Genomic changes driving the acquisition of multidrug resistance in *Candida glabrata*

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Abstract

Candida glabrata is an important opportunistic human fungal pathogen that represents an important clinical challenge, in part due to its ability to acquire resistance to antifungal drugs. In this thesis, we studied genomic and phenotypic changes occurring in genetically distinct *C. glabrata* strains during adaptation to azoles (fluconazole), echinocandins (anidulafungin), or to different combinations of the two drugs. To this end, we combined an experimental evolution approach with sequencing of target gene regions and whole genomes. Our results revealed that the adaptation to one or the two drugs was mediated by specific patterns of mutations in a narrow set of nine genes and sometimes accompanied with chromosomal changes. The decrease in susceptibility was generally associated with mild fitness costs, which could explain the observed persistence of the resistance phenotype. Importantly, we discovered modifications in genes involved in the ergosterol pathway (a target of azoles) appearing during adaptation to anidulafungin, which resulted in the appearance of cross resistance to fluconazole.

Resumen

Candida glabrata es un importante patógeno fúngico que infecta humanos y que representa un gran desafío clínico, en parte debido a su capacidad para adquirir resistencia a fármacos antimicóticos. En esta tesis, estudiamos los cambios genómicos y fenotípicos que ocurren en cepas genéticamente distintas de *C. glabrata* durante su adaptación a azoles (fluconazol), equinocandinas (anidulafungina) o a diferentes combinaciones de estos dos fármacos. Para ello, combinamos el uso de la evolución experimental con la secuenciación de regiones de genes diana y genomas completos. Nuestros resultados muestran que la adaptación a uno o a los dos fármacos está mediada por patrones mutacionales específicos en un grupo reducido de nueve genes y que, a veces, la adaptación viene acompañada de cambios a nivel cromosómico. La disminución de la susceptibilidad a fármacos está generalmente asociada a detrimentos leves en la eficacia biológica, lo que podría explicar la alta persistencia del fenotipo de resistencia. Es importante destacar que descubrimos que durante la adaptación a anidulafungina aparecieron modificaciones en los genes involucrados en la vía de síntesis del ergosterol (una de las dianas de los azoles), lo que resultó en la aparición de resistencia cruzada al fluconazol

Preface

Life-threatening infections caused by fungal pathogens are too often underestimated as serious threats for global healthcare. Fungal infections can range from common superficial skin rashes to life-threatening invasive mycoses causing the death of around one and a half million people annually (Brown et al. 2012). Systemic mycoses especially affect immunocompromised patients who are often susceptible to fungal infections and whose numbers have been growing in past years (Oren and Paul 2014). One of the main challenges for fighting human fungal diseases relates to the fact that, as eukaryotic organisms, fungi share many similarities with their host cells, which hinders the development of antifungal compounds. As a consequence, there are less than a handful commercially available drug families able to treat fungal infections in humans. Finally, and similarly to the case of antibiotic resistance in bacteria, the number of fungal clinical isolates that are resistant to one or several antimycotics is rapidly increasing, which poses a serious medical concern and brings the urgent need to understand this process. However, as compared to the acquisition of antibiotic resistance in bacteria, our knowledge about how drug resistance emerges and evolves in fungi is lagging behind.

Candida species are among the most frequent human fungal pathogens (Turner and Butler 2014). Although *Candida albicans* is the most common causative agent of candidiasis, the incidence of other species such as *Candida glabrata* is growing (Pfaller et al.

2019). *C. glabrata* is an emerging pathogen species that is only distantly related to *C. albicans* and show important phenotypic differences that result in different virulence mechanisms (Gabaldón and Carreté 2016). Furthermore, *C. glabrata* is particularly problematic as it is intrinsically less susceptible to azoles and easily acquires resistance to azoles or/and echinocandins resulting in medically relevant multidrug resistance.

Whole genome sequencing and comparative genomics are helping us to recognize the existence of a broad genomic variability between and within species as well us to understand how this variability affects important traits such as virulence or drug resistance. By combining in vitro evolution, phenotypic screening and genomic analysis, we are capable of uncovering relationships between genomic changes and important relevant phenotypes. In the frame of this thesis, we set out to unravel resistance-conferring mutations and mutational paths that lead to the acquisition of resistance. Our results provide a foundation to better comprehend the process of emergence of antifungal drug resistance and can contribute to a better clinical management of fungal diseases. A detailed understanding of mechanisms of resistance and discoveries of important contributors to its evolution is essential for developing new diagnostic approaches able to detect the drug resistance and creating effective new therapeutic strategies prepared to prevent and overcome resistance.

Thesis overview

The overall aim of the present thesis was to shed light onto the genomic changes driving the adaptation to antifungal drugs in the emerging pathogen *Candida glabrata* by using an approach that combined experimental evolution with gene and genome sequencing.

The thesis is divided into nine different chapters, which I briefly introduce here:

Chapter 1 is a literature review focused on the evolutionary emergence of drug resistance in *Candida* opportunistic pathogens. It provides information on the current knowledge about the genetic bases and possible evolutionary paths that may lead to the emergence and selection of a resistance phenotype in *Candida* species, as well as a discussion of techniques enabling their study.

Chapter 2 presents the importance of fungal research followed by a detailed introduction to *Candida glabrata.* A particular focus is placed on the comparison of *C. glabrata* with *Saccharomyces cerevisiae* and *Candida albicans,* indicating essential traits related to the focus of this thesis.

Chapter 3 provides an introduction to the methods relevant for the thesis. It involves the description of the antifungal drug susceptibility test, the techniques used to obtain and compare sequences of DNA fragments and entire genomes – i.e. resulting from target and whole genome sequencing, and the genome engineering method, CRISPR-

Cas9, which we used to prove the correlation between evolved mutations and the phenotype.

Chapter 4 outlines the main objectives of the thesis.

Chapter 5 presents the main results and analysis of the in vitro evolution of antifungal drug resistance in *C. glabrata* and the resulting mutants. It includes the analysis of the alterations in susceptibility levels to the two antifungal drugs used in the experiments and of the genomic alterations emerging in the evolved strains.

Chapter 6 provides a deeper analysis of the genetic changes observed after evolution to anidulafungin. The focus is put on the analysis of the *FKS* genes (echinocandin targets), the *CNE1* gene (possibly implicated in the loss of resistance to anidulafungin), as well as of other genes involved in ergosterol biosynthesis, such as *ERG3*.

Chapter 7 reports an investigation of the evolutionary stability of the acquired antifungal drug resistance and the underlying mutations.

Chapter 8 is a summarizing discussion. It includes the discussion of potential benefits of our approach for in vitro evolution and for testing the levels of susceptibility, as well as of the identified mutational changes driving the appearance of antifungal drug resistance in *Candida glabrata*, possible prevention of acquisition of resistance and the clinical implications of our results

Chapter 9 provides the conclusions of the thesis.

Finally, the **Appendix** provides a list of studies in which I have participated during my PhD.

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Part I

Introduction

1 Evolutionary emergence of drug resistance in *Candida* opportunistic pathogens

Ksiezopolska Ewa, and Toni Gabaldón. 2018. "Evolutionary Emergence of Drug Resistance in *Candida* Opportunistic Pathogens." Genes 9 (9). https://doi.org/10.3390/genes9090461.

1.1 Abstract

Fungal infections, such as candidiasis caused by *Candida*, pose a problem of growing medical concern. In developed countries, the incidence of *Candida* infections is increasing due to the higher survival of susceptible populations, such as immunocompromised patients or the elderly. Existing treatment options are limited to few antifungal drug families with efficacies that vary depending on the infecting species. In this context, the emergence and spread of resistant *Candida* isolates are being increasingly reported. Understanding how resistance can evolve within naturally susceptible species is key to developing novel, more effective treatment strategies. However, in contrast to the situation of antibiotic resistance in bacteria, few studies have focused on the evolutionary mechanisms leading to drug resistance in fungal species. In this review, we will survey and discuss current

knowledge on the genetic bases of resistance to antifungal drugs in *Candida* opportunistic pathogens. We will do so from an evolutionary genomics perspective, focusing on the possible evolutionary paths that may lead to the emergence and selection of the resistance phenotype. Finally, we will discuss the potential of future studies enabled by current developments in sequencing technologies, in vitro evolution approaches, and the analysis of serial clinical isolates.

Keywords: *Candida*; antifungal drugs; drug resistance; evolution

1.2 Introduction

From the estimated 1.5 million fungal species, around 300 have been reported to present virulence towards humans, even if sporadically ("Stop Neglecting Fungi" 2017). Fungal pathogens can cause life threatening invasive infections (e.g., fungaemia, meningitis), chronic conditions (e.g., pulmonary aspergillosis, asthma), and recurrent superficial infections (e.g., oral and vaginal candidiasis). Globally, fungi can affect millions of people every year, and the overall death toll has been estimated to be around 1,350,000 deaths per year (Brown et al. 2012). Species belonging to the genera *Candida*, *Aspergillus*, and *Cryptococcus* are the most prevalent cause of invasive infections, with *Candida* being responsible for the most common invasive fungal disease in developed countries—candidiasis (Bassetti et al.2016). Population-based studies have estimated the incidence rate of candidiasis to be two to 14 cases per 100,000 inhabitants, and candidemia (*Candida* bloodstream infection) affects

more than 250,000 persons worldwide every year, leading to more than 50,000 deaths (Kullberg and Arendrup 2015). In addition, candidemia brings a substantial economic burden, involving, on average, three to 13 days of hospitalization in the US, with total associated costs ranging from $6000 to $29,000 (Morgan et al. 2005). A large study of more than 1800 clinical fungal isolates from 31 countries found that 82% of the fungal infections in 2013 were caused by *Candida* (Castanheira et al. 2016). Currently, the effective treatment of candidiasis is limited by two major factors, namely the difficulty of fast and accurate diagnostics of the invasive agent, and the limited number of therapeutic options.

Candidiasis is usually diagnosed late. Firstly, fungal infections are generally considered only after antibiotic treatments fail to reduce fever. Secondly, standard diagnostic approaches require blood cultures, which are slow and can have a low sensitivity. For instance, some studies reported sensitivities as low as 17% (Nguyen et al. 2012) or 45% (Fortún et al. 2014). Furthermore, although the four most common *Candida* (*C. albicans*, *C. glabrata*, *C. parapsilosis*, and *C. tropicalis*) can account for more than 80% of the cases, there is a long list with over 30 *Candida* that have been identified as candidemia agents (Fortún et al. 2014; Gabaldón, et al. 2016), and the list keeps expanding. Added to the difficulty of a fast and accurate diagnosis, doctors face severe limitations with regards to treatment options. Currently, there are only four major classes of antifungals in clinical use: azoles, polyenes, echinocandins, and pyrimidine analogs (Odds et al. 2003). This situation alarmingly decreases the chances of a successful treatment and increases the possibilities of a fatal

outcome if the infecting pathogen is resistant to one or multiple drugs. Limitations in diagnostic methods further enhance the problems of a few therapeutic options, as different species may show diverse resistance profiles. Thus, diagnostics of the infecting agent, along with susceptibility tests, should be used to inform the choice of therapy (discussed below). Over the last years, the intensive use of some antifungal drugs, such as azoles, has promoted a shift in the epidemiology of candidiasis, in which the incidence of *C. albicans* has decreased in favor of other species that are naturally less susceptible to this drug, such as *C. glabrata*.

To the problem of the intrinsic variation of drug susceptibility among different *Candida*, we need to add the emerging issue of acquired resistance, which refers to the ability of yeasts to evolutionarily develop mechanisms that lower their susceptibility towards a given drug (Fraimow and Abrutyn 1995). This process generally involves mutations ranging from chromosomal re-arrangements to point mutations. These mutations can affect drug resistance in different ways, ranging from directly interfering with the binding of the drug to its target to inducing gene expression changes that promote physiological states that reduce drug susceptibility. In this regard, an enhanced capacity to form biofilms can result in the acquisition of resistance, as these structures promote yeast survival upon exposure to the drug (Sardi et al. 2013; Rodrigues et al. 2017).

The emergence of resistant strains, including those becoming resistance to multiple drugs, has been increasingly reported in recent years (Pfaller et al. 2014, 2013; Lockhart et al. 2017). In addition, it

has been demonstrated that such resistance phenotypes can develop over the course of an infection, and in response to treatment, which adds yet another threat to patients (Pfaller 2012).

Despite the clinical and economic relevance of drug resistance in the context of yeast infections, this subject remains poorly studied, at least in comparison with the similar issue of antibiotic resistance in bacterial pathogens. Although parallels can be established, the evolutionary mechanisms underlying the emergence of resistance in fungi and bacteria are markedly different. While drug resistance in bacteria generally involves the transference, between strains or species, of genetically mobile elements such as genomic islands (Mohammad 2014), in fungi, resistance commonly appears via genetic alterations within a lineage. Still, we are far from having a broad understanding of how resistance towards antifungal drugs emerges in the context of infection or commensalism in yeast pathogens. Fortunately, recent developments in sequencing technologies are enabling us to catalog and trace the origins of mutations conferring resistance to antifungal drugs in different species. In this review, we aim to summarize our current knowledge on how drug resistance is genetically determined in *Candida* opportunistic pathogens, and how it can be acquired in the course of evolution. In doing so, we will focus on how the advent of genomics technologies is allowing us to study these processes on unprecedented levels of scale and resolution, and how possible future studies could help us further our understanding of the evolutionary emergence of drug resistance in yeasts.

1.3 Major Antifungal Drugs and Their Mechanisms of Actions

The development of new antifungal drugs is challenging, as fungi are eukaryotic organisms that share many basic cellular processes with us. This evolutionary relatedness makes the finding of specific targets difficult and increases the likelihood of undesired secondary effects. Existing antimycotic drugs target processes that are highly divergent between fungi and the human host, such as the ergosterol synthesis pathway. Here, we will briefly summarize the main mechanisms of action of the major antifungal drug classes (**Table 1.1, Figure 1.1A**).

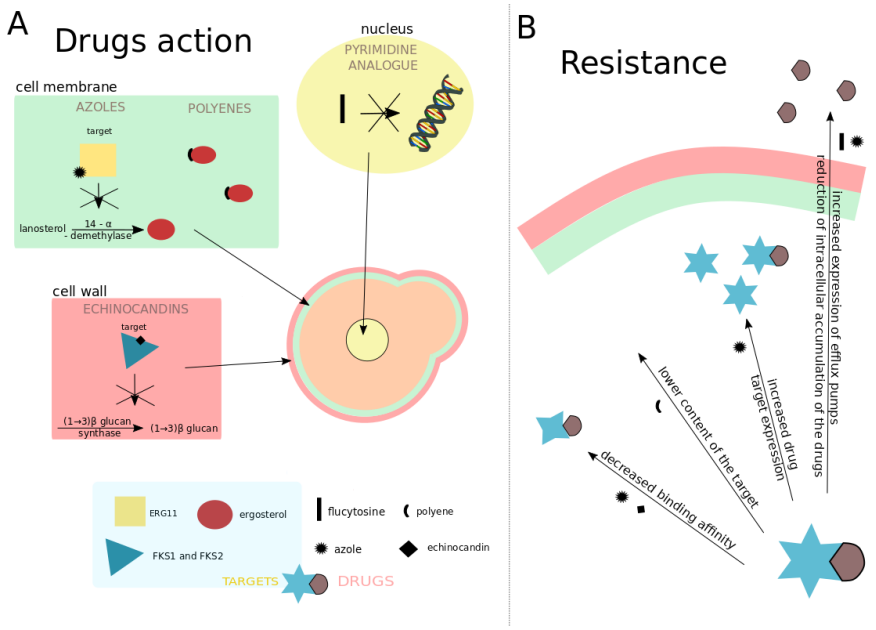


Figure 1.1. Antifungal drug actions and resistance mechanisms in *Candida*. (**A**)—action mechanisms of azoles, polyenes, echinocandins, and the pyrimidine analog in different parts of the cell. Colored shapes indicate target enzymes or molecules, with the name of the coding gene or the molecule, respectively, indicated in the light blue box at the bottom. Black shapes indicate different drug classes and a pyrimidine analog, flucytosine, with their correspondence indicated in the light brown box at the bottom. Mechanisms of actions are schematically indicated (see text) with colors and arrows indicating the main cellular location of the effect of the drug. (**B**)—most common resistance mechanisms caused by mutations. Targets are generically represented by blue stars and drugs by a brown shape. Different mechanisms causing resistance are indicated by arrows with light orange boxes indicating types of drugs for which this mechanism has been observed. Drug shapes are as in **A**.

Table 1.1. Modes of action of common antifungal drugs. Columns indicate, in this order: major classes of antifungal drug; drugs in clinical use; modes of action.

