgcaagtc	3’ *HCS1* guide
*HCS1*_guide-F2	CCCAAAAAACTAGCGCAACTgttttagagctagaaatagcaagttaaa	5’ *HCS1* guide
*HCS1*_guide-R2	AGTTGCGCTAGTTTTTTGGGcaaattaaaaatagtttacgcaagtc	3’ *HCS1* guide
*HCS1*_ repair-F	TACTTCTTCTTCTGAAACACAGAGTAGACATACTAACTGTAAACGTACATAGTCTCATTACCATTCTACAAACCAACCgaagcttcgtacgctgcaggtc	5’ *HCS1* repair
*HCS1*_repair-R	AAGATAGTTAATCTTAATAGTTACTACTGCCATTATCAAGCTATGCTGCTTCTCTTTATGTATTACCACTGGATTTtctgatatcatcgatgaattcgag	3’ *HCS1* repair
*HCS1*_externalF	TCAAATATCAAATTTAATGTTAAGTATTC	5’ *HCS1* external checking
*HCS1*_externalR	GTATCATCACGGAATTTAGTTAAAT	3’ *HCS1* external checking
*RAP1*_guide-F1	TTGAGAGGTCATACGGGGAAgttttagagctagaaatagcaagttaaa	5’ *RAP1* guide
*RAP1*_guide-R1	TTCCCCGTATGACCTCTCAAcaaattaaaaatagtttacgcaagtc	3’ *RAP1* guide
*RAP1*_guide-F2	ATGCTGATGAAGAAGCTGCAgttttagagctagaaatagcaagttaaa	5’ *RAP1* guide
*RAP1*_guide-R2	TGCAGCTTCTTCATCAGCATcaaattaaaaatagtttacgcaagtc	3’ *RAP1* guide
*RAP1*_repair-F	ATTAACACATCCAAACATCAATAACTTATTCTTTTTTTTGTCTTTTTTGTCGTCTGTATTTTTTTTGCAACCAATAAAGTgaagcttcgtacgctgcagg	5’ *RAP1* repair + S2 PFA site
*RAP1*_repair-R	CATAATTTCATTCTCCCTGATTAACCCTTTAATAATAAAGTTACTTCTTTCTCTGTAAGGTCCTTTCTTTTTTTTTtctgatatcatcgatgaattcgag	3’ *RAP1* repair + S2 PFA site
*RAP1*_internal-F	GTTGAAAAAGGGTTCCCTAC	5’ *RAP1* internal checking
*RAP1*_internal-R	CCTGCTTCATCACGAATTAA	3’ *RAP1* internal checking
*RAP1*_external-F	GGGTACAATTCTTTTTCTTTTATTTC	5’ *RAP1* external checking
*RAP1*_external-R	GAAAAAACACTTTAGATACCTTTTGA	3’ *RAP1* external checking

Table S2: *Candida albicans* strains used in this study.

Strain	**Parent**	**Description**	**Source**
TRY3	SN148	SN148::CIP10-VPR-TRY3	Shrivastava et al., 2023
C5_04410C_A	SN148	SN148::CIP10-VPR C5_04410C_A	Shrivastava et al., 2023
C3_01220W_A	SN148	SN148::CIP10-VPR C3_01220W_A	Shrivastava et al., 2023
C1_04510W_A	SN148	SN148::CIP10-VPR C1_04510W_A	Shrivastava et al., 2023
C2_05640W_A	SN148	SN148::CIP10-VPR C2_05640W_ A	Shrivastava et al., 2023
HAP41	SN148	SN148::CIP10-VPR HAP41	Shrivastava et al., 2023
HAP42	SN148	SN148::CIP10-VPR HAP42	Shrivastava et al., 2023
TRY6	SN148	SN148::CIP10-VPR TRY6	Shrivastava et al., 2023
C2_02530W_A	SN148	SN148 ::CIP10-VPR C2_02530W_A	Shrivastava et al., 2023
C2_01420C_A	SN148	SN148::CIP10-VPR C2_01420C_A	Shrivastava et al., 2023
C4_07150W_A	SN148	SN148::CIP10-VPR C4_07150W_A	Shrivastava et al., 2023
UGA33	SN148	SN148::CIP10-VPR UGA33	Shrivastava et al., 2023
NRG2	SN148	SN148::CIP10-VPR NRG2	Shrivastava et al., 2023
ACE2	SN148	SN148::CIP10-VPR ACE2	Shrivastava et al., 2023
C1_13440C_A	SN148	SN148::CIP10-VPR C1_13440C_A	Shrivastava et al., 2023
RFX1	SN148	SN148::CIP10-VPR RFX1	Shrivastava et al., 2023
ADR1	SN148	SN148::CIP10-VPR ADR1	Shrivastava et al., 2023
PHO4	SN148	SN148::CIP10-VPR PHO4	Shrivastava et al., 2023
CR_05880W_A	SN148	SN148::CIP10-VPR CR_05880W_A	Shrivastava et al., 2023
C2_08950W_A	SN148	SN148::CIP10-VPR C2_08950W_A	Shrivastava et al., 2023
GLN3	SN148	SN148::CIP10-VPR GLN3	Shrivastava et al., 2023
ASH1	SN148	SN148::CIP10-VPR ASH1	Shrivastava et al., 2023
C1_11690W_A	SN148	SN148::CIP10-VPR C1_11690W_A	Shrivastava et al., 2023
SFU1	SN148	SN148::CIP10-VPR SFU1	Shrivastava et al., 2023

GAT1	SN148	SN148::CIP10-VPR GAT1	Shrivastava et al., 2023
C3_05050W_A	SN148	SN148::CIP10-VPR C3_05050W_A	Shrivastava et al., 2023
C1_11690W_A	SN148	SN148::CIP10-VPR C1_11690W_A	Shrivastava et al., 2023
C3_01220W_A	SN148	SN148::CIP10-VPR C3_01220W_A	Shrivastava et al., 2023
C4_07150W_A	SN148	SN148::CIP10-VPR C4_07150W_A	Shrivastava et al., 2023
MET4	SN148	SN148::CIP10-VPR MET4	Shrivastava et al., 2023
MET28	SN148	SN148::CIP10-VPR MET28	Shrivastava et al., 2023
MET32	SN148	SN148::CIP10-VPR MET32	Shrivastava et al., 2023
erg3Δ/Δ	SN148	*erg3::ARG4/erg3::ARG4*	This study
hcs1Δ/Δ	SN148	*hcs1::ARG4/hcs1::ARG4*	This study
rap1Δ/Δ small colony	SN148	*rap1::ARG4/rap1::ARG4*	This study
rap1Δ/Δ big colony	SN148	*rap1::ARG4/rap1::ARG4*	This study
erg3Δ/Δ hcs1Δ/Δ	*hcs1Δ/Δ*	*hcs1::ARG4/hcs1::ARG4 erg3::HIS1/erg3::HIS1*	This study
rap1Δ/Δ erg3Δ/Δ small colony	*erg3Δ/Δ*	*erg3::ARG4/erg31::ARG4 rap1::HIS1/rap1::HIS1*	This study
rap1Δ/Δ erg3Δ/Δ big colony	*erg3Δ/Δ*	*erg3::ARG4/erg31::ARG4 rap1::HIS1/rap1::HIS1*	This study
rap1Δ/Δ hcs1Δ/Δ small colony	*hcs1Δ/Δ*	*hcs1::ARG4/hcs1::ARG4 rap1::HIS1/rap1::HIS1*	This study
rap1Δ/Δ hcs1Δ/Δ big colony	*hcs1Δ/Δ*	*hcs1::ARG4/hcs1::ARG4 rap1::HIS1/rap1::HIS1*	This study

Appendices

Appendix 1. The Adr1 transcription factor directs regulation of the ergosterol pathway and azole resistance in *Candida albicans*

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The Adr1 transcription factor directs regulation of the ergosterol pathway and azole resistance in *Candida albicans*

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ABSTRACT Transcription factors (TFs) play key roles in cellular regulation and are critical in the control of drug resistance in the fungal pathogen *Candida albicans*. We found that activation of the transcription factor C4_02500C_A (Adr1) conferred significant resistance against fluconazole. In *Saccharomyces cerevisiae*, Adr1 is a carbon-source-responsive zinc-finger transcription factor required for transcription of the glucose-repressed gene *ADH1* and of genes required for ethanol, glycerol, and fatty acid utilization. Motif scanning of promoter elements suggests that Adr1 may be rewired in fungi and govern the ergosterol synthesis pathway in *C. albicans*. Because previous studies have identified the zinc-cluster transcription factor Upc2 as a regulator of the ergosterol pathway in both fungi, we examined the relationship between Adr1 and Upc2 in sterol biosynthesis in *C. albicans*. Phenotypic profiles of either *ADR1* or *UPC2* modulation in *C. albicans* showed differential growth in the presence of fluconazole; either *adr1* or *upc2* homozygous deletion results in sensitivity to the drug, while their activation generates a fluconazole resistant strain. The rewiring from ergosterol synthesis to fatty acid metabolism involved all members of the Adr1 regulon except the alcohol dehydrogenase Adh1, which remains under Adr1 control in both circuits and may have been driven by the lifestyle of *S. cerevisiae*, which requires the ability to both tolerate and process high concentrations of ethanol.

IMPORTANCE Research often relies on well-studied orthologs within related species, with researchers using a well-studied gene or protein to allow prediction of the function of the ortholog. In the opportunistic pathogen *Candida albicans*, orthologs are usually compared with *Saccharomyces cerevisiae*, and this approach has been very fruitful. Many transcription factors (TFs) do similar jobs in the two species, but many do not, and typically changes in function are driven not by modifications in the structures of the TFs themselves but in the connections between the transcription factors and their regulated genes. This strategy of changing TF function has been termed transcription factor rewiring. In this study, we specifically looked for rewired transcription factors, or *Candida*-specific TFs, that might play a role in drug resistance. We investigated 30 transcription factors that were potentially rewired or were specific to the *Candida* clade. We found that the Adr1 transcription factor conferred resistance to drugs like fluconazole, amphotericin B, and terbinafine when activated. Adr1 is known for fatty acid and glycerol utilization in *Saccharomyces*, but our study reveals that it has been rewired and is connected to ergosterol biosynthesis in *Candida albicans*.