Antifungal drug class	Drugs	Mode of action
Azoles	Fluconazole	Inhibitor of lanosterol 14α – demethylase
	Voriconazole	
	Posaconazole	
	Itraconazole	
	Ketokonazole	
	Clotrimazole	
	Econazole	
	Miconazole	
Echinocandins	Caspofungin	Inhibitor of 1,3–β–glucan synthase
	Anidulafungin	
	Micafungin	
Polyenes	Amphotericin B	Binding ergosterol
	Nystatin	
Pyrimidine analogue	5 – Flucytosine	Inhibitor of DNA/RNA synthesis

Azoles are heterocyclic compounds containing at least one nitrogen atom as part of the ring. Common azoles used as antimycotic agents include the triazoles: fluconazole, voriconazole, and posaconazole. These drugs act by targeting the cytochrome P450 enzyme-lanosterol 14α-demethylase, that converts lanosterol to ergosterol. In yeast, this enzyme is encoded by the *ERG11* gene. Similar to cholesterol in animals, ergosterol is the main membrane sterol in most fungal species, holding an important role in controlling membrane fluidity (Weete et al. 2010). As a result of the action of azoles, the *Candida* cell membrane is depleted of ergosterol and accumulates other toxic 14α-methylated sterols. Subsequently, this causes the decrease in membrane fluidity and, in most of the cases, inhibits cell growth (Sheehan et al. 1999). Fluconazole is the azole drug most widely used for the treatment of *Candida* infections. Its utility is attributed to its high bioavailability, high water solubility, and low affinity to plasma proteins (Andriole 2000). Unfortunately, the fungistatic character of

fluconazole and its extended, perhaps excessive, use is inevitably leading to an increasing selection in favor of resistant yeast isolates.

Echinocandins are amphiphilic lipopeptides, and products of cyclopentamine. They can be formed during the fermentation of some fungi such as *Zalerion arboricola* or *Aspergillus nidulans* var. *echinulatus*, but nowadays, they are produced semi-synthetically for clinical use. The most common representatives of this class of drugs are: caspofungin, micafungin, and anidulafungin. Echinocandins inhibit the biosynthesis of an essential component of the fungal cell wall, the 1,3-ß-glucan. In *Candida*, they target two subunits of the 1,3-ß-glucan synthase, encoded by the *FKS1* and *FKS2* genes (Sucher et al. 2009), and eventually cause cell lysis. The fungicidal character against most *Candida*, their target not being present in mammalian cells, the lack of clinically significant drug-drug interactions, and the absence of adverse effects make this antifungal drug class considerably attractive for the treatment of fungal infections. Echinocandins were approved for medical use in 2002 and they are applied as a first line antifungal drug along with fluconazole. Due to their safety profile, better outcomes, and the emergence of azole-resistant species, echinocandins are currently the preferred agents for most episodes of candidemia and invasive candidiasis, with the exception of those affecting the central nervous system, the eye, and the urinary tract (Pappas et al. 2016).

Polyenes are poly-unsaturated organic compounds that contain at least three alternating double and single carbon–carbon bonds. Their antimycotic action is mediated by direct binding to and removal of

ergosterol present in the fungal cell membrane. This results in the loss of membrane permeability, subsequent membrane leakage, and eventually cell death (Kathiravan et al. 2012). In the 1950s, the polyene amphotericin B deoxycholate was the first approved successful antifungal drug (Perfect 2017). Nowadays, amphotericin B continues to be broadly used despite its high toxicity, which results from structural similarities between ergosterol and human cholesterol. Due to this toxicity, the use of amphotericin B in high concentrations may be harmful and cause damage to human tissues, such as the kidneys (Groll et al.1998).

Pyrimidine analogs are nucleosides that mimic the structure of natural pyrimidines. The only pyrimidine analog with antimycotic properties currently in use for human treatment is flucytosine, which has the potential to convert into 5-fluorouracyl and further to 5-fluorodeoxyuridine inside the fungal cell (Chandra et al. 2009). Subsequently, 5-fluorodeoxyuridine interferes with DNA, RNA, and protein synthesis. The transformation of flucytosine into 5-fluorouracyl is catalyzed by the action of the fungal enzyme cytosine deaminase (encoded by the yeast gene *FCY1*), which is not present in humans. Although the most effective and safest antimycotic in the health system (WHO 2015), it is not used in monotherapy due to the rapid development of resistance towards this drug (Kathiravan et al. 2012; Charlier et al. 2016).

1.4 Natural Susceptibility to Antifungals among *Candida*

Out of the 30 different species of *Candida* able to infect humans, *C. albicans*, *C. glabrata*, *C. parapsilosis*, and *C. tropicalis*, generally in this order, account for up to 80% of candidiasis cases. Although infections with *C. albicans* are still the most common, epidemiology is shifting towards non-albicans *Candida*, wherein the specific relative incidences are being time- and space-dependent (Quindós 2014) (for detailed geographical variation see (Pappas et al. 2018). When highlighted on a phylogenetic tree, *Candida* opportunistic pathogens belong to distinct lineages, which are interspersed with non-pathogenic relatives (Gabaldón et al. 2016) This implies that the ability to infect humans emerged several independent times during evolution. As a consequence, different *Candida* may use different mechanisms for evasion of the host immune system and exhibit different virulence-related phenotypes (Silva et al. 2012). Accordingly, various *Candida* present distinct susceptibility profiles towards antifungal drugs and different trajectories to acquire resistance when exposed to antifungals. Here, we will briefly survey known antifungal susceptibility characteristics of the main *Candida* pathogens.

How microorganisms respond to a drug is assessed experimentally by means of susceptibility tests. Levels of susceptibilities are indicated by the minimum inhibitory concentration (MIC), which is defined as the lowest concentration of the tested compound at which 50% (MIC_{50}), 90% (MIC_{90}), or complete growth inhibition of the

microorganism is observed. Susceptibility tests are commonly used in epidemiological studies, in studies comparing in vitro activities of existing and new antimycotic drugs, in guiding therapy strategy, and in monitoring the emergence of resistance.

Epidemiological studies, performed on globally sampled clinical isolates, reveal differential susceptibility patterns among *Candida*. They indicate how frequently isolates of a species are resistant to different drugs, which reflects intrinsic characteristics of the species (**Table 1.2**). For instance, *C. glabrata* and *C. krusei* have a naturally low susceptibility to azoles, while *C. parapsilosis* strains tend to have a lower susceptibility to echinocandins (Sanguinetti et al. 2015; Maiken et al. 2014). There is a growing number of rarely occurring *Candida* being reported to have lower susceptibilities to one or several drugs. Species naturally more tolerant to azoles include the above mentioned *C. glabrata* and *C. krusei*, as well as a long list of less common species such as *C. ciferrii*, *C. guilliermondii*, *C. inconspicua*, *C. humicola*, *C. lambica*, *C. lipolytica*, *C. norvegensis*, *C. palmioleophila*, *C. rugosa*, and *C. valida*. Among the species more tolerant to echinocandins, besides *C. parapsilosis*, we can find *C. orthopsilosis*, *C. metapsilosis*, *C. guilliermondii*, *C. lipolytica*, and *C. fermentati* (Sanguinetti et al. 2015; Maiken et al. 2014; Garcia-Effron et al. 2008). Finally, *C. lusitaniae*, *C. guilliermondii*, *C. glabrata*, and *C. krusei* have a generally lower susceptibility to polyenes (Spampinato and Leonardi 2013; Rex et al. 2000). Importantly, an intrinsic multidrug resistant *Candida auris* has been recently reported as an emerging cause of healthcare-associated infections worldwide in at least a dozen countries on four continents during 2009–2015

(Vallabhaneni et al. 2017). Infections caused by this species can have high mortality rates ranging from 30–60% (Chowdhary et al. 2016). Very often, strains of this emerging species are resistant to the three major drug classes: polyenes, azoles, and echinocandins. Indeed, up to 96% of *C. auris* may exhibit resistance to fluconazole, an exceptionally high value compared to 0.5–2% for *C. albicans*, 4–9% for *C. tropicalis*, 2–6% for *C. parapsilosis*, and 11–13% for *C. glabrata* (Lockhart et al. 2017; Cleveland et al. 2012; Pfaller et al. 2015). For these reasons, *C. auris* has been highlighted by the American and European centers for disease control (CDC and ECDC) as a cause of major concern.

Table 1.2. Intrinsic susceptibility patterns in *Candida* and *Saccharomyces cerevisiae*. Letters indicate susceptibility categories based on EUCAST (European Committee on Antimicrobial Susceptibility Testing) breakpoints: S—Susceptible, I—Intermediate, R—Resistant. In the absence of an established breakpoint, X indicates species with elevated minimum inhibitory concentrations (MICs) compared with *Candida albicans*. The four most common *Candida* are indicated in bold. (adapted from (Arendrup 2014)).

	Fluconazole	Echinocandins	Amphotericin B
C. albicans	S	S	S
C. auris	X	X	X
C. cifferrii	X		
C. dubliniensis	S	S	S
C. duobushaemulonii	X	X	X
C. fermentati		X	
C. glabrata	I	S	S
C. guilliermondii	X	X	
C. haemulonii	X	X	X
C. humicula	X		
C. inconspicua	X		
C. krusei	R	S	S
C. lambica	X		
C. lipolytica	X	X	
C. lusitaniae			X
C. metapsilosis		X	
C. norvegensis	X		
C. orthopsilosis		X	
C. palmioleophila	X		
C. parapsilosis	S	I	S
C. pseudohaemulonii	X	X	X
C. rugosa	X		
C. tropicalis	S	S	S
C. valida	X		
S. cerevisiae	X		

1.5 Epidemiological Studies Report Increasing Levels of Resistance

Worryingly, the picture of resistance levels across *Candida* isolates is not a static one. Rather, epidemiological studies are showing a steady rise in the amount of reported resistant isolates, even among naturally susceptible species. For example, an increase in fluconazole resistance in naturally susceptible species such as *C. parapsilosis*, *C. guilliermondii*, *C. lusitaniae*, *C. sake*, and *C. pelliculosa* was observed in a population-based surveillance programme comprising more than 250,000 *Candida* strains isolated between 1997 and 2007 (Pfaller et al. 2010). Often, for naturally susceptible species, both the relative amount of resistant strains and the overall MIC levels in clinical isolates increase after the continuous use of a given antifungal drug (Lortholary et al. 2011). Furthermore, the acquisition of resistance towards one drug in species that are intrinsically resistant to another one is not uncommon and leads to dangerous multidrug resistance (MDR). An example of this would be the acquisition of resistance to echinocandins by species like *C. glabrata* or *C. krusei*, which already exhibit a lower natural susceptibility towards azoles. The increased use of antifungals during the last 15 years correlates with an alarming development of MDR, especially in *C. glabrata* (Farmakiotis et al. 2014). For example, a large study assessing more than 1300 isolates from 80 USA hospitals indicated that 32.9% of the *C. glabrata* isolates classified as non-susceptible to echinocandins were also resistant to fluconazole, and that, overall, 1.7% of the strains presented MDR (Vallabhaneni et al. 2015).

Similarly, the CDC/SENTRY antimicrobial surveillance program reported a rise from 0 to 11% in the fraction of fluconazole-resistant strains that were also less susceptible to echinocandins between the studies performed in 2001–2004 and 2006–2010 (Pfaller et al. 2012). Other studies on *C. glabrata* report that 14% of fluconazole-resistant strains exhibit resistance to at least one echinocandin and a total of 3.5% of MDR cases were noted in Duke University hospital (Alexander et al. 2013), 7% of MDR at MD Anderson Cancer Center (Farmakiotis et al. 2014), and a resistance to azoles in 36% echinocandin-resistant strains was indicated in a five-year surveillance study in the USA (Pham et al. 2014a). Importantly, instances of cross-resistance towards amphotericin B and azoles or echinocandins in *Candida* have also been reported (Krogh-Madsen et al. 2006; Martel et al. 2010; Eddouzi et al. 2013; Forastiero et al. 2013).

Other studies have shown that while the fraction of *C. glabrata* infecting strains resistant to caspofungin in the United States is significant (10%) (Farmakiotis et al. 2014), in Europe, it is much lower, with 0% reported in studies performed in Italy and Spain (Bassetti et al. 2013); 2.1% in Lombardy, Italy (Tortorano et al. 2013); and 2% in Turkey (Kiraz et al. 2010). These differences in distribution of the resistant *Candida* may result from regional differences in either species or strain distributions or in antifungal use and prophylaxis protocols. MDR in species with no intrinsic tolerance to drugs is rare, probably because it requires multiple steps, each associated with a fitness cost. However, MDR is not restricted to *C. glabrata* or *C. auris*. Other examples include *C. kefyr* (Fekkar

et al. 2013), *C. lusitaniae* (Asner et al. 2015), and *C. albicans* (Morio et al. 2012; Jensen et al. 2015; Martel et al. 2010). Hence, the threat is real, and instances of increasing occurrence, natural resistance, and ease in acquisition of resistance should raise much more awareness. Azoles and echinocandins are the two most used antifungal drugs in hospitals, and the emergence of combined resistance to both of them severely hampers our ability to treat fungal infections.

1.6 Mutations Leading to Secondary Acquisition of Resistance

High genomic plasticity is one of the characteristics of *Candida* yeasts that enables their fast adaptation to varying environments (Anderson and Bennett 2016; Selmecki et al. 2010; Carreté et al. 2018). Upon exposure to drugs, the yeast cell population is subjected to a strong selection towards the subset of cells that can better adapt to the stressing conditions (Henry et al. 2000). Eventually, this selection pressure can lead to the increase in frequencies of mutant alleles that confer enhanced resistance to the administered drug, resulting in a population not responding to the treatment anymore. This can occur during long hospitalization periods and prolonged treatments (Ben-Ami 2018). Besides the overall use and exposure to antifungals (often used in prophylactic measures), other factors that promote the acquisition of resistance and a treatment failure include the use of sub-therapeutic concentrations, drug sequestration in the biofilm matrix, and poor control of infections (Morio et al. 2017; White et al. 1998). Mechanisms of acquired resistance mostly fall into two classes (**Figure 1.1B**): (i) mutations leading to increased

expression of the target or the alteration of its binding affinity towards the drug; and (ii) mutations leading to reduced intracellular accumulation of the drug by means of increasing the activity or expression of drug efflux pumps or, conversely, reducing the import of the drug (Shapiro et al. 2011). Below, we survey the current knowledge on known mechanisms of resistance towards the main classes of antimycotics (**Table 1.3, Figure 1.1B**).

Table 1.3. Genetic bases of resistance towards common antifungal drugs. Columns indicate, in this order: drug class, mode of resistance, genes involved, species for which this resistance mode has been found (with four major pathogenic species in bold), and comments.