KEYWORDS rewiring, Adr1, ergosterol, *Candida albicans*, alcohol and fatty acid metabolism

Candida albicans is an opportunistic fungal pathogen that is responsible for a variety of fungal infections in humans. In healthy people, this yeast resides as a commensal in niches such as the gastrointestinal tract, but it can cause mucosal, cutaneous, and systemic infections in immunocompromised individuals (1). The prevalence of resistance to antifungal agents has significantly increased in the past few decades, and this resistance has important implications for mortality, morbidity, and health care in the community (2). The development of new antifungal drugs is challenging, as fungi are eukaryotic organisms that share many basic cellular processes with humans, and this evolutionary relatedness makes the identification of specific targets difficult and increases the likelihood of undesired secondary effects. Consequently, existing antifungals tend to target processes that are divergent between fungi and the human host.

The azole class of antifungals, including fluconazole, targets the ergosterol pathway, inhibiting a step not found in the pathway for the host-specific sterol cholesterol. Azoles are generally effective for the management of *C. albicans* infections, but due in part to the fungistatic nature of the drugs, long-term treatment often results in the emergence of azole resistance, ultimately resulting in therapeutic failure (3–5). These azole antifungals bind and inhibit the activity of the enzyme lanosterol 14-alpha-demethylase encoded by *ERG11* (6). Apart from azoles, allylamines (which target Erg1), polyenes (which target ergosterol itself), morpholines (which target Erg2), and statins (which target HMG1/2) also target elements of the sterol pathway (7). As many drugs target the *C. albicans* sterol pathway, genetic changes that perturb the pathway can lead to multi-drug resistance (8, 9).

A promising approach for drug development is to identify synergistic targets that can enhance the antifungal effect of currently available drugs (10). Transcription factors (TFs) play a key role in determining how cells function and respond to different

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environments, and approximately 4% of *C. albicans* genes encode transcription factors (11), making them the single largest family of proteins in the pathogen. TFs in *C. albicans* coordinate critical cellular functions, including biofilm formation (12), drug resistance (13), and the transition from a commensal to a pathogenic lifestyle (14).

Most transcription factors are conserved in that they fall into a limited number of groups of structurally similar proteins, such as the zinc finger, the basic helix loop helix, and the leucine zipper classes. However, evolutionary changes in transcription networks are an important source of diversity across species, driven primarily not by major changes in the structures of the factors themselves but in the connections between the transcription factors and their regulated genes. There are many incidences where researchers have identified structurally equivalent transcription factors regulating different genetic circuits in different organisms (15–18); this phenomenon has been called "rewiring." Studies suggest that this rewiring happens at a relatively constant rate, and for two species that have diverged for 100 million years, only a fraction of transcription factor/target gene combinations will likely have remained conserved (19, 20). *C. albicans* belongs to the same family as *Saccharomyces cerevisiae*, but the two fungi are suggested to have diverged as long ago as 300 million years, allowing for considerable rewiring. While studies of TFs have tended to focus on similarities between these two species, it has been estimated that only 16% of the regulator-target gene connections are preserved between the *C. albicans* and *S. cerevisiae* (21).

In our study, we have activated a group of transcription factors for *C. albicans* for which there was limited information and which had the potential to be rewired. Among our tested set of TFs, we found that C4_02500C_A activation gives resistance to several cell-membrane-targeting drugs. This resistance arises because C4_02500C_A is a central regulator of the ergosterol pathway in *C. albicans*. Further analysis shows that this TF is the ortholog of *S. cerevisiae* Adr1 and that the two proteins play distinct cellular roles in the two species.

Results

Fusion of different transcription factors to the strong activation domain VP64

In *S. cerevisiae*, the fusion of VP16 to the N terminus of Gal4 resulted in the hyper-activation of Gal4 (22, 23), and the VP64 fusion has been used to successfully activate transcription factors in both plants (24) and animals (25). We have used a similar strategy in *C. albicans*. Fusing a tetrameric version of the VP16 trans-activating domain (VP64) to the DNA-binding domains of different *C. albicans* transcription factors was found to be potent in transcriptional activation (26), so we constructed plasmid CIPACT-VPR containing the VP64 module and a multiple cloning site downstream of the *ACT1* promoter of the CIPACT plasmid (Fig. S1A).

To test the activation strategy, we chose three well-studied transcriptional factors— the bZIP TF Gcn4 (null mutant gives three amino-triazole sensitivity), the TEA/ATTS (homeo-domain) TF Tec1 (null mutant blocks hyphal development), and the leucine bZIP TF Cap1 (involved in fluconazole resistance). The Gcn4 construct generated resistance to three amino-triazole, consistent with upregulation of the Gcn4 target *HIS3* (Fig. 1A). The Tec1 construct triggered filamentation under yeast morphology growth conditions (Fig. 1B), and the Cap1 activation construct enhanced resistance to fluconazole (Fig. 1C).

We next selected 30 different TFs based on their phylogenetic uniqueness, their possible involvement in drug resistance, and their potential for functioning in rewired circuits. After generating these 30 TF-VP64 fusions, we first investigated their involvement in antifungal drug resistance (Fig. 1D). We selected three drugs for our preliminary screening—fluconazole, posaconazole, and amphotericin B. All three drugs target the cell membrane; fluconazole and posaconazole target lanosterol 14-alpha-demethylase (Erg11), an enzyme of the ergosterol pathway, whereas amphotericin B targets the end-product ergosterol. We identified two transcription factor fusions, encoded by C4_02500C_A and C6_00010W_A, that gave resistance to all three drugs. As the C4_02500C_A fusion created a higher level of resistance than the C6_00010W_A fusion, we prioritized C4_02500C_A for further study (Table 1).

Activation of C4_02500C_A confers multi-drug resistance

We subsequently tested whether the VP64 fusion to Orf19.2752 (C4_02500C_A) could trigger resistance to a variety of drugs— fluconazole, posaconazole, terbinafine, nystatin, caspofungin, anidulafungin, and amphotericin B (Fig. S1A and B). The fusion of C4_02500C_A to VP64 increases the minimum inhibitory concentration (MIC) as well as the minimal fungicidal concentration of fluconazole, amphotericin B, and terbinafine (Fig. S2). It also increased the MIC for these drugs, as well as for posaconazole and nystatin (Fig. S2B), by more than threefold. However, for the drugs caspofungin and anidulafungin that target the cell wall, there was no change in the MIC or growth rate for the activated strain relative to the control. Thus, activation of C4_02500C_A did not cause general drug resistance but did seem effective in generating resistance to cell-membrane-targeting drugs.

Orf19.2752 (C4_02500C_A) is an ortholog of *S. cerevisiae* Adr1

Because TFs are frequently conserved across species, we searched for orthologs of the *C. albicans C4_02500C_A* gene. We found it to be highly similar to the *S. cerevisiae ADR1* gene; the two proteins have about 50% sequence identity, and the N-terminal DNA-binding domain is highly conserved between them (Fig. S2C).

In *S. cerevisiae*, Adr1, acting through a conserved binding motif 5′RCCCCM3′, is required for transcriptional regulation of ethanol, glycerol, and fatty acid utilization (27, 28). Due to the highly conserved DNA-binding domains of the two orthologs, we searched for this ScAdr1-binding motif upstream of *C. albicans* ORFs. We found 221 genes with this motif in their predicted promoter regions using Meme-suite software, as described in the Materials and Methods. Of the genes with this promoter motif, a significant number (one-tenth, or 20 genes) were implicated in ergosterol biosynthesis (Data File S1), while a further one quarter (52 genes) were categorized as having an unknown function. However, in contrast to the situation in *S. cerevisiae*, this motif is not enriched in ethanol, glycerol, and fatty acid metabolism genes in *C. albicans*. Because of the large number of motif-containing genes in the pathway of sterol biosynthesis, it appeared that CaAdr1 might instead be linked to sterol production.