Antifungal drug class	Mode of resistance	Gene	Species	Comments
Azoles	drug target overexpression → increased concentration of lanosterol 14α – demethylase	*ERG11*	*C. albicans* *C. parapsilosis* *C. tropicalis* *C. krusei*	overexpression regulated by *UPC2*
	drug target alteration → decreased lanosterol 14α – demethylase binding affinity for the drug	*ERG11*	*C. albicans* *C. parapsilosis* *C. tropicalis* *C. krusei* *C. auris*	
	aneuploidy	*ERG11, UPC2, TAC1*	*C. albicans*	
	loss of heterozygosity	*ERG11, TAC1* , *MRR1*	*C. albicans*	
	drug counteraction → inactivation of C5 sterol desaturase leading to alterations in the ergosterol synthetic pathway →reduction of ergosterol and accumulation of other sterols	*ERG3*	*C. albicans*	
	overexpression of drug transporter (efflux pumps)	*CDR1, CDR2, SNQ2, ABC1*	*C. albicans* *C. parapsilosis* *C. tropicalis* *C. krusei* *C. glabrata*	ATP binding cassete (ABC transporter), regulated by *TAC1, PDR1*
		MDR1, TPO3	*C. albicans* *C. parapsilosis* *C. tropicalis* *C. glabrata*	Major facilitator family (MFS transporter), regulated by *MRR1*
Echinocandins	drug target alteration→ decreased glucan synthase processivity for the drug	*FKS 1*	see Table 1.4	
		FKS 2		
Polyenes	point alteration→ decreased ergosterol content in cells	*ERG3*	*C. albicans*	cross resistance to azoles
		ERG6	*C. glabrata*	
Pyrimidine analogue	point alteration→ inactivation of cytosine permease affecting drug uptake	*FCY2*	*C. lustinae* *C. glabrata*	
	point alteration→ inactivation of cytosine deaminase leasing to alterations in the metabolism of 5-fluorocytosine	*FCY1*	*C. glabrata*	
	point alteration→ inactivation of uracyl phosphoribosyl transferase leading to alterations in the metabolism of 5-fluorocytosine	*FUR1*	*C. albicans*	

Resistance towards azoles can involve various mechanisms, namely: (i) changes in the biosynthesis of sterols, resulting in their substitution for ergosterol; (ii) overexpression of the target enzyme, leading to sufficient levels of activity in the presence of the antifungal drug; (iii) overexpression of drug efflux pumps that diminish the

intracellular concentration of the drug; and (iv) changes in the target gene sequence, leading to the reduction in the binding affinity of the protein to the drug (Shapiro et al. 2011; Lupetti et al. 2002). Acquired resistance to this group of antimycotics seems to be a result of mutations selected by the pressure exerted by the drug (Rodrigues et al. 2017). The adaptation is said to appear gradually during continuous contact with the antifungal (Rodrigues et al. 2017). In *C. albicans*, acquisition of resistance is often related to point mutations in the *ERG11* gene, encoding the enzyme targeted by azoles (Xiang et al. 2013; Flowers et al. 2015). Out of 140 different point mutations described for this gene, 21 have been directly associated with fluconazole resistance (Berkow and Lockhart 2017). Additionally, inactivation of the protein encoded by the *ERG3* gene has also been found to confer azole resistance (Morio et al. 2012). Furthermore, *ERG3* mutations result in the reduction of ergosterol and accumulation of other sterols, often leading to cross-resistance to polyenes (Cowen et al. 2014). Other factors contributing to decreased susceptibility to azoles in *C. albicans* involve the increased expression of *ERG11* due to activating mutations in the gene encoding its zinc-finger transcriptional regulator *UPC2* (MacPherson et al. 2005); overexpression of the drug efflux pumps, including multidrug resistance gene *MDR1* (controlled by the transcription factor *MRR1*) (Morschhäuser et al. 2007); or Candida drug resistance 1 and Candida resistance 2 (*CDR1/CDR2*) genes. Importantly, the deletion of either of the *CDR1/2* genes leads to the loss of the resistance phenotype (Tsao et al. 2009), and the upregulation of these pumps can be attributed to at least 17 different mutations in their

transcriptional regulator *TAC1* (Siikala et al. 2010; Coste et al. 2009). Finally, gross genomic changes such as aneuploidy or the loss of heterozygosity have also been associated with increased azole resistance in *C. albicans*. For instance, aneuploidy in chromosome 5, containing *ERG11*, its transcriptional regulator *UPC2*, and the efflux pump regulator *TAC1*, results in altered susceptibilities (Coste et al. 2006), as is also the case for the loss of heterozygosity in regions encoding *ERG11*, *TAC1*, or *MRR1* (Coste et al. 2006; Selmecki et al. 2006). Another recent study added elevated copy numbers of chromosomes 3 and 6 to the list of genome rearrangements associated with fluconazole resistance in *C. albicans* (Hirakawa et al. 2017).

Resistance to azoles in *C. parapsilosis* has been attributed to mutations in the transcription factor gene *MRR1* (Zhang et al. 2015) and *ERG11* (Y132F, either alone or in combination with an R398I) (Souza et al. 2015; Berkow et al. 2015; Grossman et al. 2015) , and overexpression of *CDR1*, *MDR1*, and *ERG11* (Souza et al. 2015; Berkow et al. 2015; Silva et al. 2011). However, alternative or additional mechanisms for azole resistance may await discovery in *C. parapsilosis* (Berkow et al. 2015). For *C. tropicalis*, point mutation (again Y132F) (Tan et al. 2015), overexpression (Jiang et al. 2013), and deletion mutations in *ERG11* (Eddouzi et al. 2013) have been described as causes for azole resistance. In addition, in vitro induced resistance unveiled the presence of increased expression of multidrug transporter genes of two different families, the ABC transporters and the major facilitators, *CDR1* and *MDR1*, respectively. Yet, there is no conclusive proof that these mechanisms are acting in clinical isolates (Barchiesi et al. 2000). The main

mechanism of resistance to azoles in *C. krusei* appears to be the reduced susceptibility of 14α-demethylase to fluconazole (Orozco et al. 1998). However, overexpression of *ERG11* (Tavakoli et al. 2010) and the ABC transporter, *ABC1*, has also been related to fluconazole resistance in this species (Lamping et al. 2009). In addition, reduced susceptibility to other types of azoles has also been linked to point mutations in *ERG11* (Silva et al. 2016). For *C. auris*, little is known on the precise contribution of *ERG11* mutations to fluconazole resistance. Nevertheless, some geographically distinct clades with reduced sensitivity seem to carry mutations in this gene (e.g., Y132F, K143R and F126T), which has been implicated in reduced azole susceptibility in other species (Lockhart et al. 2017). So far, there is no information on the altered expression of efflux pumps being connected with resistance in this microorganism.

In contrast to other species, azole resistance in *C. glabrata* is generally not associated with alteration in *ERG11* (Sanguinetti et al. 2015; Sanglard et al. 1999; Carreté et al. 2018; Vermitsky and Edlind 2004), but rather with mutations in the *PDR1* transcription factor, which cause the differential expression of downstream targets (Vermitsky and Edlind 2004). *PDR1* belongs to a pleiotropic-drug resistance (PDR) network of regulators responsible for the transcriptional upregulation of genes encoding drug efflux pumps, such as the *CDR1*, *CDR2*, and *SNQ2* (Vermitsky and Edlind 2004; Kołaczkowska and Kołaczkowski 2016). Alterations in this transcription factor have been described as the main mechanism for the enhancement of azole resistance in *C. glabrata*, with efflux pumps often induced during azole therapy (Tumbarello et al. 2008).

Another possible resistance mechanism in *C. glabrata* may involve the major facilitator superfamily (MFS) transporter, *TPO3*, as its depletion results in increased sensitivity to fluconazole and clotrimazole (O'Brien et al. 1989). Alternative mechanisms for azole resistance in *C. glabrata* involve 'petite mutants', which are characterized by a lack of mitochondrial DNA and mitochondrial dysfunction, and which also show upregulation of ABC transporter genes, improved fitness, and increased resistance towards azoles (Ferrari et al. 2011). Furthermore, mutations in 27 genes involved in transport (*PDR5* and *PDR16*), retrograde signaling (RT2), RNA polymerase II transcription, calcium homeostasis, ribosomal biogenesis, mitochondrial function, and cell wall signaling have been suggested to confer fluconazole resistance in *C. glabrata* (Kaur et al. 2004). Another study included calcium signaling as essential for the survival of azole treatment and its absence has the potential to change the character of fluconazole from fungistatic to fungicidal (Miyazaki et al. 2010). However, there may still be alternative routes to the acquisition of resistance in *C. glabrata*, as there have been at least 78 genes suggested to be implicated in *C. glabrata* resistance to fluconazole and voriconazole (Salazar et al. 2018).

Acquisition of resistance towards echinocandins is not as common as towards azoles, yet it is far from rare and is significantly linked with prior exposure to the drugs (Vallabhaneni et al. 2015). In *C. glabrata*, this phenomenon has increased from 2–3% to more than 13% in a 10-year period (Alexander et al. 2013) and can be present in up to one-third of isolates in the US (Vallabhaneni et al. 2015; Pham et al. 2014a). *Candida* can evade the activity of echinocandins by

mutations in particular regions (called hotspots) in the *FKS1* gene and in the case of *C. glabrata*, as well in *FKS2* (**Table 1.4**) (Arendrup and Perlin 2014; Garcia-Effron et al. 2008). Overall mutations in the target genes result in the reduction of the binding affinity of the antifungal drug (Balashov et al. 2006). Notably, many of these resistance-causing sequence variations are constitutive in species showing a higher intrinsic tolerance towards echinocandins (e.g., *C. parapsilosis*, *C. orthopsilosis*, *C. metapsilosis*, *C. guilliermondii*, and *C. lipolytica*) (Arendrup and Perlin 2014; Garcia-Effron et al. 2008). It has been suggested that these *FKS* polymorphisms reduce the affinity to echinocandins of the glucan synthase by two to three orders of magnitude compared to the wild-type enzyme (Castanheira et al. 2010; Garcia-Effron et al. 2009). What is more, the degree of susceptibility towards echinocandins depends on the position and specificity of the mutation (Arendrup and Perlin 2014). For example, in *C. albicans*, amino acid substitutions S641P and S645Y in *FKS1* and in *C. glabrata* S629P in *FKS1*, S663P, and F659S in *FKS2* are associated with reduced activity of the drug and much higher MICs, whereas F559Y in *FKS2* in *C. glabrata* reduces susceptibility to a lesser degree (Arendrup 2014; Garcia-Effron et al. 2009; Castanheira et al. 2014). Additionally, in *C. glabrata*, the expression of the *FKS2* gene has been shown to be calcineurin-dependent, and the resistance phenotype can be reversed upon the application of calcineurin inhibitors such as FK506 (Katiyar et al. 2012). Altered susceptibility to echinocandins is also connected with stress responses that result in paradoxical growth of the microorganism at high concentrations of

the drugs and elevated cell wall chitin content (Huang et al. 2016; Walker et al. 2010).

Table 1.4. Point mutations in hotspots of *FKS1* and *FKS2* genes connected with resistance towards echinocandins in *Candida* and *Saccharomyces cerevisiae*. Columns indicate, in this order: organism, with the four major pathogens indicated in bold; if applicable, intrinsically lower susceptibility (X); and for *FKS1* and *FKS2* hotspots, respectively, the starting amino acid position and the sequences of interest. One letter codes are used for the amino acid sequence, with colors pointing to sites that are mutated. Mutations are marked: red as strong, orange as weak, green as silently acquired or naturally occurring, blue as naturally intrinsic proven or possibly related to the intrinsic lower susceptibility, and violet as naturally occurring of unknown impact. Further, * indicates the codon involving a mutation or deletion and ** codon involving a mutation or a stop codon (adapted from: (Arendrup and Perlin 2014)).

organism		*FKS1*				*FKS2*			
		starting AA	HOT SPOT 1 AA	starting AA	HOT SPOT 2	starting AA	HOT SPOT 1 AA	starting AA	HOT SPOT 2 AA
C. albicans		641	FLTLSLRDP	1357	DWIRRYTL				
C. dubliniensis		641	FLTLSLRDP	1357	DWIRRYTL				
C. glabrata		625	FLILSLRDP	inaccurate	DWVRRYTL	659	F*LILSLRDP	1374	DWIR**RYTL
C. kefyr		inaccurate	F*LTLSLRDP	inaccurate	DWVRRYTL				
C. krusei		655	FLILSIRDP	1364	DWIRRYTL				
C. lusitaniae		inaccurate	FLTLSLRDP	inaccurate	DWIRRYTL				
C. tropicalis		inaccurate	FLTLSLRDP	inaccurate	DWIRRYTL				
C. parapsilosis	X	652	FLTLSLRDA	1369	DWIRRYTL				
C. metapsilosis	X	inaccurate	FLTLSLRDA	inaccurate	DWIRRYTL				
C. orthopsilosis	X	inaccurate	FLTLSLRDA	inaccurate	DWVRRYTL				
C. guiliermondii	X	632	FMALSLRDP	1347	DWIRRYTL				
C. lypolytica	X	662	FLILSLRDP	1387	DWIRRCVL				
S. cerevisae		639	FLVLSLRDP	1353	DWVRRYTL	658	FLILSLRDP	1372	DWVRRYTL

Resistance to amphotericin B in *Candida* is still rare. When it occurs, it is generally connected with a decrease in the levels of ergosterol in the cell membrane. Lower abundance of the enzyme has been observed in polyene-resistant species, which has been attributed to mutations in *ERG2* (Jensen et al. 2015; Hull et al. 2012), *ERG3* (Martel et al. 2010), *ERG5* (Martel et al. 2010), *ERG6* (Vandeputte et al. 2008), and *ERG11* (Martel et al. 2010) genes, which encode enzymes involved in ergosterol synthesis. Decreased susceptibility towards flucytosine has been associated with point mutations in *FCY1*, *FCY2*, and *FUR1* genes and the deletion of *FPS1* and *FPS2*

genes (Spampinato and Leonardi 2013; Hope et al. 2004; Chapeland-Leclerc et al. 2005; Vandeputte et al. 2011; Costa et al. 2015). Changes in *FCY2* interfere with the drug uptake and alterations in *FCY1* and *FUR1* inactivate enzymes involved in the pyrimidine pathway, while the absence of *FPS1* and *FPS2* reduced the accumulation of the drug in the cell. Such resistance mechanisms have been observed in *C. albicans* (Hope et al. 2004), *C. lusitaniae* (Chapeland-Leclerc et al. 2005), and *C. glabrata* (Vandeputte et al. 2011; Costa et al. 2015).

Despite the many described mutations conferring resistance to azoles or echinocandins in *Candida*, the list of possible mutations conferring resistance is probably not exhausted. Several observations suggest that unknown mechanisms remain to be discovered. For example, overexpression of the azole target gene *ERG11* has not always been associated with point mutations in its *UPC2* regulator (Berkow et al. 2015; Jiang et al. 2013; Flowers et al. 2012) , suggesting that other regulators may play a role. We also have little knowledge on the ability of the cell to uptake and transport the drugs to their targets, and so far undiscovered mutations might modulate these processes. Finally, resistant strains with no known resistance-conferring mutations in target genes have also been reported (Castanheira et al. 2016; Shapiro et al. 2011), implying the presence of yet undiscovered mechanisms. Importantly, it is not unreasonable to think that resistance might involve more than one single mechanism. Moreover, a gradient of resistance levels can exist, with some mutations conferring greater phenotypic effects than others (Sasse et al. 2012). Finally, mutations can also have synergistic or antagonistic effects

with respect to the resistance phenotype. In this regard, epistatic effects between different mutations and possible synergistic effects have not been explored.

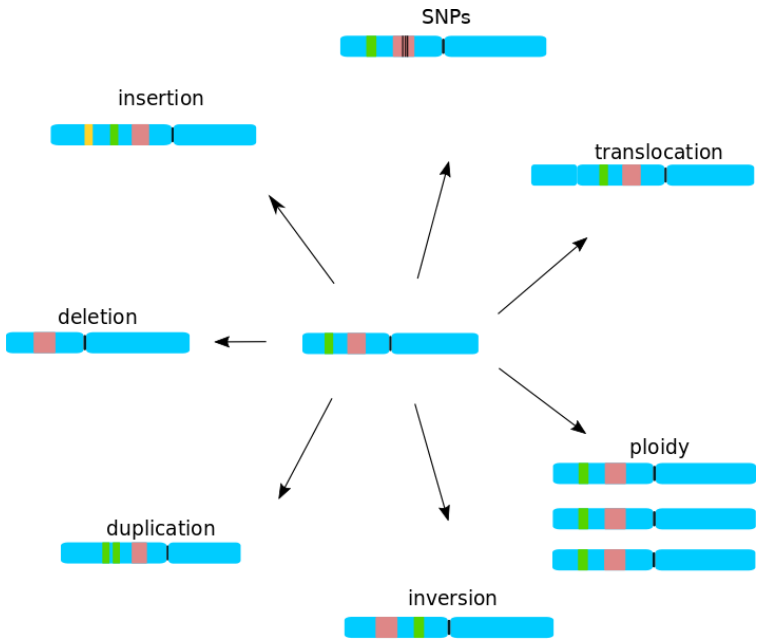
Acquired resistance limits the usefulness of species identification to define the therapeutic strategy and brings in the need to additionally perform susceptibility tests to monitor the resistance profile of the infecting strains. However, this is problematic, expensive, and time-consuming, as it requires isolation and culturing of strains before the test can be performed. Furthermore, highly standardized tests like EUCAST (European Committee on Antimicrobial Susceptibility Testing) or CSLI (Clinical and Laboratory Standards Institute) are not universally applicable. For example, these methods are not recommended for testing the susceptibility towards caspofungin (an echinocandin), given a lack of reproducibility across laboratories or even drug batches (Espinel-Ingroff et al. 2013) and the paradoxical growth of *Candida* at concentrations above MIC (Chamilos et al. 2007). In such cases, molecular methods to directly test for the presence of resistance-conferring mutations are an attractive alternative to direct susceptibility testing and, in some cases, they may even present an advantage. For instance, it has been observed that the detection of mutations in *FKS* genes has a greater predictive power than susceptibility tests regarding the risk of echinocandin therapy failure among patients infected by *C. glabrata* (Shields et al. 2012).

1.7 Evolutionary Paths for the Emergence of Resistance

In contrast to the acquisition of antibiotic resistance in bacteria, the evolutionary processes by which yeasts can acquire resistance to antifungal drugs are only barely known. Cataloging mutations that can confer a resistance phenotype (see above) is only a first step towards understanding the mechanisms leading to the emergence of resistance. Processes that drive genome evolution include single-point mutations; gene duplications, deletions, inversions, and insertions; chromosomal rearrangements; aneuploidies; the loss of heterozygosity; and finally, horizontal gene transfer and/or hybridization **(Figure 1.2**). We know very little about mutation rates or frequencies of such evolutionary events in pathogenic *Candida*. Moreover, such mutations appear in the context of evolving populations, and factors such as the size of the population or the possibility of exchanging genetic material through mating and recombination, can influence the pace at which an infecting population can adapt to the drug. In addition, how a drug actually affects the pathogen may constrain the ways in which the yeast can adapt to it. For instance, fungistatic drugs that stop the growth but do not kill the pathogen open a window of opportunity for mutations to appear. Another issue contributing to the emergence of resistance involves the dosage regime. In vivo studies in mice have indicated that more frequent applications of low dosages of fluconazole, compared to less periodic and higher dosages, lead to less frequent outgrowth of resistant *C. albicans* strains (Andes et al. 2006).