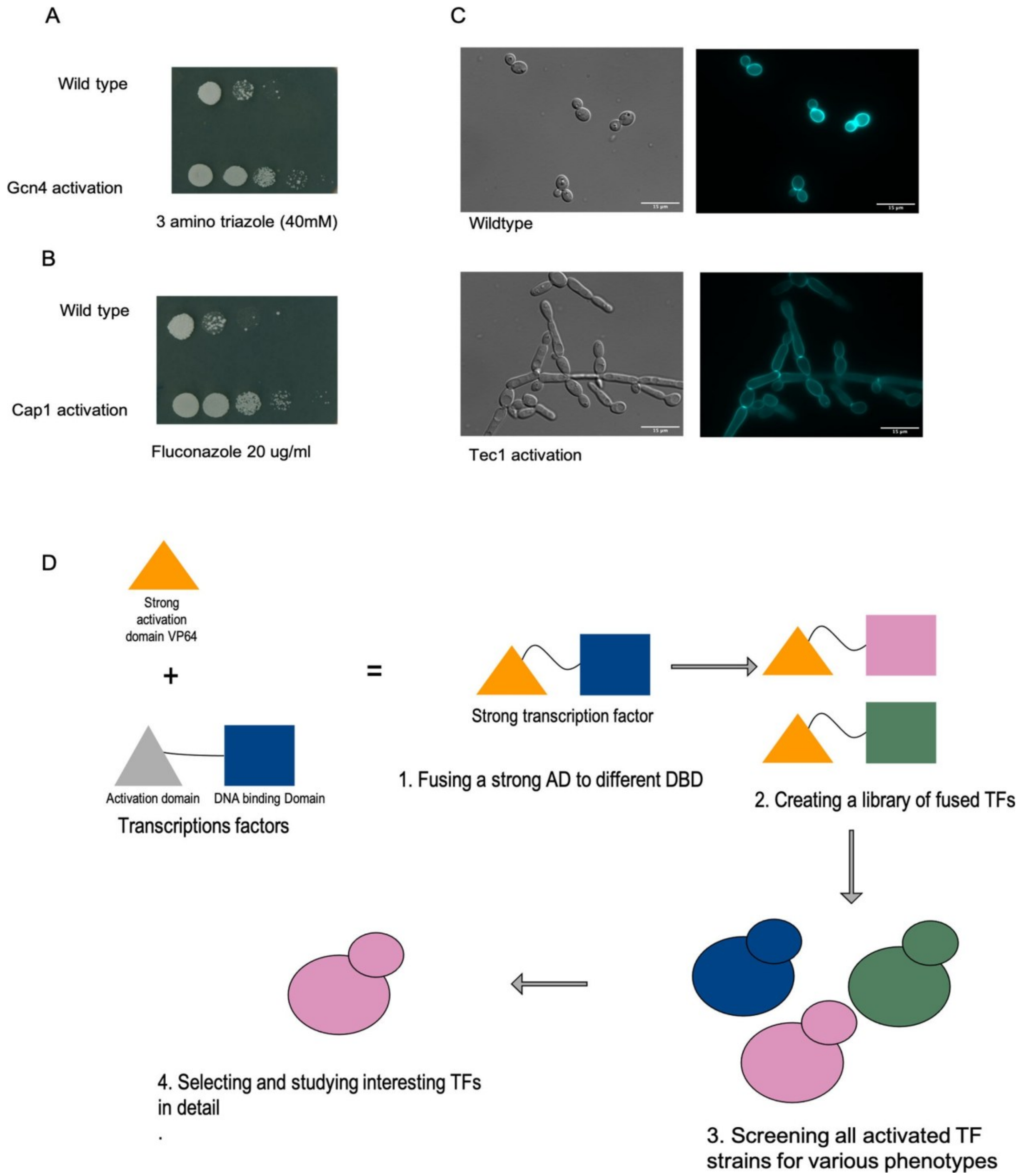


FIG 1 (A) VP64-Gcn4 chimeric transcription factor generates resistance to 3-amino-triazole (3AT). To construct the Gcn4-Vp64 fusion, we PCR amplified the Gcn4 DNA-binding domain and ligated it in the CIP-ACT-CYC plasmid in-frame with VP64 at the N terminus. After transforming this plasmid into *C. albicans*, we observed resistance to 3AT consistent with the VP64 module activating the transcription factor. (B) VP64-Tec1 TF triggers hyphal elongation in YPD media. Tec1 is a transcription factor implicated in the morphological switch from the *C. albicans* yeast form to the hyphal form. A construct containing the fusion of the N terminus of Tec1 to the VP64 module triggers elongated cellular growth. (C) VP64-Cap1 allows growth in SC media containing fluconazole. Cap1 is a poorly characterized transcription factor in *C. albicans* that gives resistance to azoles through Mdr1; activation of Cap1 upregulates MDR1 expression. Fusion of the VP64 module with Cap1 increased cellular tolerance to the azole fluconazole. (D) Schematic representation of

the workflow involved in activating the transcription factors. Based on the success of the control constructs, we selected a set of transcription factors for fusion constructs and characterized the consequences of these fusions through phenotypic analysis.

TABLE 1 Screening of activated transcription factors in the presence of different drugs[a]

Transcription factor	Fluconazole	Posaconazole	Amphotericin B	Caspofungin
Try3 Orf19.1971	−	−	+	−
C5_04410C_A Orf19.3928	−	−	−	−
C3_01220W_A Orf19.1729	+	++	−	−
C1_04510W_A Orf19.6845	++	++	+	+
C2_05640W_A Orf19.6874	−	−	−	−
Hap41 Orf19.740	−	−	+	−
Hap42 Orf19.1481	++	++	+	+
Try6 Orf19.6824	−	−	−	+++
C2_02530W_A Orf19.1577	−	−	−	−
C2_01420C_A Orf19.1447	+	+	++	−
C4_07150W_A Orf19.3088	+	+	+	+
Uga33 Orf19.7317	−	−	−	−
Nrg2 Orf19.6339	++	++	++	++
Ace2 Orf19.6124	−	−	−	−
C1_13440C_A Orf19.4972	−	−	−	−
Rfx1 Orf19.3865	−	−	+	++
Grf10 Orf19.4000	+	−	+	+++
C4_05680W_A Orf19.1253	+	+	−	++
CR_05880W_A Orf19.6626	++	++	+	−
C2_08950W_A Orf19.211	+	+	−	+
C5_04280C_A Orf19.3912	−	−	−	−
C4_02500C_A **Orf19.2752 (Adr1)**	**+++**	**+++**	**+++**	**+**
C2_10660W_A Orf19.5343	+	+	+	+
C1_11690W_A Orf19.1150	+	+	−	−
C1_10020W_A Orf19.4869	−	−	+	++

C4_05880W_A Orf19.1275	−	−	−	−

(*Continued on next page*)

TABLE 1 Screening of activated transcription factors in the presence of different drugs[a] (*Continued*)

Transcription factor	Fluconazole	Posaconazole	Amphotericin B	Caspofungin
Try4 Orf19.5975	−	−	−	+++
C4_05880W_A Orf19.1275	−	−	−	−
Met4 Orf19.5312	−	−	−	+
Gcn4	+	+	+	−
Cap1 (control)	+++	+++	++	+
Tec1 (control)	++	++	+	+

[a]The optical densities are represented by plus signs (+), where + indicates growth equivalent to that of the positive control, ++ indicates a higher OD growth than + and +++ higher than ++ (−<+<++<+++ OD at 600 nm after 24 h) whereas - indicates no growth. Results focused on in this paper are in bold.

Adr1 DNA-binding motif

We investigated potential direct 5′NRCCCCM3′ binding using ChEC-seq analysis. We fused the MNase cassette to Adr1 and used the calcium-activated nuclease to identify potential binding targets for Adr1. These results identified direct binding to several genes with the 5′NRCCCCM3′ motif in their promoters, including Mrr2, Adh1, Ecm22, Erg5, Erg28, Cdr1, and Adr1 itself. These genes were also upregulated in the Adr1-activation RNA-seq data set, suggesting that Adr1 may control 5′NRCCCCM3′ motif-bearing genes and thus be directly involved in transcriptional regulation of the ergosterol biosynthesis pathway (Fig. 2B and C). As well, the transcription factor Mrr2 was dramatically upregulated; this could help explain the observed multi-drug resistance phenotype, as Mrr2 acts to upregulate the *CDR* transporter-encoding genes, and our RNA-seq analysis shows that both *CDR1* and *CDR2* are more highly expressed in the Adr1-VP64 fusion strain than in the control (Fig. 2B). To establish if the fluconazole resistance is a direct result of this upregulation of Mrr2, we deleted *MRR2* in the Adr1-activated strain. Deletion of *MRR2* had essentially no effect on fluconazole resistance driven by activation of Adr1, suggesting that the upregulation of the Mrr2 TF was in fact not critical in creating the azole-resistance phenotype (Fig. S1B). Similarly, amphotericin B resistance was not affected by *MRR2* deletion. However, the deletion did impact the terbinafine resistance, suggesting that the observed allylamine resistance could be mediated through Mrr2 upregulation (Fig. S1D).

To further validate that Adr1 binds to the promoter sequence 5′NRCCCCM3′, we put a GFP reporter construct under control of the *ERG 11* promoter, the *MRR2* promoter, and, to serve as a control, the *ACT1* promoter. We introduced these constructs into both wildtype and *adr1* deletion strains. We found that the *adr1* deletion strain generated a much weaker GFP signal from the *ERG11* and *MRR2* promoters than the signals from the wildtype strain, while the expression of the *ACT1* construct did not differ between the two strains (Fig. 2D). These results supported the requirement of Adr1 for the promoter function of *ERG11* and *MRR2*. We then disrupted the NRCCCCM sequence in the *ERG11* and *MRR2* GFP reporter constructs and saw background GFP signals from both the wildtype and *adr1* deletion strains. This confirms that NRCCCCM is the sequence through which the Adr1 transcription factor functions (Fig. 2D).

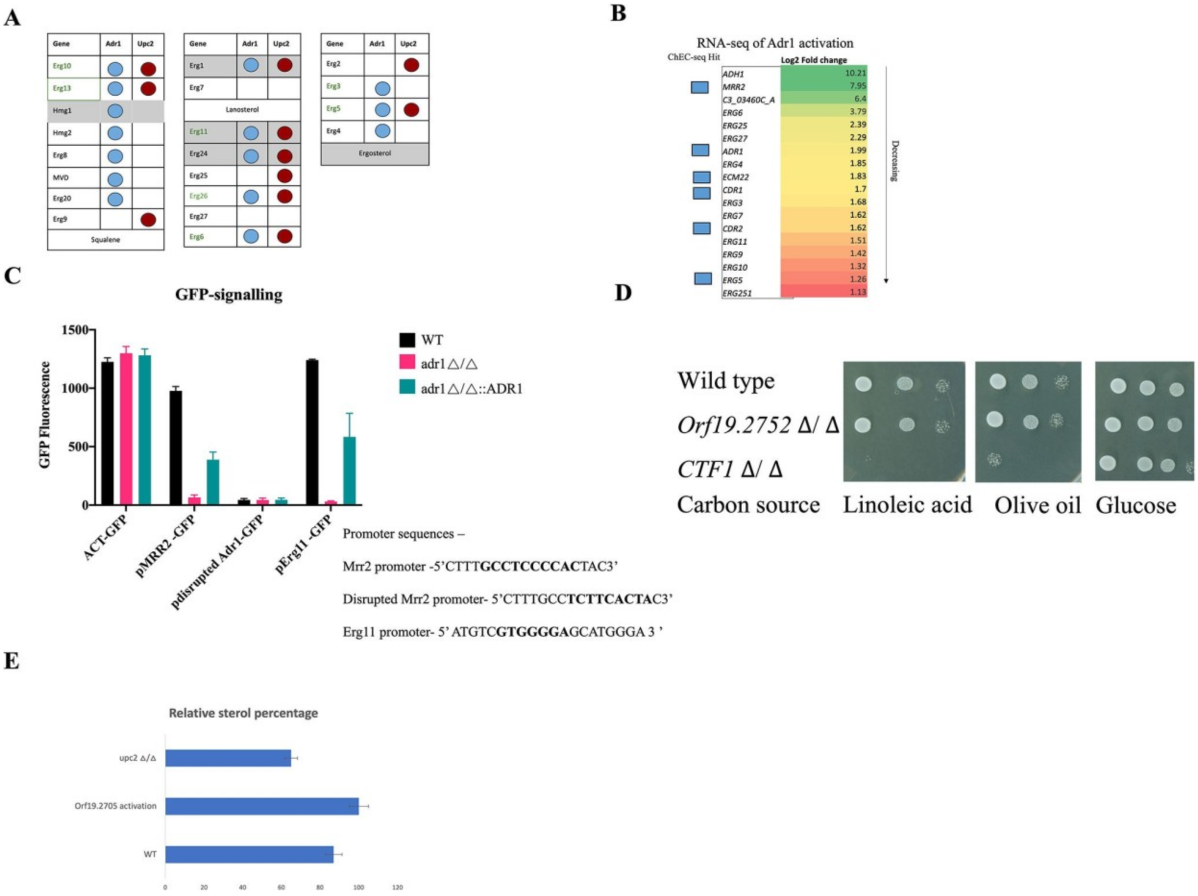


FIG 2 (A) The presence of the Adr1 motif upstream of ergosterol genes. The presence of the candidate Adr1 motif is represented by a blue circle. We found that most of the ergosterol genes have the candidate Adr1 motif 5′′NRCCCCM3′ in their promoter regions. (B) The transcriptomic profile of selected genes in the Orf19.2752-VP64 fusion strain shows upregulation of ergosterol genes. After activation of the Adr1 transcription factor, we did an RNAseq comparison of the activated strain and the wild type. We identified various ergosterol pathway genes that were upregulated and observed high expression of the genes for the transcription factor Mrr2 and the alcohol dehydrogenase Adh1. The full gene set is found in Fig. S2 along with the FPMK values. We also confirmed the binding of Adr1 by performing ChEC-Seq analysis; significantly bound genes are noted. (C) GFP reporter signaling. We measured, in both *adr1Δ/Δ* and wild-type strains, GFP driven by the ACT1 promoter, the ERG11 promoter with the potential Adr1-binding site 5′NRCCCCM3′, the MRR2 promoter with the potential Adr1-binding site 5′NRCCCCM3′, and disrupted the MRR2 promoter lacking the 5′NRCCCCM3′-binding site. We constructed GFP in the CIPACT-CYC plasmid by PCR and homology, replaced the ACT1 promoter with the ERG11 or MRR2 promoters, and disrupted Mrr2 in the wild-type strain CAI4 and the adr1 deletion strain as described previously. We quantified the GFP signaling using a BioTek Cytation 5. (D) Deletion of

ADR1 (ORF19.2752). We deleted the ADR1 gene and checked the resulting strain for fatty acid, glycerol, and alcohol utilization. We found that it does not have any effect on growth on a fatty acid substrate, while a CTF1 deletion strain shows impaired growth on either linoleic acid or olive oil as a substrate. (E) Sterol estimation of wild-type and the Orf19.2752-activated strain. We isolated the sterols from overnight-grown *C. albicans* by the pargolol-hexane extraction method. The extracted sterols showed a four-peak spectral absorption pattern characteristic of ergosterol and 24(28)-DHE. The activated strain showed an approximately twofold increase in measured sterols.

CaAdr1 influences sterol metabolism

Azole drugs target Erg11 of the ergosterol pathway in *C. albicans* (29), and upregulation of Erg11 is one of the known mechanisms for drug resistance against fluconazole (30, 31). To test if the *ADR1* gene of *C. albicans* was involved in sterol metabolism, we deleted the gene and checked the consequences of loss of function; consistent with a role in sterol biosynthesis, *ADR1* deletion causes slight sensitivity to cell membrane targeting drugs like fluconazole (Fig. S1C and D; Fig. S2A). The complementation of *adr1* with the native protein or the Vp64-activated version restored the drug sensitivity (Fig. S1C). However, unlike the situation with *ScADR1*, the deletion of *CaADR1* did not block growth on fatty acid substrates (Fig. 2). By contrast, deletion of the gene for the transcription factor Ctf1, identified as a regulator of fatty acid metabolism genes in the pathogen (32), completely blocked *C. albicans* growth on linoleic acid and severely compromised growth on olive oil (Fig. 2D). This suggests that in *C. albicans*, Ctf1 is controlling fatty acid utilization, while Adr1 is not involved. In *S. cerevisiae*, ScAdr1 was found to be haplo-insufficient for ethanol, glycerol, and fatty acid metabolism. Similarly, in *C. albicans*, CaAdr1 is haplo-insufficient for fluconazole resistance, as the heterozygote showed sensitivity to fluconazole relative to the WT but was clearly more resistant than the homozygous null (Fig. S1C).

We directly checked the sterol content of the Adr1-activated and wild-type strains by extracting sterols with the organic solvents pargolol and hexane, followed by spectrophotometric assessment. Activated CaAdr1 enhances the production of ergosterol (Fig. 2E). We also directly assessed the transcriptional consequences of Adr1 activation through RNAseq analysis. *ADH1*, which encodes the alcohol dehydrogenase that oxidizes ethanol to acetaldehyde, was the most highly upregulated gene in our profile, and intriguingly, the orthologous gene in *S. cerevisiae* is a direct target of ScAdr1 (33).

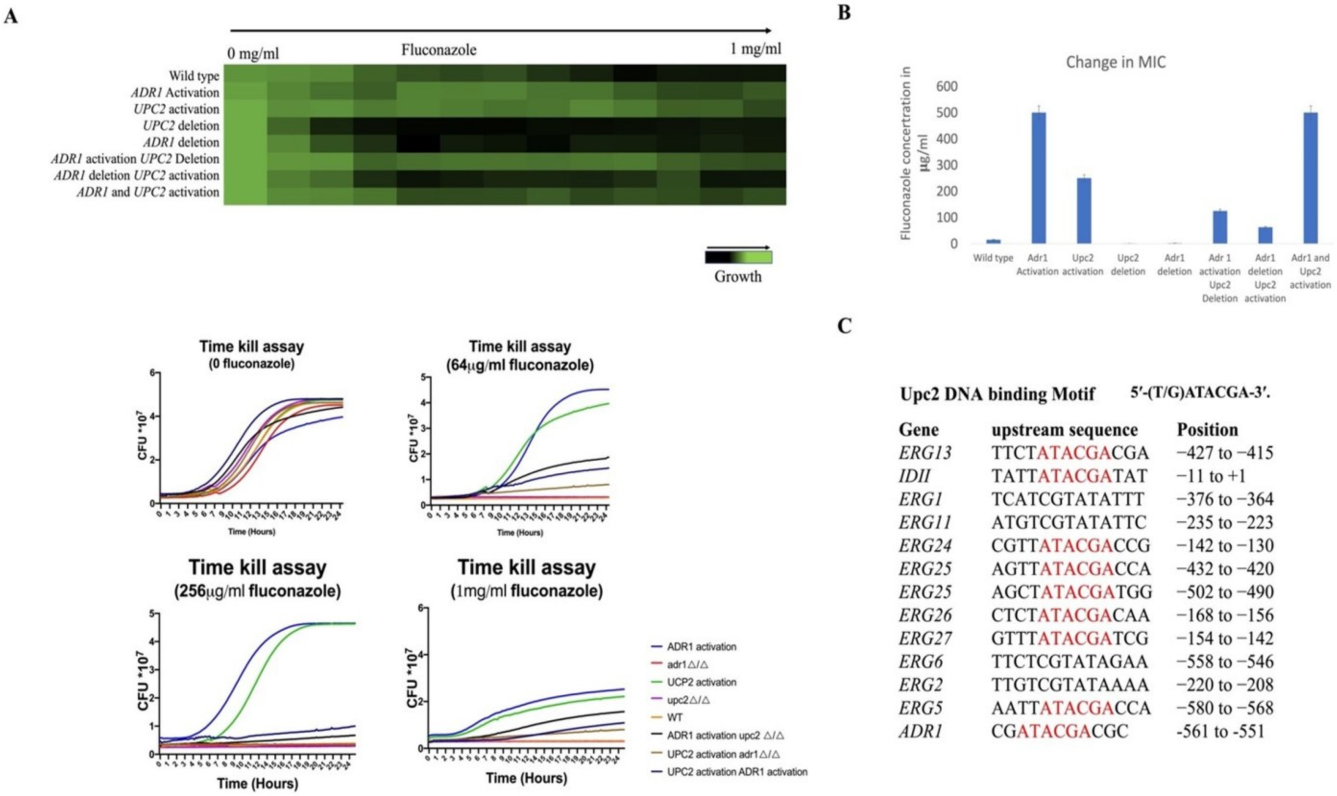
Adr1 and Upc2 roles in azole response

Consistent with the presence of the candidate Adr1-binding motif in their promoters, we found that the expression level of most of the ergosterol pathway genes, including Erg11, was upregulated by the activated Adr1 construct. This increase in ergosterol pathway gene expression was, however, not associated with upregulation of the classic ergosterol biosynthesis pathway transcription factor Upc2, which functions as a key regulator of the pathway in both *S.*

cerevisiae and *C. albicans* (34–36). Therefore, it appears that Adr1 activation of the *C. albicans* ergosterol pathway genes is likely direct (Table S1), and thus the fluconazole resistance generated by the Adr1VP64 fusion protein may be due to the generalized increase in the expression of these ergosterol biosynthetic pathway genes. We then asked whether the fluconazole resistance generated by Adr1 activation was fully independent of Upc2. First, we compared fluconazole resistance levels in strains with Adr1 activated and with Upc2 activated, as well as in strains with deleted *UPC2* or *ADR1*. As shown in Fig. 3A and B, both Upc2 activation and Adr1 activation gave similarly high levels of resistance to fluconazole, while both Adr1 deletion and Upc2 deletion conferred sensitivity to fluconazole, with the Upc2 deletion strain being somewhat more sensitive. Second, we assessed the resistance to fluconazole of the doubly activated strain; in this case, the strain grew poorly in the absence of the drug but was resistant to fluconazole at similar levels to that of the single-activated mutants (Fig. 3A and B). Finally, we investigated the behavior of cells with activated Adr1 that lacked functional Upc2 and cells with activated Upc2 that lacked Adr1. We observed that loss of Upc2 had essentially no effect on fluconazole resistance caused by activation of Adr1, suggesting that the effect of Adr1 on drug resistance is independent of Upc2, while loss of Adr1 significantly compromised fluconazole resistance caused by activation of Upc2 (Fig. 3A and B). This is consistent with part of the effect of Upc2 activation on azole resistance working through Adr1. We checked the upstream sequences of the *UPC2* and *ADR1* genes for regulatory motifs and found that the promoter sequence of the *ADR1* gene contains a potential *UPC2* motif (Fig. 3C), consistent with Adr1 being part of the Upc2 regulon, while the *UPC2* promoter lacks any potential Adr1-binding motif.