Finally, various evolutionary outcomes might be driven by different selection strategies that influence the way in which the relative frequencies of drug-resistant genotypes increase within a population.

Figure 1.2. Possible genomic changes in the evolution of yeast genomes. The blue shape represents a chromosome with two arms separated by a centromere (black-line); red, green, and yellow stripes represent genomic regions. The variation may be a result of single nucleotide polymorphisms (SNPs), chromosomal rearrangement (translocation or ploidies), gene-insertion, deletion, duplication, or inversion.



It has been suggested that some mutations or genomic re-arrangements may generally precede the appearance of point mutations, conferring a more efficient and stable resistance. Such stepwise models try to explain how resistance can appear rapidly in infecting populations that are supposedly kept at low densities by the antifungal treatment. In this regard, large genomic re-arrangements such as aneuploidies are good candidates because they result in the

concerted over- or under-expression (depending on whether there is a gain or loss of chromosomes) of several genes, they are well tolerated by the cells, and they are rather common, particularly under stress conditions (Duesberg et al. 2001; Selmecki et al. 2009; Sionov et al. 2010). For example, azole resistance in *C. albicans* has been associated with a specific segmental aneuploidy comprising the two left arms of chromosome 5 flanked by a single centromere, an isochromosome 5L [i(5L)] (Selmecki et al. 2006). This region carries the *ERG11* and *TAC1* genes involved, respectively, in ergosterol synthesis and drug efflux (Selmecki et al. 2008). Interestingly, the acquisition of aneuploidies during in vitro evolution experiments carried out in the presence of fluconazole has also been associated with overall advantages in fitness (Selmecki et al. 2009). Yet, azole-induced aneuploidies were lost during cultivation in a stress-free environment and were thus considered providers of raw genetic material in the process towards the acquisition of resistance. This ease for chromosomal changes also demonstrates and emphasizes the genomic plasticity of *Candida*.

Genome rearrangements have also been suggested to play a role in the adaptation of *C. glabrata* to stressful conditions (Healey et al. 2016). Early studies from 1997 already reported whole chromosome duplications bearing the *ERG11* gene in azole-resistant strains (Marichal et al. 1997). Other investigations led to similar claims based on differences among karyotypes of serial clinical isolates of the species exhibiting an increased resistance to antifungals (Ahmad et al. 2014). Yet, chromosomal aberrations were also observed in the

same world-wide used *C. glabrata* reference strain obtained from various laboratories and cultivated under non-stressful conditions (Bader et al. 2012). Similarly, in a recent whole genome sequencing analysis, aneuploidies containing genes involved in drug resistance were not associated with increased resistance profiles (Carreté et al. 2018), again excluding a direct effect of chromosomal changes on antifungal drug resistance.

Thus, aneuploidies are acknowledged to play a role in mediating drug resistance in *C. albicans*, but the impact of this phenomenon in other species is not clear. On the other hand, alternative genomic changes involving copy-number variation (CNV), including short segmental CNV (Selmecki et al. 2010) and loss of heterozygosity (LOH) (in heterozygous species such as *C. albicans*), are also proposed to drive fast adaptation (Coste et al. 2006; Dunkel and Morschhäuser 2011). Furthermore, the appearance of 'hypermutator phenotypes' resulting from mutations in DNA repair genes has been proposed to precede the appearance of resistance in bacteria (Woodford and Ellington 2007). Similarly, hypermutator phenotypes resulting from mutations in the DNA mismatch repair gene *MSH2*, have been suggested to enable fast adaptation to drugs in *Cryptococcus neoformans* and *C. glabrata* (Boyce et al. 2017; Healey et al. 2016). However, at least for *C. glabrata*, other studies have cast doubts on the hypothesis that variations in *MSH2* generally precede the appearance of other resistance-conferring mutations. For instance, some of these mutations were found to be ancient polymorphisms within different *C. glabrata* clades, and to be equally widespread among non-resistant isolates (Carreté et al. 2018). Consistent with this, *MSH2* non-

synonymous polymorphisms can be locally common, irrespective of the susceptibility of the isolates, as found for 69% (57/83) of susceptible clinical isolates in India (Singh et al. 2018).

Recent research has drawn attention to the existence of phenotypic variation within a genetically homogeneous population. This is particularly important for *C. glabrata* and its ability to undergo exposure to azoles. In this case, the so-called heteroresistance phenotype refers to the observation of the coexistence of various levels of resistance to antifungal drugs within a clonal cell population (Ben-Ami et al. 2016). This trait may be a reason for the high natural tolerance of the species towards the drug, and it can actually be a mechanism that buys time until the appearance of mutations that confer a stable and constitutive resistance. The mechanism of heteroresistance is still poorly understood, as is its potential relationship with the evolutionary paths leading to antifungal resistance in *C. glabrata*. To complicate things further, heteroresistance might cause false outcomes in susceptibility tests, which may result in the misidentification of potentially resistant isolates as susceptible and even in fatal treatment failure (Ben-Ami et al. 2016). Along with heteroresistance, we would like to mention the concepts of tolerance and persistence. Tolerance has been described as the ability of an organism to grow at concentrations higher than the MIC, in contrast to antifungal resistance that reflects the increase in MIC independent of the microorganisms' capacity to survive at drug concentrations higher than this value (Fridman et al. 2014; Delarze and Sanglard 2015). Furthermore, tolerance is reversible and results from epigenetic mechanisms, while resistance

is an inheritable property determined by genes and their mutations (Delarze and Sanglard 2015). It has been observed that strains exhibiting tolerance are more prone to cause clinically persistent infections than strains having the same MIC but not being tolerant (Rosenberg et al. 2018). It has been also suggested that drug tolerance is an evolvable phenotype, which is distinct from and does not correlate with antifungal drug resistance (Rosenberg et al. 2018). Finally, persistence occurs when microorganisms are not only able to withstand the antifungal therapy, but can also cause a relapse, even after a successful one (Rosenberg et al. 2018).

1.8 Whole Genome Sequencing of Serial Isolates to Track the Emergence of Resistance

The evolutionary paths leading to the appearance of resistance have been extensively studied in bacteria by means of whole genome sequencing of serial clinical isolates and in vitro evolution studies. Fortunately, nowadays, those approaches are also being increasingly introduced in the research of fungal pathogens. Sequencing the entire genome of a microorganism has never been so easy. Next-generation sequencing and comparative genomics not only allow us to record the footprints of genetic evolution of new species, but also help us in tracking the genomic changes that follow the emergence of a phenotype of interest. One of the initial studies on the genetic bases of yeast adaptation to antifungal drugs in a human host by means of genome sequencing was performed by Ford et al. (Ford et al. 2015). In this study, sequencing was used to assess changes in the frequency of variants in *C. albicans* isolates sampled consecutively from the

same patient and shown to acquire resistance by the end of the treatment. The study indicated persistent and recurrent LOH and SNPs in 166 genes as the main modifications associated with decreased fluconazole susceptibilities. More specifically, LOH was found on chromosome 3, in regions comprising *CDR1* and *CDR2* (efflux pumps coding genes) and *MRR1* (encoding the regulator of the *MDR1* major facilitator superfamily efflux pump) (Schubert et al. 2011); and on chromosome 5 with genes encoding the drug target *ERG11*, and *TAC1* (transcription factor that positively regulates the expression of *CDR1* and *CDR2*) (Coste et al. 2006). Other mutations were found in cell adhesion genes (e.g., *ALS3,5* and *7* and *HYR3*), as well as genes involved in filamentous growth (e.g., *FGR14*, *FGR28*, and *EFH*) and biofilm formation (e.g., *BCR1* and *YAK1*), indicating that resistance was co-evolving with virulence. On the other hand, although aneuploidies were present and may be important adaptive intermediates (see above), they did not seem to correlate with the resistance phenotype. The authors maintain the suggestion that these variations ease the survival until more stable and/or less costly mutations arise. Additionally, it is also possible that serial isolates from the same patient can result in resistance caused by different trajectories (Selmecki et al. 2008). Nine serial clinical *C. albicans* isolates obtained from a patient that underwent antifungal treatment were observed to acquire resistance by multiple and competing mechanisms (Selmecki et al. 2008). This emphasizes the urge to understand the dynamics of emergence of the resistance, including the evolutionary trajectories, the rates at which different mutations

arise, and the potential relationships between processes mediating the adaptive mechanisms.

Genome sequencing of serial clinical isolates has also been applied to *C. glabrata*. A recent study sequenced and compared the genomes of two *C. glabrata* clinical isolates obtained from the same patient separated by 50 days of azole treatment (Vale-Silva et al. 2017). The identified genetic differences comprised 17 non-synonymous SNPs, including one gain of function substitution in the *PDR1* gene (L280F) and small-sized indels mainly affecting adhesin-like genes. Despite all the effort, which included the use of advanced PacBio long-read sequencing, the only significant mutation that was found was among those already known to confer azole resistance (Ferrari et al. 2009). The rest of the observed genetic alterations were attributed to fitness or accidental mutations. Acquired resistance of *C. glabrata* to echinocandins was also analyzed by whole genome sequencing of serially isolated strains obtained from a patient subjected to caspofungin treatment (Singh-Babak et al. 2012). This study identified non-synonymous mutations in the drug target gene *FKS2* and in other eight genes (the orthologs of *S. cerevisiae MOH1*, *GOH1*, *CDC6*, *TCB1/2*, *DOT6*, *MRPL11*, *SUI2*, and *CDC55*). Yet, the functions of the orthologs in *S. cerevisiae* of these eight genes suggested that they were not directly related to the resistance phenotype, but rather that they might be connected to the adaptation of *C. glabrata* to the host or, alternatively, they might compensate for the effect of *FKS2* mutations. Additionally, changes in the *FKS2* gene were associated with the highest increase in echinocandin resistance and a considerable cost in fitness. Finally, another study

in *C. glabrata* used a whole genome sequencing approach, but only searched for mutations in genes suggested to play a role in resistance (Biswas et al. 2017). More specifically *FKS1* and *FKS2* in echinocandin resistance; *FCY1*, *FCY2*, *FUR1*, *FPS1*, and *FPS2* in fluorocytosine resistance; and *ERG9*, *ERG11*, *CDR1*, *PDR1*, *FLR1*, and *SNQ2* in azole resistance. Interestingly, the results uncovered specific mutations in *FKS1* (S629P) and *FKS2* (S663P) present only in the echinocandin-resistant strains. In contrast, mutations present in marker genes for azole resistance, *PDR1* and *CDR1*, were present in both azole-susceptible and resistant isolates, which again underscores the need for further investigations.

1.9 In Vitro Evolution Studies

Although in vivo studies performed on patient samples are clinically more relevant than in vitro ones, they come with disadvantages. In in vivo studies, the results are not easily replicable, the population size parameters are not controlled, and usually only the mutational composition of the final isolate is assessed. These limitations make the use of alternative in vitro approaches a promising tool to unravel the evolutionary paths leading to the emergence of resistance. This so-called 'experimental evolution' approach enables the control of conditions and exact measurement of relevant parameters. Moreover, samples can be stored at intermediate time points and the experiment can be re-started with alternative conditions from any point, thus allowing researchers to 'retape' evolution. Furthermore, the order of occurrence of adaptive mutations—i.e., the evolutionary trajectory—can be tracked. Several studies have shown a high consistency

between results obtained in vivo and in vitro in yeasts like *C. albicans* and *S. cerevisiae* (Cowen et al. 2002; Anderson et al. 2003). In vitro evolution experiments coupled with whole genome sequencing have been extensively used to understand the emergence of antibiotic resistance in bacteria (Bryant et al. 2012), but their use in the field of antifungal drug resistance is still in its infancy.

There are two main approaches for in vitro evolution experiments: batch serial transfer and continuous culture. In the first one, the sample is grown on selective solid or liquid media, and a fraction of it is repeatedly and serially transferred to a fresh medium. Then, the culture passes by different growth phases, which implies that the amount of nutrients in the medium diminishes with time. On the other hand, in a continuous culture system, the physiological state of the cells, the growth conditions, and the environment, including nutrient concentrations, are kept constant. Both methods have been successfully used to study the emergence of drug resistance in *Candida* yeasts (Huang et al. 2011; Cowen et al. 2000). The important advantages of a serial dilution system over the continuous culture are related to lower costs, the use of generally available laboratory equipment, and, most importantly, the feasibility of conducting experiments involving a high number of replicates in parallel, enabling a comprehensive analysis of a variety of changes, mechanisms, and evolutionary trajectories of adaptation. For example, Cowen et al. (Cowen et al. 2000) serially propagated six experimental populations of *C. albicans* derived from a single colony for 330 generations in medium supplemented with fluconazole at a concentration doubling their MIC. This in vitro

evolution experiment resulted in the selection of azole-resistant isolates evolved by different mechanisms and exhibiting distinct levels of resistance and different expression patterns for azole-associated genes (*CDR1*, *CDR2*, *ERG11*, and *MDR1*). Another study compared the evolution of experimental *C. albicans* populations evolved under the presence of fluconazole with those without this stress (Huang et al. 2011). Similar to the previous study, multiple resistant mutants appeared rapidly in independent lineages. Moreover, they found that most adaptive mutants with increased fitness under drug exposure did not show significant fitness defects in the absence of the drug. These studies show the great potential of in vitro evolution studies for uncovering evolutionary paths leading to the emergence of resistance. Yet, such studies are scarce and limited to a few species and drugs, which suggests that many alternative adaptation pathways remain unknown. Hence, we believe that the use of in vitro evolution approaches, coupled with whole genome sequencing, should be extended in future studies. An example of an in vitro experimental evolution and a follow-up analysis is presented in **Figure 1.3.**

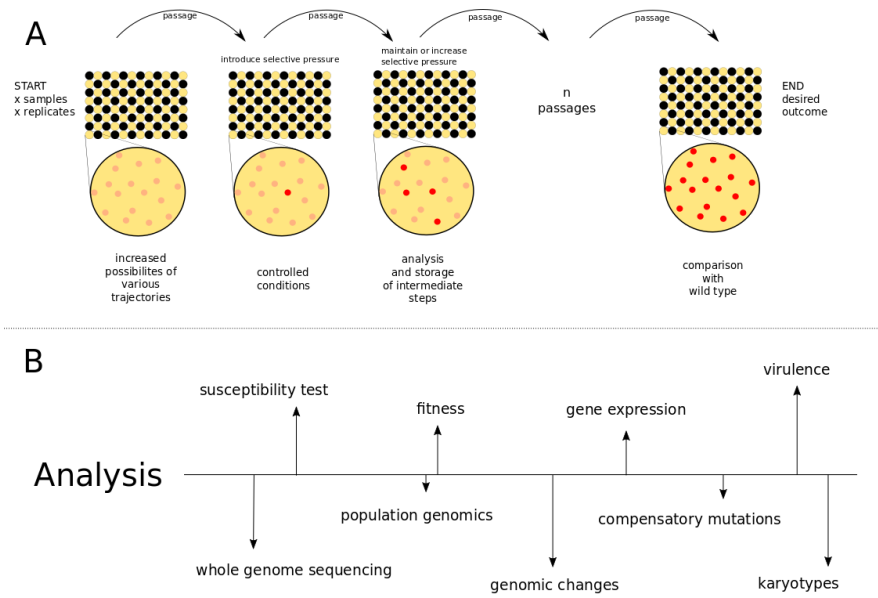


Figure 1.3. Schematic representation of an in vitro evolution experiment. (**A**) and possible follow-up analysis (**B**). (**A**)–96-well plate can be inoculated in a checkerboard manner with up to 48 samples (sample—yellow well, blank—black well), allowing many possible combinations of strains and replicates. Initially, all cells within each population are expected to be genetically identical (enlarged well—pink circles). Next, the samples are introduced to a selective condition (for example antifungal drug). The amount of the sample (or number of growth cycles), the interval of the passages, and the amount of selective pressure between the passages can be set up and controlled as preferred. Ideally, each transfer favors a selection of mutants with a desired phenotype (red dots in the enlarged well). Storage and/or analysis of the samples can be performed as preferred, e.g., after each passage. The experiment is finished after a certain amount of time or when the desired phenotype is present in the evolving population. (**B**)–Further analysis subsequent to the in vitro evolution experiment may involve analysis of the phenotype (top) or genotype (bottom). These analyses can include, among others: drug susceptibility; fitness measurement (ability to replicate and survive in a given environment); assessment of levels of gene expression; virulence test (ability to infect or damage a host); whole genome sequencing; population genomics (large-scale comparison of DNA sequences of populations); identification of specific genomic changes (see **Figure 1.2**), with the possibility of determining compensatory mutations; karyotypes (changes in chromosome numbers or large genomic re-arrangements).