Phylogenetic analysis

We examined a phylogenetic profile of the Ascomycota and characterized the upstream sequences of all ergosterol biosynthesis genes for the regulatory motifs associated with both the Adr1 and the Upc2 transcription factors. In *C. albicans*, our data suggest that Adr1 works together with Upc2 to control the ergosterol biosynthesis pathway, with Adr1 also controlling the expression of genes such as *ADH1,* which encodes the alcohol dehydrogenase that oxidizes ethanol to acetaldehyde, and *MRR2,* which encodes a stress-responsive transcription factor. The more basal filamentous fungi also have candidate binding motifs for both Adr1 and Upc2, the promoters of the ergosterol biosynthesis genes. However, the presence of the Adr1 motif in the promoter of *MRR2* is very specific to the CTG clade species (Data file S1).



Upc2 DNA binding Motif 5′-(T/G)ATACGA-3′.

Gene	upstream sequence	Position
ERG13	TTCTATACGACGA	−427 to −415
IDII	TATTATACGATAT	−11 to +1
ERG1	TCATCGTATATTT	−376 to −364
ERG11	ATGTCGTATATTC	−235 to −223
ERG24	CGTTATACGACCG	−142 to −130
ERG25	AGTTATACGACCA	−432 to −420
ERG25	AGCTATACGATGG	−502 to −490
ERG26	CTCTATACGACAA	−168 to −156
ERG27	GTTTATACGATCG	−154 to −142
ERG6	TTCTCGTATAGAA	−558 to −546
ERG2	TTGTCGTATAAAA	−220 to −208
ERG5	AATTATACGACCA	−580 to −568
ADR1	CGATACGACGC	-561 to -551

FIG 3 (A) Adr1 and Upc2 influence the response to fluconazole. Fluconazole MIC assay of ADR1 deletion, ADR1 activation, ADR1 activation in UPC2 deletion, ADR1 deletion in UPC2 activation and double activation, followed by the time kill assay. (B) Fold change in MIC from Adr1 and Upc2 activation and deletion.

Graphical representation of the change in minimum inhibitory concentrations of various drugs in the Orf19.2752-activated strain. (C) Upc2 DNA-binding motif upstream of various regulons, including ADR1. Upstream regions of genes, including the transcription factors Adr1, Upc2, Mrr2, and Mrr1, as well as the structural genes for the ergosterol biosynthesis pathway, were examined for candidate TF-binding motifs. We identified a potential Upc2-binding motif upstream of the ADR1 gene.

In the evolutionary trajectory leading to *S. cerevisiae*, it appears that the Adr1 TF was repurposed to control alcohol, fatty acid, and glycerol utilization, taking over from Ctf1 orthologs that do the job in the filamentous fungi and the CTG clade species. Based on the search of promoter motifs, we identified that the Upc2 binding motif is found in the promoters of ergosterol pathway genes throughout the fungi, along with the Adr1 motif. But after *Candida guilliermondii* in the phylogeny, the Adr1 motif signal weakens, leaving only Upc2 with a strong signal associated with the ergosterol pathway. The Adr1-binding motif signal connecting the ergosterol pathway genes in the CTG clade appears to be gradually transferred to genes involved in the control of alcohol and fatty acid utilization in *S. cerevisiae* and its relatives (Data file S1).

DISCUSSION

One of the common approaches to investigating the regulatory networks controlling drug resistance in fungal pathogens is through comparison with the *S. cerevisiae* circuits; this approach has led to the discovery of many TFs responsible for drug resistance in both *S. cerevisiae* and *C. albicans*, including Fcr1, Ndt80, Mrr1, and Upc2 (34, 37–40). However, these two fungi diverged as long ago as 300 million years, and for species that diverged by such an evolutionary distance, most of the DNA-binding patterns of a given regulator in one species are unlikely to be preserved in the other species (20). Overall, genome-wide correlations converge on about 10% overlap for species with this level of divergence (20), and therefore, there is a significant probability that many of the TFs responsible for drug resistance will be different between *C. albicans* and budding yeast. To identify candidate TFs with *Candida*-specific roles in drug resistance, we selected a set of TF-encoding genes that were either not found in *S. cerevisiae* or predicted to have potentially changed function between the two species. We identified 30 such TFs and activated them to identify potential roles in drug resistance (as well as other cellular processes) (Table 1). Among these transcription factors, Orf19.2752 activation resulted in clear resistance to a set of drugs generally targeting the cell membrane; activation of this transcription factor generated resistance to azoles, allylamines, and polyenes. Sequence comparisons established that *C4_02500C_A (ORF19.2752)* was the *C. albicans* ortholog of the *S. cerevisiae* gene *ADR1*, a gene not linked to drug resistance in budding yeast. These two transcription factors share a highly conserved DNA-binding domain.

In *S. cerevisiae*, Adr1 is involved in the transcriptional regulation of genes involved in the catabolism of ethanol, glycerol, and fatty acids (27, 28) and is proposed to act through a candidate DNA-binding motif, 5′NRCCCCM3′, in the promoter regions of these genes (41). Interestingly, in *C. albicans*, this same DNA-binding motif is enriched in the upstream regions of the ergosterol pathway genes, whereas it is generally absent from the promoters of the ethanol, glycerol, and fatty acid utilization genes of this pathogen. This suggests that Adr1 may have been rewired from the ergosterol biosynthesis pathway in other fungi to the metabolic utilization of ethanol, glycerol, and fatty acids in *S. cerevisiae*. Further investigation established that activation of CaAdr1 generated transcriptional upregulation of most of the ergosterol pathway genes in the pathogen. However, Upc2, the well-established regulator of the Erg-pathway genes in both *C. albicans* and *S. cerevisiae*, was not transcriptionally upregulated, suggesting that in *C. albicans*, Adr1 activation of the ergosterol pathway genes was not going through Upc2.

To further investigate the proposed Adr1 DNA-binding motif, we performed a ChEC-seq analysis (42, 43) and found that several drug-resistance-related genes, including Mrr2, Adh1, Ecm22, Erg5, Ddr48, Erg28, Cdr1, and Adr1, were both upregulated in the RNAseq analysis and were ChEC-seq hits. Previous *in-silico* analysis of a number of TFs had shown a weak but statistically significant overlap in the genes in *S. cerevisiae* and *C. albicans* containing the Adr1 motif in their promoters (44). This is consistent with our ChEC-seq analysis; while many genes have been rewired between the two species, some genes, like *ADH1*, are under Adr1 control in both species, suggesting that Adr1 might have multiple roles in both fungi. However, the bulk of the circuit of ethanol, fatty acid, and glycerol metabolism controlled by Adr1 in *S. cerevisiae* is under the control of Ctf1 in *C. albicans* (32), as Adr1 deletion did not affect growth on substrates like olive oil and linoleic acid, whereas Ctf1 deletion gives a clear auxotrophy. Apart from Adh1, in our RNA-seq as well as CheC-seq results, we found transporter, metabolism, and stress-responsive genes, including oxidative stress. Previously, it was reported that amphotericin B resistance might be due to upregulation of oxidative stress genes (45).

These data underline the multiple circuit restructurings involved in the control of these pathways in different fungi. In *S. cerevisiae*, Cat8 and Adr1 both appear to have been rewired to connect to the module controlling alcohol, acetic acid, and fatty acid utilization, Adr1 from the ergosterol circuit, and Cat8 from gluconeogenesis (ScSip4). Another event is the disappearance of the Ctf1 TF from the *S. cerevisiae* genome, as there is no apparent ortholog of Ctf1 in *S. cerevisiae*. This loss could be facilitated by the transfer of the Ctf1 regulon to Adr1 control in *S. cerevisiae*. In *S. cerevisiae*, Upc2 gains a paralog (Emc22) and apparently unique control over the ergosterol pathway (46, 47).

In *C. albicans*, Adr1 activation causes upregulation of many ergosterol pathway members, including Erg11 (target of azoles), Erg1 (target of allylamines), Erg2 (target of morpholines), HMG1/2 (target of statins), and causes increases in ergosterol itself (target of polyenes), which has the potential to generate multi-drug resistance. The activation of Adr1 dramatically enhances the MIC of fluconazole, amphotericin B, terbinafine, and statins. We created a series of strains to determine how Upc2 and Adr1 are influencing the ergosterol pathway. Activation of either Upc2 or Adr1 enhanced azole resistance, while deletion of either gene created azole sensitivity. Activation of both TFs at the same time caused poor growth, perhaps due to the disturbed circuits or due to the activation of an Erg3-driven side branch of the pathway generating the toxic 14-methylergosta-8,24(28)-dienol. However, the relative resistance to azoles remained similar to the individually activated strains, suggesting that the actions of the two TFs are not additive or synergistic. Another known stress associated with ergosterol is hypoxia, and therefore, we tested both Adr1 activation and *adr1* deletion under hypoxia conditions (48, 49). We did not find any changes in the *adr1* deletion strain under hypoxia, while the upregulated allele somewhat improved growth (Fig. S2F). Previously, the *upc2* deletion strain has been reported to show a significant growth defect under hypoxia (50), so this distinction will need further investigation.

The fluconazole resistance caused by activation of Upc2 is significantly dependent on the presence of Adr1, but loss of Upc2 had very little effect on the resistance profile of strains with activated Adr1. In addition, there is a potential Upc2 DNA-binding site in the promoter region of *ADR1*, while *UPC2* has no candidate site for Adr1. These results are consistent with Upc2 serving as a master regulator of ergosterol biosynthesis, controlling ERG gene expression both directly and through regulation of the Adr1 TF, which also serves as an activator of ERG gene expression. In the absence of Upc2, Adr1 is sufficient to ensure ERG expression, although response to azole drugs is compromised by loss of either TF. A strain containing deletions of both *adr1* and *upc2* was very slow growing and also very sensitive to fluconazole.

Among the highly upregulated genes resulting from Adr1 activation is the gene encoding Mrr2, itself a TF involved in the expression of the multi-drug-resistance-regulating membrane transporter Cdr1. However, even though *CDR1* expression was somewhat upregulated in the Adr1-activated strain, the Mrr2 upregulation did not seem critical for the observed fluconazole resistance because deletion of *MRR2* in the Adr1-activated strain did not compromise this resistance.

While it appears that in filamentous fungi and the CTG clade species Adr1 is linked to ergosterol biosynthesis, in the Saccharomycotina, it has been shifted to control the pathway for ethanol, glycerol, and fatty acid utilization (51, 52), replacing Ctf1 that controls the process in the non-Saccharomycotina species. This transfer appears so complete that the Ctf1 factor has been lost in the Saccharomycotina. Intriguingly, throughout this transition, Adr1 has maintained a role in the control of the expression of the alcohol dehydrogenase catalyzing the oxidation of ethanol to acetaldehyde (*ADH1* in *C. albicans*, *ADH2* in *S. cerevisiae*). To deal with ethanol toxicity, in *S. cerevisiae*, ethanol is modified into unsaturated lipids and ergosterol (51). The rewiring of Adr1 from the ergosterol pathway to the ethanol utilization process (41, 52) may have been driven by the shift to the Crabtree-positive lifestyle of *S. cerevisiae*, requiring the ability to both tolerate and process high concentrations of ethanol.

Conclusion

Sterol biosynthesis is critical for fungal biogenesis and a target for many antifungal drugs. We have identified the TF Adr1 as a key regulator of sterol biosynthesis in the fungal pathogen *C. albicans*, where it works in concert with the zinc cluster TF Upc2. We suggest that Upc2, when bound to ergosterol, remains inactive in the cytoplasm but is activated when there is depletion of ergosterol, whether by environmental factors or due to the presence of drugs targeting the ergosterol pathway. Activated Upc2 goes to the nucleus and turns on key players of the ergosterol biosynthetic pathway as well as the TFs Mrr1 and Adr1. Adr1 aids in regulating the ergosterol pathway genes and turns on the TF Mrr2. Thus, activation of Upc2 and Adr1 leads to the coordinated expression of the ergosterol biosynthesis pathway, as well as the activation of the phospholipid transporters Cdr1 and Cdr2, which can also function to export antifungal drugs (Fig. 4).

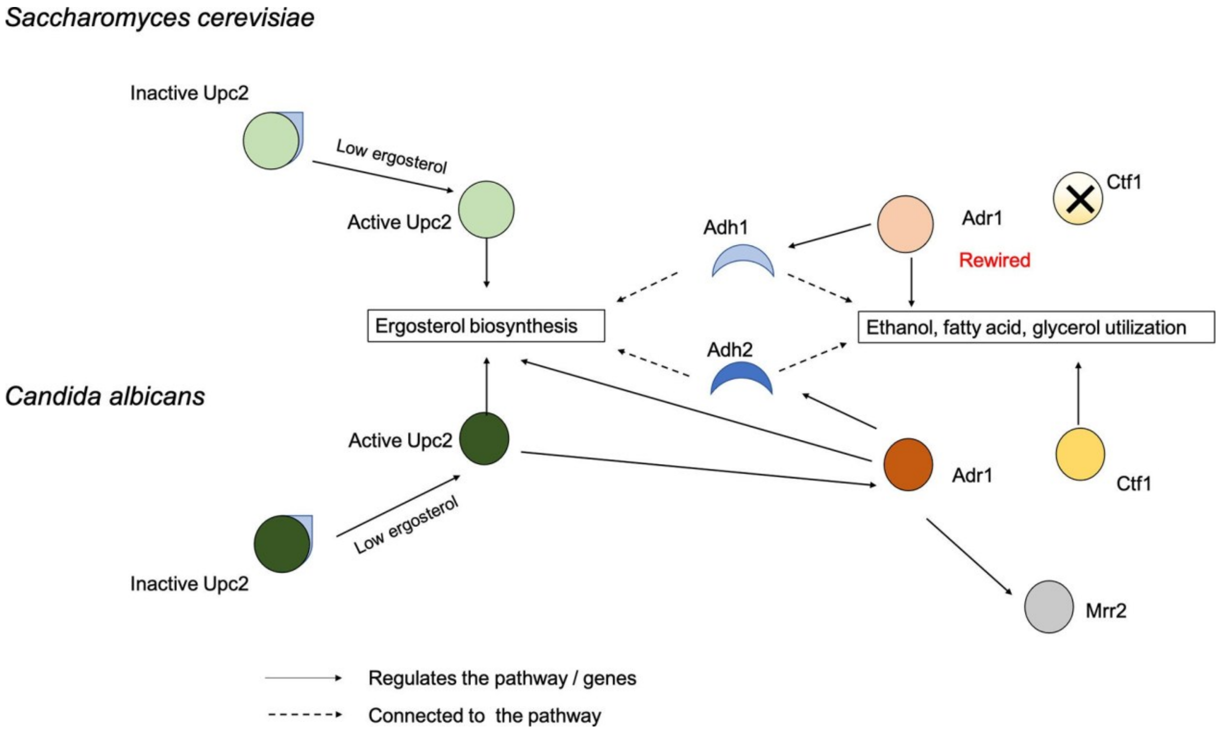


FIG 4 Proposed model of the multiple rewiring of transcription factors between *S. cerevisiae* and *C. albicans*, along with coordination of Upc2, Adr1, and Mrr2 during the presence of azoles or depletion of ergosterol, leading to drug resistance in *C. albicans*. In both *S. cerevisiae* and *C. albicans*, the Upc2 transcription factor binds to ergosterol and remains inactive in the cytosol. Depletion of ergosterol changes Upc2 to an active form, which enters the nucleus and initiates the transcription of the genes for ergosterol biosynthesis. In *C. albicans*, activated Upc2 also triggers expression of the Adr1 transcription factor, which further serves to direct expression of ergosterol biosynthesis genes as well as the alcohol metabolism gene Adh1 and the Mrr2 transcription factor. In *S. cerevisiae*, Adr1 has been rewired to control other parts of the alcohol utilization circuit in addition to alcohol dehydrogenase, as well as both fatty acid and glycerol utilization circuits.

MATERIALS AND METHODS

Bioinformatics analysis

Sequences of genes Ca*ADR1* and *ScADR1* were obtained from the Candida Genome Database (CGD; http://www.candidagenome.org/) and the Saccharomyces Genome Database (https://www.yeastgenome.org/). Gene orthogroup assignments for all predicted protein-coding genes across 23 ascomycete fungal genomes were obtained from the Fungal Orthogroups Repository (53) and maintained by the Broad Institute.

DNA sequence motifs were identified using the web-based motif-detection algorithm MEME (http://meme.sdsc.edu/meme/intro.html) (54). For more stringent motif identification, we used MAST hits with an *E*-value of <50. An *E*-value of 500 corresponds roughly to a *P*-value of 0.08 in our analysis, and an *E*-value of 50 roughly corresponds to a *P*-value of 0.008. We also used AME (http://meme-suite.org/tools/meme), which identifies known motifs throughout the *Candida* upstream sequences.

Protein domains and linear motifs were detected from each individual TF protein sequence using INTERPROSCAN, PFAM, and ELM motif definitions. For ChEC-seq analysis, we used Integrative Genomics Viewer (IGV) (https://igv.org) and MEME-ChIP software.

Strains and culture conditions

For general growth and maintenance of the strains, the cells were cultured in fresh YPD medium [1% (wt/vol) yeast extract, 2% (wt/vol) Bacto peptone, 2% (wt/vol) dextrose, and 80 mg/L uridine with the addition of 2% (wt/vol) agar for solid medium] at 30°C. For drug assays, we used synthetic dextrose (SD) medium [0.67% (wt/vol) yeast nitrogen base, 0.15% (wt/vol) amino acid mix, 0.1% (wt/vol) uridine, 2% (wt/vol) dextrose, and 2% (wt/vol) agar for solid media] along with the various concentrations of drugs in liquid and solid media.

Gene knockouts using CRISPR

All *C. albicans* mutants were constructed in the wild-type strain CAI4. The protocol used for the CRISPR-mediated knockout of *ADR1*, *CTF1*, and *UPC2* was adapted from (55); we used *URA3* replacements in our study. CRISPR-mediated knockouts used the lithium acetate method of transformation with the modification of growing transformants overnight in liquid YPD at room temperature after removing the lithium acetate-PEG. *C. albicans* transformants were selected on SD-URA plates.

Activation of transcription factors

For the activation module, the *ACT1* promoter and VP64 were amplified by PCR, and homology was created by primer extension such that there is an Mlu I site between *ACT1* and VP64. This ligated CIPACT-VP64 plasmid was transformed into *Escherichia coli* using the calcium chloride method. High-throughput equipment at the Concordia Genome Foundry, the Biomek FXP Automated Workstation, otherwise known as the Biomek FXP liquid handler, was used to insert different DNA-binding domains to create and later screen this library.

Plasmids extracted from colonies that were determined to have the guide sequence successfully cloned were then used to transform *C. albicans* using a lithium acetate transformation protocol. pCIPACT1 was linearized by StuI-HF digestion, and 1–2 µg of the linearized plasmid was used in the transformation. *C. albicans* transformants were selected on SD-URA plates.

RNA seq analysis

The CaAdr1 and SC5143 strains were grown in SC media overnight at 30°C, diluted to an OD600 of 0.1 in YPD at 30°C, and then grown to an OD600 of approximately 1.0 on a 220-rpm shaker. Total RNA was extracted from two biological replicates using the Qiagen RNeasy Minikit protocol, and RNA quality and quantity were determined using an Agilent bioanalyzer. Paired-end sequencing (150 bp) of extracted RNA samples was carried out at the Quebec Genome Innovation Center located at McGill University using an Illumina miSEQ sequencing platform. Raw reads were pre-processed with the sequence-grooming tool cutadapt version 0.4.1 (56) with the following quality trimming and filtering parameters: `--phred33 --length 36 -q 5 --stringency 1 -e 0.1`. Each set of paired-end reads was mapped against the *C. albicans* SC5314 haplotype A, version A22, downloaded from the CGD (http://www.candidagenome.org/) using HISAT2 version 2.0.4. SAM tools were then used to sort and convert SAM files. The read alignments and *C. albicans* SC5314 genome annotations were provided as input into StringTie v1.3.3 (57), which returned gene abundances for each sample. Raw and processed data have been deposited in NCBI's Gene Expression Omnibus (58).