1.10 Conclusions

Fungi pose a growing clinical threat, and we have limited drugs to combat them. The problem of resistance to antifungal drugs is highly prevalent and has increased over the last years. Currently, 20–30% of candidemia cases involve species with intrinsic resistance to either fluconazole or echinocandins (Arendrup 2014). This is a significant change as *C. albicans*, naturally susceptible to all drugs, used to account for 85% of the cases before the advent of antimycotics (Arendrup 2014). The main driver of this change involves the use, and overuse, of antifungal drugs in the clinics. Resistance can be based on diverse mechanisms, which can vary from species to species. *C. glabrata* is a good illustrating example of how a well-understood mechanism in one species does not necessarily apply to other species. Emerging and dangerous species, like multidrug-resistant *C. auris*, pose a constant threat and we are likely to witness the rise of new such multidrug-resistant pathogenic yeasts in the near future.

Next to the complexity of varying natural susceptibilities across species, we need to consider the process of secondary acquisition of resistance in otherwise susceptible yeasts. Such cases are being increasingly reported and have brought around an urgent need to develop more efficient ways to assess and monitor the microorganisms' response to a drug, also during the course of the infection. More studies on the underlying processes of resistance and evolutionary pathways that result in drug adaptation are needed, as well-understood molecular mechanisms do not always completely

account for the high levels of resistance observed in many clinical isolates. Fortunately, technical developments such as next-generation sequencing are allowing us to interrogate mutational processes at unprecedented levels of scale and resolution. Promising discoveries are being disclosed during the analysis of serially isolated clinical strains with acquired resistance and light is being shed on the complex landscape of mutations and genomic re-arrangements that lead to the emergence of the phenotype. Along with comprehensive sequencing and the comparison of clinical samples, several laboratories are approaching the issue by the use of controlled, experimental evolution experiments. Ongoing results are showing that rather than a single, established path, there is an array of possible trajectories by which a microorganism can adapt to drugs. Understanding the molecular and evolutionary mechanisms responsible for the development of drug resistance in common and emerging yeast pathogens will undoubtedly contribute to the development of novel target-specific drugs or resistance-blocking supplements. In addition, research on the genetic bases of resistance also has the potential to ultimately lead to novel diagnostic tools that would allow detecting particular resistant profiles from genetic hallmarks.

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2 *Candida glabrata* - the "other" *Candida* pathogen

2.1 Fungal infections and candidiasis

Despite the constant alarms raised by clinicians and scientists (Brown et al. 2012), fungi are an underappreciated clinical threat. Important issues with regard to fungal infections include the absence of licensed antifungal vaccines; the lack of fast and accurate diagnosis of mycoses (Wickes and Wiederhold 2018); and the limited availability of therapeutic options restricted to few antifungal drug classes, which are expensive, toxic (Mourad and Perfect 2018), and too frequently connected with intrinsic and acquired resistance. Furthermore, financial support for research in fungal diseases is lower as compared to that of other infectious diseases that cause similar mortalities (Rodrigues and Albuquerque 2018; Rodrigues 2016). Although the immunocompromised patients, whose numbers are increasing, are persons the most prone to fungal infections, the Global Action Fund for Fungal Infections (GAFFI) has also stressed the destructive impact of fungal diseases for those with intact immune systems (GAFFI 2015). GAFFI estimates that all fungal diseases combined cause more annual deaths than either tuberculosis or malaria, with the global death toll of fungal infections estimated around 1.5 million people each year (Bongomin et al. 2017). In addition, it is estimated that over 300 million people of all ages suffer from a serious fungal infection each year globally; skin mycoses are almost as common as

headaches and dental caries; and more than 1 million eyes go blind each year due to fungal keratitis (GAFFI 2016).

Species of the genera *Candida*, *Aspergillus*, *Pneumocystis* and *Cryptococcus* are the most common causes of life-threatening fungal infections ("Stop Neglecting Fungi" 2017) and invasive candidiasis (i.e. infection caused by *Candida* species) is the most frequent invasive fungal infection (IFI) associated with health care (Gonzalez-Lara and Ostrosky-Zeichner 2020). Blood infections caused by *Candida* species (candidemia) are considered grievous IFI, causing increasing concerns in the healthcare system especially when affecting patients with compromised immune system, including those infected with HIV, cancer patients, organ transplant recipients, as well as elderly, neonates, and patients requiring invasive therapies (Pfaller et al. 2012; Astvad et al. 2018; Lamoth et al. 2018; Ramos-Martínez et al. 2017). Both the high occurrence of the disease and the rising numbers of species and strains resistant to the few available antifungal drugs are an important source of clinical concern (Arendrup 2014; Verweij et al. 2016). Detailed information on major antifungal drugs and their mechanisms of actions, natural susceptibility to antifungals among *Candida*, mutations leading to secondary acquisition of resistance and evolutionary paths for the emergence of resistance are described in **Chapter 1**.

From the 37 species of *Candida* that have been identified as etiological agents of candidiasis, five account for the vast majority of cases (>90%) according to a recently conducted study (Pfaller et al. 2019). Updated global epidemiology data ranks *C. albicans* as the

most abundant source of candidiasis (46.9%), followed by *C. glabrata*, (18.7%), *C. parapsilosi*s (15.9%), *C. tropicali*s (9.3%), *C. krusei* (2.8%), and other *Candida* spp. (6.5%) as reported in the SENTRY Antifungal Surveillance Program, which has investigated almost 21,000 *Candida* strains isolated in participating hospitals in the period from 1997 to 2016 (Pfaller et al. 2019). Although the relative incidence of the different species is geographically dependent, we are overall facing a continuous shift towards a higher prevalence of non *Candida albicans* species, which correlates with increased antifungal drug resistance and with the increasing use of antifungal drugs (Arendrup 2013; Bailly et al. 2016; Hachem et al. 2008; Bodey et al. 2002; Imhof et al. 2004; Kontoyiannis and Lewis 2002). This trend emphasizes the need for research on the variety of distinct *Candida* species.

C. glabrata is the second most common *Candida* species causing bloodstream infections (Pfaller et al. 2011; Diekema et al. 2012; Guinea 2014). It accounts for one third of candidemia isolates in the US, and in Europe its abundance was estimated as high as 26% in Denmark, 27% in Belgium, or 21% in Scotland (Lamoth et al. 2018). *C. glabrata* bloodstream infections frequently affect elderly individuals, diabetic patients and solid organ transplant recipients (Guinea 2014; Pfaller et al. 2014; Khatib et al. 2016). The continuous increase in the incidence of *C. glabrata* over the last decades (**Figure 2.1**) has been linked to its ability to withstand antifungal therapy (Le et al. 2017; Singh-Babak et al. 2012), especially azole treatment (Whaley and Rogers 2016). Importantly, resistant isolates of *C. glabrata* have been reported for all classes of antifungal drugs, and

multidrug resistance is dangerously on the rise (Khan et al. 2008; Cho et al. 2015; Chapeland-Leclerc et al. 2010; Farmakiotis et al. 2014; Garnaud et al. 2015; Hull et al. 2012).

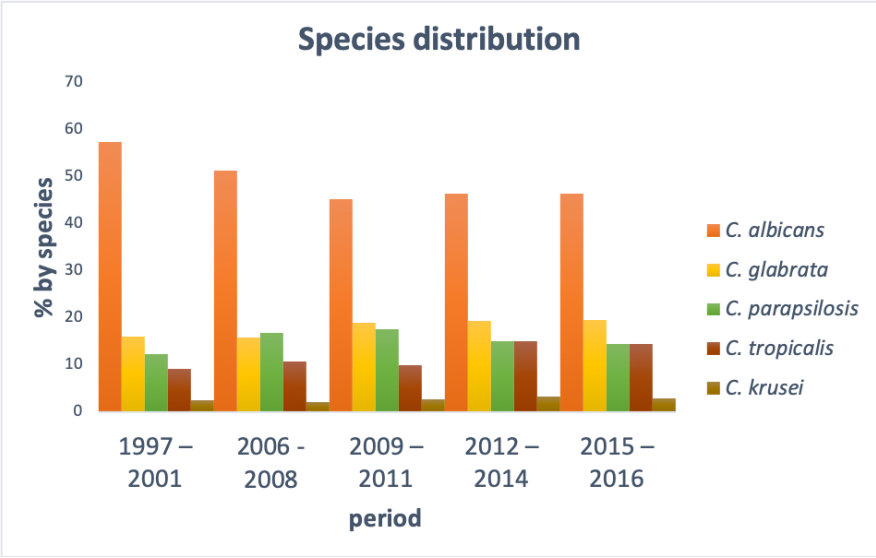


Figure 2.1. Relative amount of *Candida* species identified by the SENTRY surveyance programme from 1997 to 2016. Adapted from "Twenty Years of the SENTRY Antifungal Surveillance Program: Results for *Candida* Species From 1997–2016", M. Pfaller, 2019, Open Forum Infect Dis (Pfaller et al. 2019).

2.2 *Candida glabrata* and comparison with *Saccharomyces cerevisiae* and *Candida albicans*

Candida glabrata was first named as *Cryptococcus glabratus* more than 100 years ago, and described as a component of the human intestinal microbiota, given its initial isolation from faeces of a healthy person (Anderson 1917). Later on, the name was changed to *Torulopsis glabrata* and today, the species is called *Candida glabrata* (Hazen 1995) (although the alternative name *Nakaseomyces glabrata*

would be taxonomically more correct) and it is considered an opportunistic fungal pathogen and a serious clinical threat.

The current taxonomy of *C. glabrata* is: Kingdom Fungi, Subkingdom Dikarya, Phylum Ascomycota, Subphylum Saccharomycotina, Class Saccharomycetes, Order Saccharomycetales, Family Saccharomycetaceae, Genus *Nakaseomyces,* Clade *Nakaseomyces/Candida* and Species *glabrata* (NCBI:txid284593). *C. glabrata* is more closely related to *Saccharomyces. cerevisiae* and only distantly related to the most common *Candida* pathogen, *Candida albicans* (**Figure 2.2**). Contrary to most other *Candida* pathogens, *C. glabrata* does not belong to the CTG (or CUG) clade in which the CUG codon encodes serine as opposed to leucine (Dujon et al. 2004; Gabaldón et al. 2013). Both *C. glabrata* and *S. cerevisiae* belong to the post Whole Genome Duplication (WGD) group, a group of related lineages descending from a major genome duplication event proposed to have occurred 100–200 million years ago (Wolfe and Shields 1997), and inferred to result from an hybridization event (Marcet-Houben and Gabaldón 2015). As compared to *S. cerevisiae*, *C. glabrata* experienced more events of duplicated gene loss following the genome duplication, and thereby exhibits lower gene redundancy (Gabaldón et al. 2013; Dujon et al. 2004). Several *Nakaseomyces* species have the ability to infect humans and this trait appears to have emerged independently within the genus (Gabaldón et al. 2013). This genus contains three pathogenic species (*C. glabrata, Candida nivariensis* and *Candida bracarensis*) and three environmental species (*Candida castellii, Kluyveromyces delphensis* and

Kluyveromyces bacillisporus) (Gabaldón et al. 2013) where *C. glabrata, C. nivariensis C. bracarensis* and *K. delphensis* are referred to as the '*glabrata* group' (Gabaldón et al. 2013).

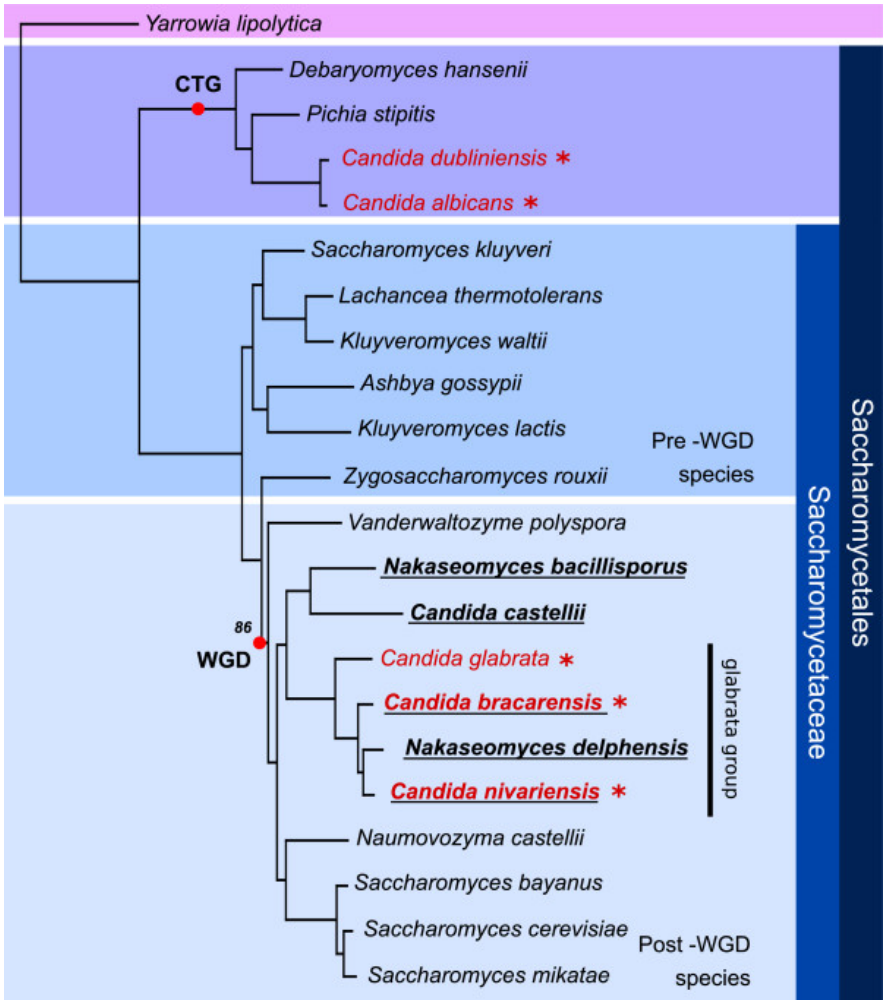


Figure 2.2. Maximum likelihood species tree of 22 Saccharomycotina species. Reprinted from " Comparative genomics of emerging pathogens in the *Candida glabrata* clade," T. Gabaldón T, 2013, BMC Genomics (Gabaldón et al. 2013).

The genomes of *C. glabrata* and the non pathogenic yeast *S. cerevisiae* share 88% of their gene synteny blocks, with only 446

(8.6%) of *C. glabrata* genes being absent from *S. cerevisiae*. It is therefore of interest to know how *C. glabrata* became a human pathogen while *S. cerevisiae* is a widely used yeast in food and industrial processes, with very few reported cases of clinical infections (Enache-Angoulvant and Hennequin 2005; de Llanos et al. 2011; Muñoz et al. 2005). Several important phenotypic differences between the species may relate to this. First, the optimal growth temperature for *C. glabrata* is 37°C, which resembles the internal temperature of the human body giving the species a survival advantage. Second, *C. glabrata* is also characterized by improved adaptation to nutrient limitation and varying levels of stress that might be caused either by other microorganisms or by the protective mechanisms of the host. Third, *C. glabrata* underwent essential cell wall remodeling resulting in superior adherence, mediated by proteins called adhesins (de Groot et al. 2013), which are important contributors to colonization of host cells and formulation of infections (de Groot et al. 2008). Fourth, as compared to *S. cerevisiae*, *C. glabrata* displays better adherence and expansion of genes mediating it (Butler et al. 2009; Gabaldón et al. 2013), which in *C. glabrata* are also implicated in the formation of biofilms (d'Enfert and Janbon 2016). Biofilms are complex extracellular matrix-embedded, multi-layered microbial structures that are formed on biotic or abiotic surfaces through the establishment of microbe-microbe and microbe-surface interactions (d'Enfert and Janbon 2016; Fanning and Mitchell 2012). The biofilms formed by *C. glabrata* are characterized by an increased antifungal resistance and persistence, not only within the host but also on the surface of

medical equipment, resulting in troublesome and difficult to eliminate sources of clinical infections.