Sterol quantification

The cells were grown overnight (16 h) at 30°C and harvested by centrifugation. Nontreated cells were maintained separately and considered controls. The cell pellets were washed twice with sterile distilled water. We followed the same method that has been described in reference (59) with slight modifications. Cell pellets were resuspended in 2.5 mL methanol, 1.5 mL potassium hydroxide [60% (wt/vol)], and 1 mL methanol-dissolved pyrogallol [0.5% (wt/vol)] and heated at 90°C for 2 h. The cell extracts were cooled, and then sterols were extracted with two rounds of treatment with 5 mL of hexane. The extracted sterols indicated a four-peak spectral absorption pattern produced by ergosterol and 24(28)-dehydroergosterol [24(28)-DHE] spectrophotometrically (DU530 life science UV spectrophotometer). Both ergosterol and

24(28)-DHE absorb at 281.5 nm, whereas only 24(28)-DHE absorbs at 230 nm. The ergosterol content is determined by subtracting the amount of 24(28)-DHE (calculated from the A230) from the total ergosterol plus the 24(28)-DHE content (calculated from the A281.5). Ergosterol content was calculated as a percentage of the wet weight of the cells with the following equations: % ergosterol + % 24(28)-DHE [(A281.5/290) × *F*] / pellet weight, % 24(28)-DHE − [(A230/518) − *F*] / pellet weight, and % ergosterol = [% ergosterol + % 24(28)-DHE] – [% 24(28)-DHE], where *F* is the factor for dilution in petroleum ether and 290 and 518 are the *E*-values (in percent per centimeter) determined for crystalline ergosterol and 24(28)-DHE, respectively.

ChEC-seq analysis

To perform the ChEC-seq analysis, we followed reference (43). We constructed the Adr1-MNase strain and grew overnight cultures of *C. albicans* Adr1-MNase-tagged and free MNase strains that were then diluted to a starting OD600 of 0.1 in 50 mL YPD medium and grown at 30°C to an OD600 of 0.7 to 0.8. Cells were washed three times with 1 mL buffer A [15 mM Tris (pH 7.5), 80 mM KCl, 0.1 mM EGTA, 0.2 mM spermine, 0.5 mM spermidine, one tablet of Roche complete EDTA-free mini protease inhibitors, 1 mM phenylmethylsulfonyl fluoride], resuspended in 800 μL buffer A containing 0.1% digitonin (Sigma), and permeabilized for 10 min at 30°C with shaking. MNase digestions were performed by adding $CaCl_2$ to a final concentration of 5 mM and incubating for different time points at 30°C. At each time point, a total of 200 μL aliquots of the ChEC digestions were transferred to a tube containing 50 μL of 250 mM EGTA to quench MNase digestion. DNA was extracted using a MasterPure yeast DNA purification kit (Epicentre, MPY80200). ChEC DNA was subjected to size selection using the Pippin Prep (SageScience) size selection system with a 2% agarose gel cassette, allowing the removal of multi-kilobase genomic DNA fragments and the enrichment of 100 to 400 bp DNA fragments.

For the GFP signaling quantification, we introduced GFP in the CIPACT-CYC plasmid by PCR, and homology was created by primer extension for enzymes XmaI and MluI. Furthermore, we replaced the *ACT1* promoter with the *ERG11* or *MRR2* promoters and disrupted Mrr2 using Kpn1 and an enzyme. The ligation was done using T4 DNA ligase and was transformed into *E. coli* using the calcium chloride method. These plasmids were further transformed into the wild-type strain CAI4 and the *adr1* deletion strain as described previously. The GFP signals were quantified using a BioTek Cytation 5.

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AUTHOR CONTRIBUTIONS

Manjari Shrivastava, Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Project administration, Resources, Software, Validation, Visualization, Writing – original draft, Writing – review and editing | Gaëlle S. Kouyoumdjian, Data curation, Methodology, Validation | Daniel Ruiz, Data curation, Methodology | Manon Henry, Data curation, Formal analysis, Methodology | Antony T. Vincent, Formal analysis, Methodology, Validation, Visualization | Adnane Sellam, Resources, Validation | Malcolm Whiteway, Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Software, Supervision, Validation, Visualization, Writing – original draft, Writing – review and editing.

ADDITIONAL FILES

The following material is available online.

Supplemental Material

Data File S1 (mBio01807-23-s0001.xlsx). All the raw data and bioinformatic analysis. **Figure S1 (mBio01807-23-s0002.tif).** Supplemental information, including the construct for activation of non-zinc cluster transcription factors. **Figure S2 (mBio01807-23-s0003.tif).** Supplemental information, including information on Orf19.2752 and fluconazole, posaconazole, terbinafine, and amphotericin B resistance.
Supplemental legends (mBio01807-23-s0004.docx). Legends for Fig. S1 and S2.

REFERENCES

1. Calderone RA, Fonzi WA. 2001. Virulence factors of *Candida albican*s. Trends Microbiol. 9:327–335. https://doi.org/10.1016/s0966--842x(01)02094-7
2. Arendrup MC, Patterson TF. 2017. Multidrug-resistant *Candida*: epidemiology, molecular mechanisms, and treatment. J Infect Dis 216:S445–S451. https://doi.org/10.1093/infdis/jix131
3. Lopez-Ribot JL, McAtee RK, Lee LN, Kirkpatrick WR, White TC, Sanglard D, Patterson TF. 1998. Distinct patterns of gene expression associated with development of fluconazole resistance in serial *Candida albicans* isolates from human immunodeficiency virus-infected patients with oropharyngeal candidiasis. Antimicrob Agents Chemother 42:2932–2937. https://doi.org/10.1128/AAC.42.11.2932
4. Morschhäuser J. 2002. The genetic basis of fluconazole resistance development in *Candida albicans*. Biochim Biophys Acta Mol Basis Dis 1587:240–248. https://doi.org/10.1016/S0925-4439(02)00087-X
5. Perea S, López-Ribot JL, Kirkpatrick WR, McAtee RK, Santillán RA, Martínez M, Calabrese D, Sanglard D, Patterson TF. 2001. Prevalence of molecular mechanisms of resistance to azole antifungal agents in *Candida albicans* strains displaying high-level fluconazole resistance isolated from human immunodeficiency virus-infected patients. Antimicrob Agents Chemother 45:2676–2684. https://doi.org/10.1128/ AAC.45.10.2676-2684.2001
6. Kelly SL, Arnoldi A, Kelly DE. 1993. Molecular genetic analysis of azole antifungal mode of action. Biochem Soc Trans 21:1034–1038. https://doi.org/10.1042/bst0211034
7. Bhattacharya S, Esquivel BD, White TC. 2018. Overexpression or deletion of ergosterol biosynthesis genes alters doubling time, response to stress agents, and drug susceptibility in *Saccharomyces cerevisiae*. mBio 9:e01291-18. https://doi.org/10.1128/mBio.01291-18
8. Prasad R, Murthy SK, Gupta V, Prasad R. 1995. Multiple drug resistance in *Candida albicans*. Acta Biochim Pol 42:497–504.
9. Prasad R, Singh A. 2013. Lipids of *Candida albicans* and their role in multidrug resistance. Curr Genet 59:243–250. https://doi.org/10.1007/ s00294-013-0402-1
10. Lu H, Shrivastava M, Whiteway M, Jiang Y. 2021. *Candida albicans* targets that potentially synergize with fluconazole. Crit Rev Microbiol 47:323–337. https://doi.org/10.1080/1040841X.2021.1884641
11. Homann OR, Dea J, Noble SM, Johnson AD. 2009. A phenotypic profile of the *Candida albicans* regulatory network. PLoS Genet 5:e1000783. https://doi.org/10.1371/journal.pgen.1000783
12. Nobile CJ, Nett JE, Hernday AD, Homann OR, Deneault J-S, Nantel A,

Andes DR, Johnson AD, Mitchell AP. 2009. Biofilm matrix regulation by *Candida albicans* zap1. PLoS Biol 7:e1000133. https://doi.org/10.1371/journal.pbio.1000133

13. Cowen LE, Nantel A, Whiteway MS, Thomas DY, Tessier DC, Kohn LM, Anderson JB. 2002. Population genomics of drug resistance in *Candida albicans*. Proc Natl Acad Sci U S A 99:9284–9289. https://doi.org/10.1073/ pnas.102291099
14. Liu H. 2001. Transcriptional control of dimorphism in *Candida albicans*. Curr Opin Microbiol 4:728–735. https://doi.org/10.1016/s1369-5274(01)00275-2
15. Tebung WA, Choudhury BI, Tebbji F, Morschhäuser J, Whiteway M. 2016. Rewiring of the Ppr1 zinc cluster transcription factor from purine catabolism to pyrimidine biogenesis in the saccharomycetaceae. Curr Biol 26:1677–1687. https://doi.org/10.1016/j.cub.2016.04.064
16. Erkenbrack EM, Davidson EH. 2015. Evolutionary rewiring of gene regulatory network linkages at divergence of the echinoid subclasses. Proc Natl Acad Sci U S A 112:E4075–E4084. https://doi.org/10.1073/pnas.1509845112
17. Oliveira-Pacheco J, Alves R, Costa-Barbosa A, Cerqueira-Rodrigues B, Pereira-Silva P, Paiva S, Silva S, Henriques M, Pais C, Sampaio P. 2018. The role of *Candida albicans* transcription factor RLM1 in response to carbon adaptation. Front Microbiol 9:1127. https://doi.org/10.3389/fmicb.2018.01127
18. Lavoie H, Hogues H, Mallick J, Sellam A, Nantel A, Whiteway M. 2010. Evolutionary tinkering with conserved components of a transcriptional regulatory network. PLoS Biol 8:e1000329. https://doi.org/10.1371/ journal.pbio.1000329
19. Carvunis A-R, Wang T, Skola D, Yu A, Chen J, Kreisberg JF, Ideker T. 2015. Evidence for a common evolutionary rate in metazoan transcriptional networks. Elife 4:e11615. https://doi.org/10.7554/eLife.11615
20. Johnson AD. 2017. The rewiring of transcription circuits in evolution. Curr Opin Genet Dev 47:121–127. https://doi.org/10.1016/j.gde.2017.09.004
21. Habib N, Wapinski I, Margalit H, Regev A, Friedman N. 2012. A functional selection model explains evolutionary robustness despite plasticity in regulatory networks. Mol Syst Biol 8:619. https://doi.org/10.1038/msb.2012.50
22. Sadowski I, Ma J, Triezenberg S, Ptashne M. 1988. Gal4-Vp16 is an unusually potent transcriptional activator. Nature 335:563–564. https://doi.org/10.1038/335563a0
23. Young ET, Dombek KM, Tachibana C, Ideker T. 2003. Multiple pathways are Co-regulated by the protein kinase Snf1 and the transcription factors Adr1 and Cat8. Journal of Biological Chemistry 278:26146–26158.
24. Ordiz MI, Barbas CF, Beachy RN. 2002. Regulation of transgene expression in plants with polydactyl zinc finger transcription factors. Proc Natl Acad Sci U S A 99:13290–13295. https://doi.org/10.1073/pnas.202471899
25. Maeder ML, Linder SJ, Cascio VM, Fu Y, Ho QH, Joung JK. 2013. CRISPR RNA–guided activation of endogenous human genes. Nat Methods 10:977–979. https://doi.org/10.1038/nmeth.2598
26. Beerli RR, Segal DJ, Dreier B, Barbas CF. 1998. Toward controlling gene expression at will: specific regulation of the erbB-2/HER-2 promoter by using polydactyl zinc finger proteins constructed from modular building blocks. Proc Natl Acad Sci U S A 95:14628–14633. https://doi.org/10.1073/pnas.95.25.14628
27. Denis CL. 1987. The effects of Adr1 and Ccr1 gene dosage on the regulation of the glucose-repressible alcohol dehydrogenase from *Saccharomyces cerevisiae*. Mol Gen Genet 208:101–106. https://doi.org/10.1007/BF00330429
28. Young ET, Kacherovsky N, Van Riper K. 2002. Snf1 protein kinase regulates Adr1 binding to chromatin but not transcription activation. J Biol Chem 277:38095–38103. https://doi.org/10.1074/jbc.M206158200
29. Flowers SA, Colón B, Whaley SG, Schuler MA, Rogers PD. 2015. Contribution of clinically derived mutations in ERG11 to azole resistance in *Candida albicans*. Antimicrob Agents Chemother 59:450–460. https://doi.org/10.1128/AAC.03470-14
30. Xiang M-J, Liu J-Y, Ni P-H, Wang S, Shi C, Wei B, Ni Y-X, Ge H-L. 2013. ERG11 mutations associated with azole resistance in clinical isolates of *Candida albicans*. FEMS Yeast Res 13:386–393. https://doi.org/10.1111/1567-1364.12042
31. Henry KW, Nickels JT, Edlind TD. 2000. Upregulation of ERG genes in *Candida* species by azoles and other sterol biosynthesis inhibitors. Antimicrob Agents Chemother 44:2693–2700. https://doi.org/10.1128/ AAC.44.10.2693-2700.2000
32. Ramírez MA, Lorenz MC. 2009. The transcription factor homolog CTF1 regulates β-oxidation in *Candida albicans.* Eukaryot Cell 8:1604–1614. https://doi.org/10.1128/EC.00206-09
33. Bertram G, Swoboda RK, Gooday GW, Gow NA, Brown AJ. 1996. Structure and regulation of the *Candida albicans* Adh1 gene encoding an immunogenic alcohol dehydrogenase. Yeast 12:115–127. https://doi.org/10.1002/(sici)1097-0061(199602)12:2<115::aid-yea889>3.0.co;2-e
34. Flowers SA, Barker KS, Berkow EL, Toner G, Chadwick SG, Gygax SE, Morschhäuser J, Rogers PD. 2012. Gain-of-function mutations in UPC2 are a frequent cause of ERG11 upregulation in azole-resistant clinical isolates of *Candida albicans*. Eukaryot Cell 11:1289–1299. https://doi.org/10.1128/EC.00215-12
35. Lohberger A, Coste AT, Sanglard D. 2014. Distinct roles of *Candida albicans* drug resistance transcription factors TAC1, MRR1, and Upc2 in virulence. Eukaryot Cell 13:127–142. https://doi.org/10.1128/EC.00245-13
36. Vasicek EM, Berkow EL, Flowers SA, Barker KS, Rogers PD. 2014. Upc2 is universally essential for azole antifungal resistance in *Candida albicans*. Eukaryot Cell 13:933–946. https://doi.org/10.1128/EC.00221-13
37. Chen C-G, Yang Y-L, Shih H-I, Su C-L, Lo H-J. 2004. CaNdt80 is involved in drug resistance in *Candida albicans* by regulating Cdr1. Antimicrob Agents Chemother 48:4505–4512. https://doi.org/10.1128/AAC.48.12.4505-4512.2004
38. Morschhäuser J, Barker KS, Liu TT, BlaB-Warmuth J, Homayouni R, Rogers PD. 2007. The transcription factor Mrr1p controls expression of the Mdr1 efflux pump and mediates multidrug resistance in *Candida albicans*. PLoS Pathog 3:e164. https://doi.org/10.1371/journal.ppat.0030164 39. Chen

C-G, Yang Y-L, Tseng K-Y, Shih H-I, Liou C-H, Lin C-C, Lo H-J. 2009. Rep1p negatively regulating Mdr1 efflux pump involved in drug resistance in *Candida albicans*. Fungal Genet Biol 46:714–720. https:// doi.org/10.1016/j.fgb.2009.06.003

40. Sellam A, Tebbji F, Nantel A. 2009. Role of Ndt80P in Sterol metabolism regulation and azole resistance in Candida albicans. Eukaryotic cell 8:1174–1183.
41. Tachibana C, Yoo JY, Tagne J-B, Kacherovsky N, Lee TI, Young ET. 2005. Combined global localization analysis and transcriptome data identify genes that are directly coregulated by Adr1 and Cat8. Mol Cell Biol 25:2138–2146. https://doi.org/10.1128/MCB.25.6.2138-2146.2005
42. Mesa-Arango AC, Trevijano-Contador N, Román E, Sánchez-Fresneda R, Casas C, Herrero E, Argüelles JC, Pla J, Cuenca-Estrella M, Zaragoza O. 2014. The production of reactive oxygen species is a universal action mechanism of amphotericin B against pathogenic yeasts and contributes to the fungicidal effect of this drug. Antimicrob Agents Chemother 58:6627–6638. https://doi.org/10.1128/AAC.03570-14
43. Tebbji F, Khemiri I, Sellam A. 2020. High-resolution genome-wide occupancy in Candida Spp. using Chec-Seq. Msphere:00646–20.
44. Whiteway M, Tebung WA, Choudhury BI, Rodríguez-Ortiz R. 2015. Metabolic regulation in model Ascomycetes–adjusting similar Genomes to different lifestyles. Trends in Genetics 31:445–453.
45. Yan Y, Liao Z, Shen J, Zhu Z, Cao Y. 2022. Nicotinamide potentiates amphotericin B activity against Candida albicans. Virulence 13:1533– 1542.
46. Yang H, Tong J, Lee CW, Ha S, Eom SH, Im YJ. 2015. Structural mechanism of Ergosterol regulation by fungal Sterol transcription factor Upc2. Nature communications 6:6129.
47. Young ET, Dombek KM, Tachibana C, Ideker T. 2003. Multiple pathways are Co-regulated by the protein kinase Snf1 and the transcription factors Adr1 and Cat8. Journal of Biological Chemistry 278:26146–26158.
48. Lane Set al. 2015. Function and regulation of Cph2 in Candida albicans. Eukaryotic cell 14:1114–1126.
49. Synnott JM, Guida A, Mulhern-Haughey S, Higgins DG, Butler G. 2010. Regulation of the hypoxic response in Candida albicans. Eukaryotic cell 9:1734–1746.
50. Maguire SL, Wang C, Holland LM, Brunel F, Neuveglise C, Nicaud J-M, Zavrel M, White TC, Wolfe KH, Butler G. 2014. Zinc finger transcription factors displaced SREBP proteins as the major Sterol regulators during Saccharomycotina evolution. PLoS genetics
51. Ma M, Liu ZL. 2010. Mechanisms of ethanol tolerance in *Saccharomyces cerevisiae.* Appl Microbiol Biotechnol 87:829–845. https://doi.org/10.1007/s00253-010-2594-3
52. Thukral SK, Eisen A, Young ET. 1991. Two monomers of yeast transcription factor ADRI bind a palindromic sequence symmetrically to activate Adh2 expression. Mol Cell Biol 11:1566–1577. https://doi.org/10.1128/ mcb.11.3.1566-1577.1991
53. Wapinski I, Pfeffer A, Friedman N, Regev A. 2007. Automatic genomewide reconstruction of phylogenetic gene trees. Bioinformatics 23:i549– i558. https://doi.org/10.1093/bioinformatics/btm193
54. Bailey TL, Johnson J, Grant CE, Noble WS. 2015. The MEME suite. Nucleic Acids Res 43:W39–W49. https://doi.org/10.1093/nar/gkv416
55. Vyas VK, Barrasa MI, Fink GR. 2015. A *Candida albicans* CRISPR system permits genetic engineering of essential genes and gene families. Sci Adv 1:e1500248. https://doi.org/10.1126/sciadv.1500248
56. Martin M. 2011. Cutadapt removes adapter sequences from highthroughput sequencing reads. EMBnet J 17:10. https://doi.org/10.14806/ ej.17.1.200
57. Pertea M, Pertea GM, Antonescu CM, Chang T-C, Mendell JT, Salzberg SL. 2015. Stringtie enables improved reconstruction of a transcriptome from RNA-seq reads. Nat Biotechnol 33:290–295. https://doi.org/10.1038/nbt.3122
58. Edgar R, Domrachev M, Lash AE. 2002. Gene expression omnibus: NCBI gene expression and hybridization array data repository. Nucleic Acids Res 30:207–210. https://doi.org/10.1093/nar/30.1.207
59. Breivik ON, Owades JL. 1957. Yeast analysis, spectrophotometric semimicrodetermination of ergosterol in yeast. J Agric Food Chem 5:360–363. https://doi.org/10.1021/jf60075a005