Despite the fact that both species cause the same disease, candidiasis, and share the genus name, *C. glabrata* and *C. albicans* are evolutionarily distant which is reflected in many phenotypic differences (**Table 2.1)**. These include different virulence mechanisms and strategies for survival within the host that may affect the treatment and course of the disease. First, *C. glabrata* cells cannot form hyphae, an important and widely known feature of *C. albican*s resulting in an active invasion of host tissues and damages during colonization (Brunke and Hube 2013; Kasper et al. 2015). Second, secreted protease activity, another *C. albicans* virulence mechanism, is lower in *C. glabrata*. Third, *C. glabrata* has a haploid genome, while *C. albicans* is naturally diploid. Haploid organisms are considered to adapt faster to different environments and be more susceptible to single-nucleotide mutations, whereas diploids adapt slower and are more prone to present large changes of genome structure (Gerstein et al. 2011; Sharp et al. 2018). Fourth, both species differ in their mating mechanisms and cell cycle, *C. albicans* being parasexual and *C. glabrata* being probably able of sexual mating but at very low frequencies and in conditions that have never been reproduced in the laboratory (Dodgson et al. 2005; Carreté et al. 2018). Fifth, *C. glabrata* is characterized by having an improved intrinsic capability to withstand high concentrations of azole antifungals (Diekema et al. 2012).

C. glabrata possesses an outstanding plastic genome, which contributes to its adaptive phenotypic flexibility (Carreté et al. 2018). The reference nuclear genome (of strain: CBS138) is composed of 12,3Mb spanning 13 chromosomes (A to M), with sizes ranging from 491,328 of the shortest chromosome A to 1455,689 bp of the longest chromosome L. Whole genome sequencing of 33 globally distributed *C. glabrata* strains revealed the presence of at least seven genetically distinct clades with evidence of inter-clade recombinations and recent global spread of previously isolated populations (Carreté et al. 2018).

The haploid character of *C. glabrata* contributes to its ability to undergo rapid genetic changes, present increased mutational rates and have higher potential for developing resistance-conferring mutations (Matsumoto et al. 2014; Dannaoui et al. 2012). This is especially favorable in stressful conditions, which often result in genetic instability caused by increased rates of genomic recombination and spontaneous mutations, that may provide new selectively advantageous phenotypes (Shor and Perlin 2015).

Genetic diversity in *C. glabrata* is also manifested by chromosomal alterations including rearrangements and duplications which occur and persist at high frequency (Carreté et al. 2018; Healey et al. 2016; Poláková et al. 2009). Karyotype variability has been shown to be present in different progenies of the same reference strain, further highlighting genome plasticity in this species (Bader et al. 2012).

Efficient colonization of the human host and of medical devices, high genomic plasticity, intrinsic low susceptibility to azoles, ability to stably adapt to azoles and echinocandins leading to multidrug resistance, and heteroresistance are, among several others, traits that make *C. glabrata* an important clinical threat. It is of high importance to understand the mechanisms and evolutionary paths by which *C. glabrata* escapes antifungal treatment, as it will not only be useful in the efforts of minimizing the spread of resistance but also in the development of new antifungal agents or cofactors, or in the modification of existing drugs to improve their efficiency.

Table 2.1. Comparison of important traits of *Candida albicans*, *Candida glabrata* and *Saccharomyces cerevisiae*.

Feature	*C. albicans*	*C. glabrata*	*S. cerevisiae*
phylogeny	CTG clade	post WGD - non - CTG clade	
ploidy	diploid	haploid	variable
optimal growth	30°C	37°C	variable
cellular morphology	yeast, pseudohyphae and hyphae	yeast, pseudohyphae(rare)	yeast, pseudohyphae
decreased azole susceptibility	absent	intrinsic	absent
expansion of adhesion genes	no	yes	no
improved ability to sustain starvation	no	yes	no
improved ability to sustain stress	no	yes	no

3 Introduction to key methodologies used in this thesis

The approach used to conduct this project can be divided into three major steps. First, the samples were subjected to in vitro evolution through which they acquired antifungal drug resistance (process described in the previous chapter). Second, the phenotypes of the evolved strains were evaluated by comparing the drug susceptibilities of the WT and mutant strains. Finally, sequencing of DNA fragments or whole genomes allowed the identification of DNA variations, which were correlated with the phenotypes. Additionally, we confirmed some of the genotype/phenotype relationships by using genomic engineering (CRISPR-Cas9).

3.1 Susceptibility and fitness measurements

How drugs affect the growth of a microorganism is measured by susceptibility tests. Antifungal susceptibility tests have become essential tools to assess the local and global disease epidemiology, determine the best treatment for a specific fungus and to identify resistance. All these elements being vital in the combat against fungal diseases (Alastruey-Izquierdo et al. 2015). Since the essence of the test lies in accurate, reliable and reproducible results, highly standardized methods have been developed to allow the comparison of susceptibility values across time and laboratories for epidemiological, clinical and research studies.

Despite a variety of commercial tests available, broth microdilution methods are still considered as the gold standards. Two organizations, the European Committee on Antibiotic Susceptibility Testing (EUCAST 2020) and the Clinical Laboratory Standards Institute (CLSI 2020) have designed standardized methods to perform antifungal susceptibility testing. Both protocols show comparable results, and both have defined antifungal breakpoints - which is the concentration of the drug used as a threshold by clinicians to classify the strains as susceptible or resistant- for some species. Broth microdilution tests involve growing the microorganisms in different antifungal drug concentrations, which allows for the determination of the Minimum Inhibitory Concentration (MIC). MIC is the lowest concentration of a drug required to inhibit the growth of an organism. Additionally, MIC can be assessed based on 50% and 90% as levels of growth inhibition, giving, respectively, values for MIC_{50} and MIC_{90}. MIC values are used to categorize the strain according to existing breakpoints, and the obtained information can be used to predict the likelihood of success or failure of the antifungal therapy (Sanguinetti and Posteraro 2017; Pfaller 2012). In practice, a fixed amount of cells is inoculated into the growth medium supplemented with serially diluted antifungal drug concentrations, and the growth of the microorganisms at each concentration is measured and compared to the growth on the medium without the drug.

Although susceptibility tests are highly standardized and widely used, *Candida* species may exhibit growth abnormalities in the presence of the drugs that may lead to misinterpretation of the final

results. One such phenomenon is called the “paradoxical effect” which refers to the ability of yeast cells to survive at high concentrations of echinocandins and which is attributed to the induction of stress responses and the increased presence of chitins in the cell wall (Wagener and Loiko 2017; Rueda et al. 2014; Shields et al. 2011; Chamilos et al. 2007; Jacobsen et al. 2007; Rueda et al. 2017; Marcos-Zambrano et al. 2017; Fleischhacker et al. 2008). Another abnormal growth phenomenon is known as the “trailing effect”, which is observed when the total inhibition of fungal growth is not achieved but the growth is rather maintained and persistent at increasing drug concentrations (Coenye et al. 2008; Zomorodian et al. 2016; Rueda et al. 2017) (**Figure 3.1**). These two anomalies can interfere with the determination of MIC and cause clinical problems when these misleading results are used to decide appropriate fungicidal treatment.

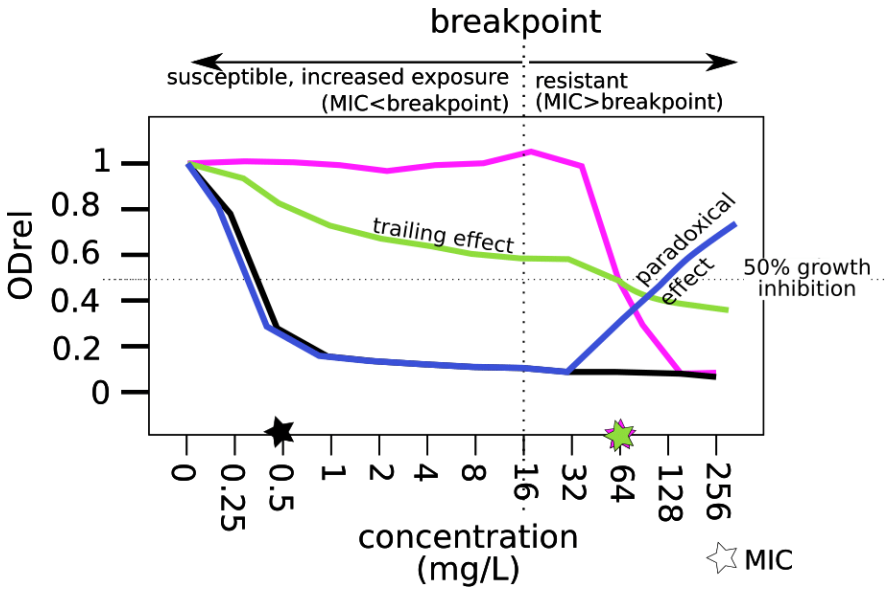


Figure 3.1. Example of possible outcomes of a susceptibility test. Representation of optical density (OD) relative to the OD when growth is measured with no drug (OD for no drug control is set as 1) at each of the concentrations of the drug with a marked breakpoint (EUCAST) and MIC where 50% of the inhibition is observed (stars). Black line shows a susceptible while pink and green resistant samples. The blue line represents an experiment showing the paradoxical effect. MICs for both of the resistant samples are the same while the green sample shows a trailing effect for which the overall resistance is lower when compared to the pink sample.

In experimental evolution studies aiming to understand resistance, as those performed in this thesis, susceptibility tests are indispensable to assess the pre- and post-evolution susceptibilities levels and the acquisition of resistance. For a more accurate evaluation, the test can include not only the final readout of the growth after 24h incubation but also intermediate growth measurements. The process can be automatized by the use of a robot handling the test plates and by performing quantifications of the growth of numerous samples

simultaneously in a high-throughput manner. Having the information of the growth at various time intervals allows for the determination of growth curves of the sample over a range of drug concentrations as well as in the absence of the drug, resulting in comprehensive fitness determinations. Thus, the differences in fitness and susceptibilities can be based on variables as, for example, the growth rate or the area under the growth curve that, combined with MIC can improve the final interpretation of the results.

3.2 Determination of small genetic variations in targeted regions of the DNA

Polymerase chain reaction (PCR) coupled to Sanger sequencing of the amplicon can be used in the detection of small genetic variants in selected genomic regions (Mullis et al. 1986; Sanger et al. 1977). PCR is a methodology that uses thermocycling, a polymerase and specifically designed single-stranded sequences (primers) to obtain multiple copies of a specific, short region of DNA. The results can be further subjected to sequencing - a process that determines the nature and order of the nucleotides in a DNA fragment of interest. Sanger sequencing is a “chain termination method” based on the detection of labelled, chain-terminating nucleotides that are selectively incorporated by a DNA polymerase during in vitro DNA replication (Sanger et al. 1977). The detected fluorescent signals are translated into a DNA sequence that will be aligned and compared with a reference sequence in a search for the genetic variations. These variations can include single-nucleotide polymorphisms (SNPs), which commonly refer to single-nucleotide substitutions where a

nucleotide is replaced with any of the other three kinds of nucleotides; as well as small insertions or deletions (indels).

These methods are often used to find mutations in genes, or regions of genes where changes are anticipated. In addition, whenever the abundance of alterations in a particular region of DNA is higher than for other genomic regions across a large number of samples, this region is designated as a mutational hotspot which could serve as a genetic marker of resistance and has interest due to its diagnostic potential. A good example where PCR and Sanger sequencing can be applied is the detection of alterations in hotspot regions within the *FKS* genes that drive the acquisition of the resistance to echinocandins in *Candida* species (Shields et al. 2019, 2012).

3.3. Determination of genetic variations in whole genomes

Although Sanger sequencing is valuable for the detection of changes in specific DNA regions, more high throughput Next Generation Sequencing (NGS) can be used to investigate genetic variants across the entire genome (Buermans and den Dunnen 2014). Among various existing NGS technology platforms, the most widely adopted for this purpose is Illumina. Illumina uses a "sequencing by synthesis" technology, where the DNA is fragmented, strands are separated and the bases at each of the fragments are identified by emitted signals appearing when complementary DNA bases are added to the template strand by a polymerase (Slatko et al. 2018). A sequencing run generates a large number of short, fragmented genomic DNA

sequences called reads. For instance, a whole flow cell of an Illumina Hiseq 2500 sequencing machine, used in this thesis, can produce about 1600 - 2000 million reads corresponding to 400-500 Gigabases of sequences (2x125bp read configuration), which is 32,520 times the size of the *C. glabrata* genome (12,3Gb). Usually several barcoded genomic libraries are pooled in the same flow cell to obtain the desired sequencing coverage per sample (i.e. 100x). These reads are further aligned (mapped) to a reference genome. Consistent discrepancies between the sequences of the aligned reads and the reference genome at a given position allows for the detection of genetic variants that distinguish the sequenced sample from the reference genome.

Another advantage of NGS over the targeted sequencing approach relates to the ability of the former to capture not only small genetic differences but also gross genomic alterations. Besides the identification of SNPs and indels that is achieved through a process called variant calling, an additional analysis can be performed to assess the number of copies of particular genes. The identification of these Copy Number Variants (CNV) is achieved by using the information and differences in the depth of sequencing coverage - the number of reads that are mapped to a given reference base (Sims et al. 2014). A depth of coverage that is twice that of the average in the genome suggests a duplication, whereas lower depth of coverage or the total absence of mapped reads suggest partial or total deletion of a gene. This approach is also used to discover complex gene rearrangements including large aneuploidies, segmental duplications and deletions.

There is a large collection of available bioinformatics tools that help the transition from raw NGS data to final, interpretable results (i.e. a table with confidently identified sequence variants) (Lee et al. 2012; Pereira et al. 2020). As the interest in whole genome sequencing increases and the technology is advancing, the protocols and pipelines are also improving leading to highly accurate results from which proper conclusions can be drawn.

3.4 Genetic modifications using CRISPR-Cas9

Genetic manipulations are important to empirically test genotype to phenotype relationships hypothesized from observational studies, and therefore improve our knowledge about the mechanisms underlying antifungal drug resistance. An obvious approach to confirm the cause-effect relationship between a specific genetic alteration and a resistance phenotype is to re-introduce this mutation (or a deletion of the given gene) in an otherwise susceptible strain and assess whether the same phenotype is obtained. Engineering of genetic modifications is a time consuming process that is not free of technical challenges and that has to be adapted to a particular species. Problematic issues may involve the ploidy of the genome, the lack of sexual cycle (such as in *C. glabrata*), low efficiency of the transformation or low rates of homologous recombination, absence of cloning vectors and selectable markers, to name a few (Samaranayake and Hanes 2011; Defosse et al. 2018). Recently, a genetic manipulation technique called CRISPR (clustered regularly interspaced short palindromic repeats)-Cas9 has revolutionized

genome editing and gained popularity in medically relevant fungi due to its simplicity, efficiency and ability to address mentioned concerns (Morio et al. 2020).

The main component of CRISPR-Cas9 technology is the Cas9 protein (Jinek et al. 2012). Cas9 is a type II RNA-guided endonuclease that causes a double-stranded break (DSB) 3 bp upstream of a protospacer adjacent motif - PAM, which for *Streptococcus pyogenes* Cas9 is any nucleotide base followed by 2 guanines – NGG. Cas9 is directed to a particular site in the genome by a single-guide RNA (sgRNA). SgRNA has two components: a 20-bp target-specific, and followed by a PAM motif in the target sequence, CRISPR-RNA (crRNA) that guides the enzyme to a target site and a trans-activating crRNA (tracrRNA). The DSB can be repaired by nonhomologous end joining (NHEJ), resulting in a potential frame shift, or by homology-directed repair (HDR) allowing for precise editing (Ceccaldi et al. 2016). HDR is based on homologous recombination using a donor DNA template that contains a desired alteration flanked by homology arms both up- and downstream of the cut site.

For a proper functioning of CRISPR–Cas9 gene editing, various aspects need to be considered. The most important are that Cas9 protein has to be correctly expressed and codon optimized, and that sgRNA translation needs to ensure its binding with Cas9. In addition, decisions on transient or permanent expression of the construct and adequate delivery system have to be made. One way to bypass all these issues is to use in vitro assembled ribonucleoprotein complexes

(RNP) (Grahl et al. 2017). In this approach, purified Cas9 protein and CRISPR RNAs are used directly for the transformation. Hence, this CRISPR-Cas9 experiment is limited to the design of a target specific crRNA and a repair construct that contains the desired genome modification. This approach has been proved successful in *C. lusitaniae*, *C. auris*, and *C. glabrata* (Grahl et al. 2017).

4 Objectives

The overarching goal of the present thesis was to understand what genomic changes underlie the acquisition of antifungal resistance in *Candida glabrata.*

More specifically the objectives are:

1 - To trace the adaptation of *C. glabrata* strains to various combinations of antifungal drug regimes.

2- To assess whether different genetic backgrounds present in natural populations of *C. glabrata* can have an impact on the ability to acquire resistance to azoles or echinocandins.

3- To assess the contribution of different genomic changes (i.e. point mutations, aneuploidies, duplications, etc) to the appearance of antifungal drug resistance.

4- To assess the stability of the acquired resistance.

Part II

Results

5 Narrow mutational signatures drive acquisition of multidrug resistance in *Candida glabrata*

Ksiezopolska, E.*; Schikora-Tamarit,M*; Beyer R., Christoph Schüller Ch.; Gabaldón T. "Narrow mutational signatures drive acquisition of multidrug resistance in *Candida glabrata" (under revision)*

* - These authors share equal first authorship.
I designed, set up and executed the in vitro evolution experiments. The design involved selection of the drugs, conditions for both parts of the evolution and strains to evolve. The set up included organization of the equipment and execution involved all the laboratory work. I performed the whole analysis of the *FKS* mutations: PCR, Sanger sequencing and interpretations of the results. I selected the interesting mutants and extracted the DNA for the whole genome sequencing and took part in the interpretations of genomic changes: SNPs and genomic rearrangements, some of which I proved experimentally (PCR and Sanger sequencing). I sequenced and analyzed all *ERG3* (including the evolutionary trajectories) and *CNE1* mutations. I set up and performed CRISPR Cas9 transformations and assessed their phenotypic consequences.

5.1 Abstract

Fungal infections are a growing medical concern, in part due to increased resistance to one or multiple antifungal drugs. However, the mechanisms underpinning evolutionary acquisition of drug resistance are poorly understood. Here we used experimental microevolution to study adaptation of the yeast pathogen *Candida glabrata* to fluconazole and anidulafungin, two widely used antifungal drugs with different modes of action. Our results show widespread ability of rapid adaptation to one or the two drugs.

Resistance, including multidrug resistance, was often acquired at moderate fitness costs, and mediated by mutations in a limited set of genes that were recurrently and almost inevitably mutated in strains adapted to each of the drugs. Importantly, we uncover a dual role of mutations in *ERG3* in resistance to anidulafungin and cross-resistance to fluconazole in a subset of anidulafungin-adapted strains. Our results shed light on the mutational paths leading to resistance and cross-resistance to antifungal drugs.

Teaser

Multidrug resistance can rapidly emerge through mutations in a reduced catalogue of genes, including those associated with cross-resistance to other drugs.

5.2 Main text

Each year, fungal infections affect over a billion people worldwide, and cause 1.5 million deaths (Bongomin et al. 2017). Current challenges to overcome this trend include the lack of fast and accurate diagnosis, and the rise of antifungal drug resistance (Consortium OPATHY and Gabaldón 2019). Acquisition of resistance is particularly worrying given the limited number of antifungal compounds available. However, we have a limited understanding of the evolutionary processes leading to drug adaptation in fungi (Ksiezopolska and Gabaldón 2018). *Candida* species are among the main causes of hospital-acquired fungal infections (Bongomin et al. 2017). *Candida albicans* is the most common cause of candidiasis, but the relative incidence of non-albicans *Candida* species is on the

rise (Pfaller et al. 2019), with *Candida glabrata* often being the second most prevalent cause of infection (Pfaller et al. 2019). Antifungal resistance in *C. glabrata* is particularly problematic, as this yeast shows a remarkable ability to adapt to both azoles and echinocandins, thus leading to multidrug resistance (MDR) (Vallabhaneni et al. 2015; Perlin 2015b; Pristov and Ghannoum 2019; Arendrup and Patterson 2017). Most antifungals commonly used against *Candida* are azoles (e.g. fluconazole, flz), fungistatic drugs that inhibit a lanosterol demethylase encoded by *ERG11* (Heimark et al. 2002), and echinocandins (e.g. anidulafungin, ani), that inhibit 1,3-β- D-glucan synthase encoded by *FKS* genes (Perlin 2007), and are fungicidal to *Candida*. Most prevalent mechanisms conferring protection against azoles in yeasts involve alterations in the target enzyme, or overexpression of drug efflux pumps (Lupetti et al. 2002). Known mechanisms of azole resistance in *C. glabrata* almost exclusively consist of gain-of-function mutations in *PDR1,* which encodes a transcriptional regulator of drug efflux pumps (Sanglard et al. 1999), whereas echinocandin resistance has been linked to non-synonymous variations in two conserved hot-spot regions of *FKS* genes (Perlin 2015a). Antifungal drug resistance, tolerance and adaptation are all related to a cell's ability to respond to stress (Cowen and Steinbach 2008). Under stress, genome maintenance and repair mechanisms are altered, which may lead to the appearance of resistance phenotypes (Healey et al. 2016; Ksiezopolska and Gabaldón 2018). Indeed, rapid adaptation to varying conditions has been attributed to a remarkable genomic plasticity in *Candida* (Carreté et al. 2018). In *C. glabrata*, a large

degree of genomic and phenotypic variation has been described between and within genetically-diverse clades (Carreté et al. 2018), and even within clonal populations infecting a patient (Carreté et al. 2019).

5.3 Results

5.3.1 *Candida glabrata* has a widespread ability to acquire drug and multidrug resistance

Here we set out to explore the evolutionary adaptation of *C. glabrata* to azoles and echinocandins using an in vitro evolution approach coupled to phenotyping and targeted gene and whole genome sequencing (**Supplementary Figure 5.1**, **Materials and Methods**). To this end, 12 strains (**Appendix 1: Data S1**), representing the seven previously described *C. glabrata* clades (Carreté et al. 2018) were subjected to increasing concentrations of antifungal drug(s) in the following regimes: fluconazole (FLZ samples, note the use of upper case for samples/conditions as opposed to lower case for the drug); anidulafungin (ANI); and both drugs in combination (ANIFLZ). In addition, to gain insight into mechanisms of cross-resistance, adaptation to serial exposure to both drugs was studied by growing isolates from the final steps of the ANI samples under the flz regime (AinF), and, conversely, final FLZ isolates under ani (FinA). Control populations of all strains were grown for the same time without any drug (YPD). The experiment comprised a total of 288 independently evolved populations. When exposed to a single drug or to the two drugs in a sequential manner, all populations survived the entire

experiment. However, when simultaneously exposed to both drugs, 21 populations (43.75%) died, including all replicates of each of two strains from clade I (CST109) and clade III (M12). Nevertheless, populations from other strains from these clades survived, indicating that low adaptation potential is strain and not clade-specific. We analyzed available sequences of these two strains (Carreté et al. 2018) and found they shared eight genes with alterations that were not altered in surviving strains within the same clades **(Appendix 1:Data S2**).

We determined susceptibility using Minimum Inhibitory Concentration (MIC) and the relative Area Under the Curve (rAUC) measurements **(Materials and Methods, Figure 5.1 A, Supplementary Figure 5.2, Supplementary Text).** All surviving strains acquired stable resistance to the exposed agent(s), that is, the resistance phenotype was kept for several generations in standard growth conditions after the removal of the selective agent (**Appendix 1: Data S3**, **Figure 5.1 B, Supplementary Figure 5.3**), indicating the phenotype is inherited and genetically encoded. Unexpectedly, we observed increased resistance to flz in a large subset of ANI samples (21/47, MIC> 256), thereby showing that adaptation to ani can frequently induce cross-resistance to flz. The reverse resistance gain to ani in FLZ samples was not observed **(Supplementary Figure 5.3)**. Increased resistance to both drugs (MDR) was often achieved, including all ANIFLZ, a majority of AinF (91.6%) and FinA (97.9%) samples and, due to the mentioned cross-resistance, in 44.7% of ANI samples (**Appendix 1: Data S3, Figure 5.1 C**). In serial drug exposure experiments, previously acquired resistance was

rarely lost during exposure to the second drug (1 FinA, and 4 AinF samples), indicating the phenotype is stable. We evaluated the fitness costs of acquired resistance using AUC values of growth curves in the absence of the drug as a proxy for fitness (fAUC) relative to the fitness of the unevolved (WT) strain (**Figure 5.1 D, Appendix 1: Data S3**). All flz-exposed samples showed a tendency to reduce fitness ($p<10^{-05}$, Kolmogorov-Smirnov test), while the mean fitness of ANI samples remained unaltered ($p>0.05$). Consistently, a small but significant negative correlation between resistance (rAUC) and fitness levels for flz, but not for ani, was detected (**Figure 5.1 E**). Nevertheless, many of the flz-exposed samples retained fitness levels within two standard deviations of the mean of YPD-exposed strains (56% of ANIFLZ, 77% AinF, 81% FLZ and 68% FinA), and only a few samples (2.9%, 5/8 of them ANIFLZ) had severely reduced fitness levels below 50% of the corresponding WT strain. These results indicate that resistance, including MDR, is often achieved at mild fitness costs.

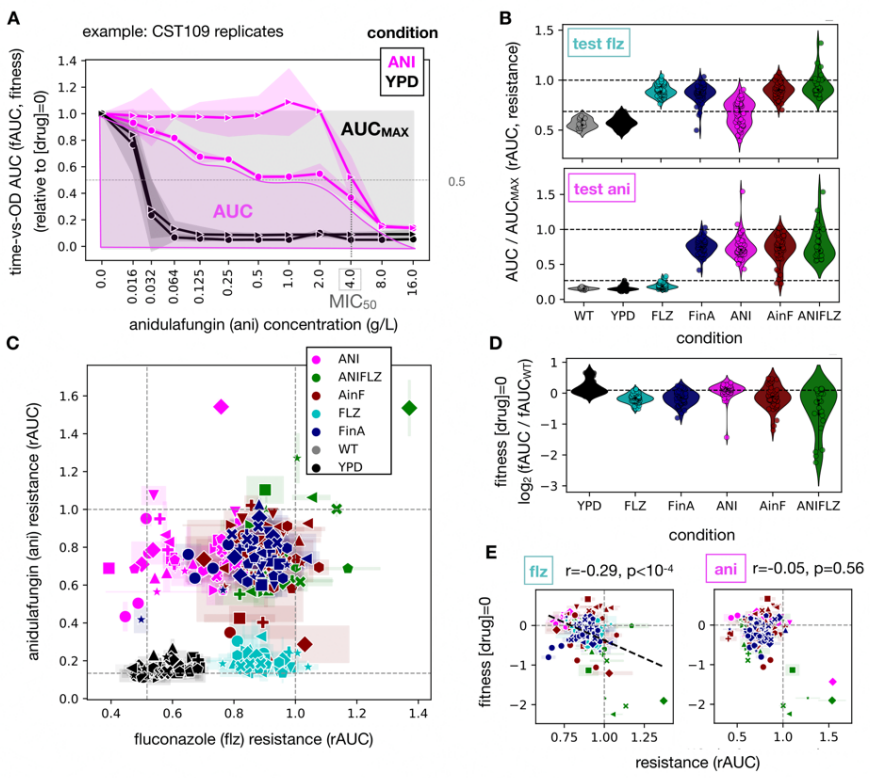


Figure 5.1. Fitness and drug resistance. (A) We measured relative fitness (the ratio between fitness in each drug concentration vs the no drug condition (control) in a time course experiment at several concentrations of flz and ani. Fitness was measured as the Area under the time-vs-optical density (OD) Curve (fAUC). The graph depicts an illustrative example of two replicates of the CST109 strain in the ANI and YPD evolution experiments. The shaded areas represent the median absolute deviation across technical replicates. As a proxy for drug resistance, we defined rAUC as the Area Under the Curve (AUC) of these data (normalized by the maximum AUC, where fitness is maintained across all the range of concentrations (AUC_{MAX})). 50% of growth inhibition, as compared to the no drug control, is marked as MIC_{50}. **(B)** Drug resistance (rAUC) for flz (top) and ani (bottom) across all samples in our experiments. Each point corresponds to an independently-evolved biological replicate. Note that some samples have an rAUC above 1.0, where fitness did not drop upon increasing drug concentration (suggesting high resistance). **(C)** The relationship between ani and flz resistance across all samples. Dashed lines indicate median rAUCs levels for each drug in the YPD samples and $rAUC_{MAX}$ (1.0). Each point corresponds to a biological replicate and the error bars reflect the median absolute deviation across technical replicates. Each marker corresponds to a different strain. **(D)** Fitness in the absence of drug (measured as the $\log_2$ fold change in fAUC (see (**A**)) between each sample and the median fAUC in the WT of the matching strain). **(E)** Fitness in the absence of drugs

is slightly correlated with the levels of flz, but not ani, resistance (rAUC). Spearman's correlation coefficient (r) and p-value are shown for flz (left) and ani (right) resistance. The correlation for flz resistance was maintained when considering only samples with mild fitness defects (fitness>-1, r=-0.22, p-value = 0.0029). Only resistant samples, defined as those with a $\log_2$ fold increase above 1 as compared to the WT **(Supplementary Figure 5.3 B)** were included in this analysis.

5.3.2 The *FKS* mutational spectrum in resistant strains expands beyond hotspot regions

We used a target sequencing approach to screen 121 ani adapted strains for mutations in the typically surveyed hotspot (HS) regions of *FKS* genes (Shields et al. 2015) (see **Materials and Methods**, **Appendix 1: Data S4**). Additionally, we selected 77 representative samples for whole genome sequencing and called small variants (SV), copy number variations (CNV) and genomic rearrangements (GR) appearing de novo in each of the evolved samples (see **Materials and Methods**, **Appendix 1: Data S5**). All 121 ani-evolved strains presented newly acquired non-synonymous (ns) mutations in the targeted *FKS* regions (**Appendix 1: Data S4**), which indicates that *FKS* mutations might be necessary for ani adaptation. Mutations preferentially occurred in *FKS2* over *FKS1*, and in HS1 over HS2 (**Figure 5.2**), suggesting a more prevalent role of these loci. Notably, 22% of *FKS* mutations were outside HS regions. Three resistant strains carried only such non-HS *FKS* mutations (**Appendix 1: Data S4**), and whole genome sequencing in these revealed no additional mutations outside *FKS* genes that could explain the resistance phenotype. These observations suggest that some of these non-HS *FKS* mutations contribute to resistance and emphasize the

importance of studying *FKS* genes beyond HS regions. We validated this observation by reintroducing two such non-HS mutations in *FKS1* and *FKS2* in a wt background, which resulted in the predicted ani-resistance phenotype (**Supplementary Figure 5.4 A**). Overall, the most frequently mutated site in ani adapted samples was *FKS2*-F659 (63 samples, 52.1%, **Appendix 1: Data S4**), with the most prevalent alteration being F659del (52 samples, 43%), which was the only *FKS* mutation in 26 samples (21.5%). This finding suggests that, as compared to replacements, amino acid deletions may more efficiently prevent the binding of the drug, and reinforces the need to consider this type of mutations. Finally, 26 samples exposed to ani (19.8%), carried a truncation in one of the *FKS* genes (two of them with a GR breaking the coding region, **Supplementary text, Supplementary Figure 5.5**), in combination with a ns mutation in the other paralog, indicating that this specific combination might facilitate adaptation.

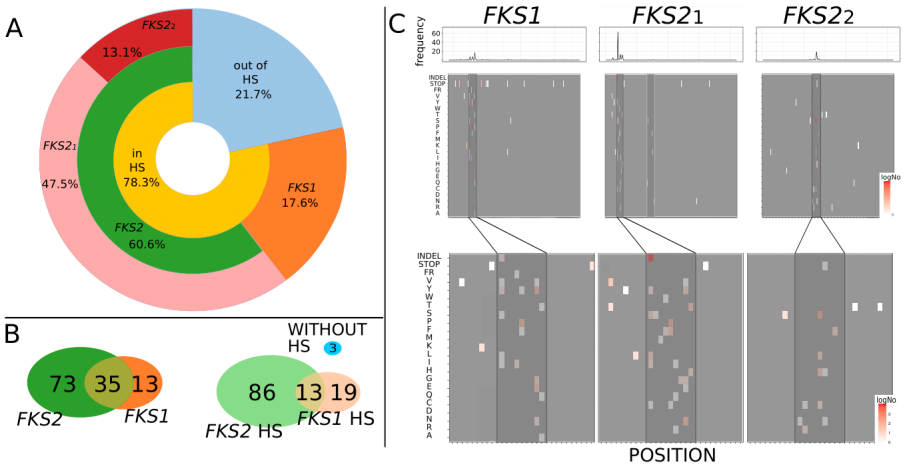


Figure 5.2. Mutational analysis of *FKS* regions. (**A**) Distribution of the mutations in studied regions of *FKS*. A non-negligible presence of mutations outside of HSs can be observed. (**B**) Distributions of samples according to the presence of mutations in particular *FKS* gene and distribution of samples according to the presence of mutations in *FKS* HSs. (**C**) Mutational signatures per sequenced regions: *FKS1*, and *FKS2*_1 and *FKS2*_2. Mutated positions are shown as highlighted boxes at the corresponding amino acid in the mutation, over a gray background. Color scale, from white to red, indicates the observed number of mutations (log scale). Darker gray boxes indicate HSs and white framed box in *FKS2*_1 marked positions with possible other mutational hot spots. The bottom part of the graph represents a zoom in HS and mutations in their close proximity.

5.3.3 Mutational landscapes in resistant strains reveal a high diversity of genetic alterations affecting a restricted set of recurrently mutated genes

The analysis of genome wide mutational patterns revealed no newly acquired SVs in YPD samples, while drug evolved strains accumulated a small number (<10) of variants **(Supplementary Figure 5.6)**. This indicates that susceptible strains are a few mutational steps away from acquiring resistance. Strains carrying distinct *MSH2* variants did not accumulate a different number of mutations, thereby supporting the notion that these represent natural,

functional variants rather than hypermutator mutations (Carreté et al. 2018). As expected (vanden Bossche et al. 1992), we found that aneuploidies were common in experiments involving exposure to flz, but they were not detected in cells exposed only to ani (**Supplementary Figure 5.7**). Total or partial aneuploidies in chromosome E (ChrE), encompassing *ERG11*, were the most common, appearing in 11/16 FLZ, 4/15 AinF, and 2/6 ANIFLZ samples. Most (10/11) FLZ samples with the ChrE aneuploidy retained it upon further exposure to ani (FinA). A partial ChrE aneuploidy could be explained by an unbalanced translocation with chromosome J **(Supplementary text, Supplementary Figure 5.5 D)**, suggesting that GR can drive drug resistance. No heterozygous variant was detected in any of the duplicated chromosomes, thereby indicating that the aneuploidies were adaptive *per se* and not because they allowed faster evolution of duplicated genes. To identify mutations likely associated with the resistance trait, we selected genes that were mutated at least twice independently in our experiment. This search identified nine genes (*ERG11, PDR1, CDR1, CNE1, EPA13, FKS1, FKS2, ERG3, ERG4,* see **Figure 5.3**). Importantly, all resistant strains carried mutations or duplications in at least one of these genes, and the subset of mutated genes largely separated samples by treatment. This strong association of acquired mutations, treatment, and phenotypes indicates that a limited set of genes is central for the acquisition of resistance. The most common altered gene under exposure to flz was *PDR1*, which was in many instances (14/37 strains) accompanied by alterations in *ERG11* (**Figure 5.3, Appendix 1: Data S5).** Although less common, five

resistant strains contained no *PDR1*-related mutations or aneuploidies (**Figure 5.3**), indicating that alternative mechanisms confer resistance on their own. These strains harbored mutations in *ERG3* (three strains, discussed below) and *ERG11* (2 strains). Importantly, *ERG11* mutations and aneuploidies in ChrE, bearing this gene, were strongly anti-correlated, with a single ANIFLZ sample carrying both alterations. In this case the mutation was present in the two alleles, suggesting that the mutation preceded the chromosomal duplication. Among *ERG11* mutations, K152 was the most altered amino acid (12/16 samples), followed by *ERG11*-Y141 (2/16 samples). Although common in other *Candida* species, these mutations have not been commonly reported in *C. glabrata* (Ksiezopolska and Gabaldón 2018). Structural analysis revealed that both altered residues were close to the azole binding pocket (**Figure 5.4**). To assess whether the catalogue of mutations found in our in vitro analysis were representative of what can be found in clinical strains, we compared this catalog with variants found in 393 *C. glabrata* clinical isolates with genomes publicly available at Candidamine (http://candidamine.org/). Our results (**Supplementary text, Supplementary Figure 5.8**) show that the overlap of specific mutations is very low. This low overlap is however expected from the actual large diversity of the identified mutations in our experiments **(Appendix 1: Data S4, S5, Supplementary Figure 5.8 B)** and is similarly low for mutations identified in actual clinical surveys -i.e. SENTRY (Pfaller et al. 2019). These results suggest that, although the set of genes recurrently mutated during the acquisition of resistance is rather

limited (nine genes), the amount of specific mutations -i.e. which residue is mutated and what type of mutation occurs- is large and highly diverse, and only partially covered by our experiment or clinical surveys.

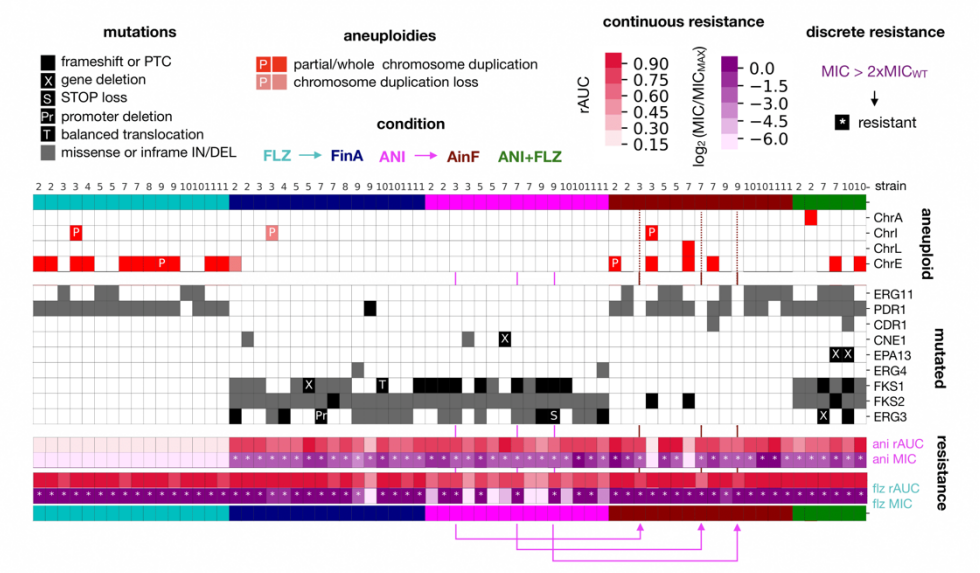


Figure 5.3. Aneuploidies and recurrently mutated genes. Each drug is associated with a particular set of mutated genes and aneuploidies. Columns represent the evolved samples, each strain indicated by a number: 2 - CST34, 3 - EB091, 4 - CST78, 5- M12, 6 - EF1237, 7 - EF1620, 8 - F15, 9 - CBS138, 10 - P35, 11 - BG2. Replicates of the same strain appear in the same order as in the experimental plate. Colors indicate the experimental condition. Blocks show, from top to bottom: chromosomal alterations, mutated genes and susceptibility data. Whole and partial (P) chromosomal duplications appearing newly in each condition are marked as red while losses are marked as light salmon boxes. Protein altering mutations (gray boxes) and losses (black boxes) of genes appearing in at least two drug-evolved samples are shown. Note that we found a balanced translocation in *FKS1* (T) and a deletion in the *ERG3* promoter region (Pr) **(see Results, Supplementary Figure 5.5 D, Supplementary text)**. PTC stands for Premature Termination Codon. Pink arrows indicate the parent-daughter relationships for three AinF samples that did not present any new alteration in recurrent genes.

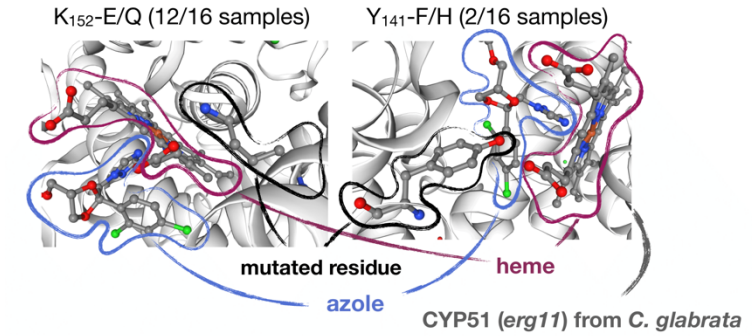


Figure 5.4. Structural localization of frequent *ERG11* mutations. Given the availability of a characterized 3D structure for Erg11p in contact with azoles (pdb id: 5JLC) we inspected the location of recurrently mutated residues and found that they are close to the azole binding pocket. The structure (pdb id: 5JLC) was visualized using SWISS MODEL (Waterhouse et al. 2018). A screenshot of the two residues in the context of itraconazole and a heme group is shown. The basic group of K152 is close to an acid group in heme, potentially establishing an electrostatic interaction that is important for stability. Importantly, Y141 is conserved with Y132, a position that has been mutated in various other azole resistant *Candida* species (Lockhart et al. 2017; Berkow et al. 2015; Tan et al. 2015). As a possible mechanism of resistance, we hypothesize that the substitution by E or Q destabilizes this interaction, thereby impairing the binding of azoles.

5.3.4 Crosstalk between echinocandin and fluconazole resistance

In the experiments of sequential exposure to the two drugs, all samples successfully adapted, in turn, to the two challenges. When adapting to the new drug, most samples (90 out of 95) retained the previously acquired resistance, resulting in MDR (**Figure 5.5 A and B**). However, three sequenced samples lost the previously acquired resistance upon the change of selective conditions (according to MIC, see **Supplementary Figure 5.3)**. These included a FinA sample and two AinF samples. This FinA sample acquired a premature termination codon in *PDR1*, which may revert the flz resistance conferred by previous mutations in this gene. In the two AinF

samples that lost resistance to ani, we found frame-shift mutations in *FKS2* downstream of the ani resistance-conferring mutations found in the parental ANI samples (**Figure 5.5 A**). Interestingly, both of the ANI parents carried only one *FKS2* mutation and alterations in *CNE1,* encoding an ER protein involved in quality control of misfolded proteins (Molinari et al. 2004). This remarkable coincidence suggests that the combination of these alterations is related to a higher propensity to lose resistance, although this hypothesis needs further study. Except for a single ChrA duplication found in one strain, most ANIFLZ samples showed mutational signatures similar to those acquired during sequential exposure to the two drugs (AinF and FinA, **Figure 5.3**). This observation suggests that the genetic basis driving acquisition of resistance to each of the drugs is similar when the two drugs are in combination.

A remarkable finding of our experiment is the cross-resistance to flz found in a significant fraction of ANI samples (see above). Whole genome sequencing of seven of these strains revealed that all of them carried alterations in *ERG3*, which encodes the C-5 sterol desaturase of the ergosterol pathway (**Figure 5.3**). This association was further explored by Sanger-based target sequencing of the *ERG3* gene in the remaining ani-evolved strains, which showed that all 21 ani-evolved strains showing cross-resistance to flz (MIC>256ug/ml) carried alterations in *ERG3* (**Appendix 1: Data S6**). Accordingly, we detected a significant association between *ERG3* ns mutations and flz resistance in ANI samples **(Figure 5.5 C and D, Supplementary Figure 5.9)**. Interestingly, the relationship between fitness and flz concentration was different as compared to FLZ samples

(**Supplementary Figure 5.9 C**)**.** This finding suggests that the quantitative contribution of *ERG3* mutations to flz resistance differs from that of *PDR1* or *ERG11* alterations. Resistance to flz is often spontaneously acquired in *C. glabrata* by partial or total loss of mitochondrial DNA, rendering a so-called *petite* phenotype (Kaur et al. 2004). However, we can discard this effect in the identified *ERG3* mutants due to the absence of deletions in the mtDNA (**Figure 5.3, Appendix 1: Data S4**), and the absence of a *petite* phenotype (**Supplementary Figure 5.10**). When *ERG3*-mutated strains were subsequently exposed to flz (AinF), three of them did not acquire additional mutations in *PDR1* or *ERG11*, nor did they present ChrE duplications, thereby suggesting that their *ERG3* mutations are indeed responsible for their survival in flz. In support of this notion, the levels of flz resistance of these three AinF samples and their respective ANI parents were similar **(see Figure 5.5A)**. However, the relationship between *ERG3* alterations and cross-resistance to flz was incomplete and mutation-dependent, as earlier work has shown that *ERG3* deletion does not affect flz resistance (Geber et al. 1995), and we found that out of 28 ANI samples harboring *ERG3* mutations, six - carrying premature stop (3), missense (2), and frameshift (1) mutations - retained wild type levels of susceptibility. Consistent with some *ERG3* alterations being selected under exposure to ani, 2 ANIFLZ and 6 FinA samples bearing *ERG3* changes additional to *PDR1* and/or *ERG11* mutations were detected (**Figure 5.3**). Incidentally, another FinA sample carried a deletion in the gene immediately upstream of *ERG3* (*CAGL0F01815g*, of unknown function), which may result in regulatory alterations of *ERG3*

through disruption of the promoter **(Supplementary text, Supplementary Figure 5.5 A)**. To investigate this relationship further we re-introduced one of the *ERG3* mutations (D122Y) in an ani-resistant background, which conferred the expected flz-resistance phenotype (**Supplementary Figure 5.4 B**). However, attempts to re-introduce *ERG3* mutations in a wt background coupled to selection of transformants in flz were unsuccessful (no other expected phenotype is known for this mutation), suggesting that this cross-resistance mechanism appears only in an ani-resistant background context. Consistent with this view, we did not observe *ERG3* mutations when cells were exposed only to flz. Additionally, we traced the order of appearance of *ERG3* and *FKS* mutations along intermediate generations in ANI strains and found equal number of cases (two each) in which either *ERG3* or *FKS* mutations predated the other one, and five cases in which both mutations are traced to the same intermediate generation (**Appendix 1**:**Data S7).** This data suggest one mutation does not necessarily predate the other one. Altogether, our results support a dual role of *ERG3* alterations in the adaptation to ani and in causing cross resistance to flz in *C. glabrata*.

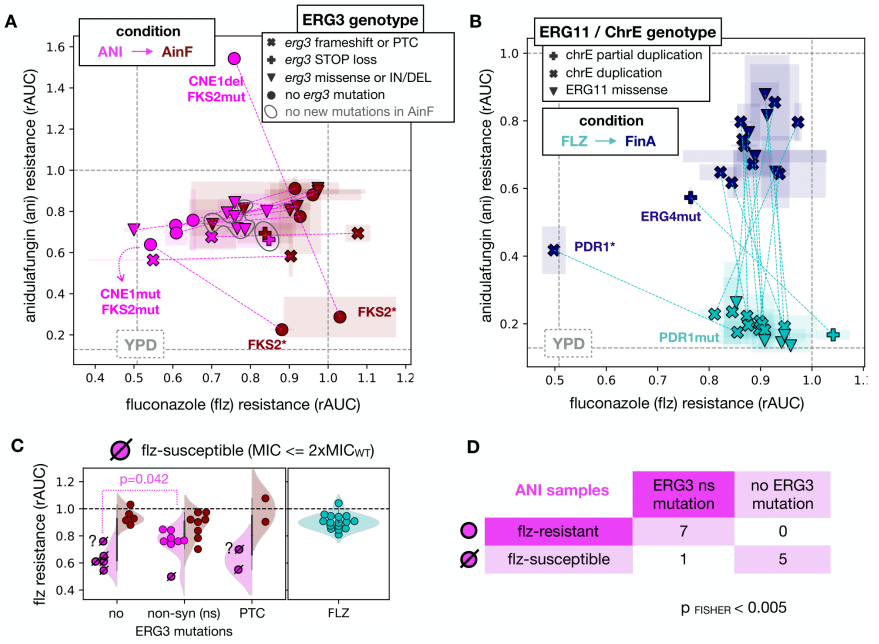


Figure 5.5. Paths to multidrug resistance. (A) Relationship between rAUC of ani and flz in ANI (pink) and AinF (red) samples. The pink dashed lines indicate parent – daughter relationship (ANI-AinF). The gray dashed lines indicate the maximum rAUC (1.0) and the median rAUC across YPD samples in each drug. The symbols represent different types of *ERG3* mutations, and the gray circles outline three samples that did not acquire any new mutation in the recurrent genes in AinF. The two ANI samples with alteration in *CNE1* which lost ani resistance due to truncations in *FKS2*(*) in AinF samples are marked. One of these FinA samples showed high ani resistance (above 1.0, meaning the fitness was higher in ani than in no drug), but also showed low basal fitness, which means that the high resistance value may be not representative. (**B)** Relationship between rAUC of ani and flz in FLZ (light blue) and FinA (dark blue) samples. The green dashed lines indicate parent – daughter relationship (FLZ-FinA). The gray dashed lines indicate the maximum rAUC (1.0) and the median rAUC across YPD samples in each drug. No acquisition of ani resistance was observed in FLZ samples but only as a result of ani (FinA). The symbols represent the presence of *ERG11* missense mutations or chromosome E aneuploidies. Two FinA samples showed a drop in flz resistance levels. One of them carried a *PDR1* premature termination codon (*), which resulted in susceptibility according to our MIC-based thresholding **(see Methods),** reduced flz resistance below the median rAUC value of YPD samples. The other sample carried *ERG4* mutation that resulted in a reduction but not a total loss of flz resistance. (**C)** Non-synonymous (including missense and STOP loss) *ERG3* mutations are associated with higher flz-resistance (rAUC) in ANI samples. The p-value corresponds to a Kolmogorov-Smirnov test. The corresponding AinF and FLZ samples are also shown for comparison of flz resistance levels. The dashed symbols represent samples that were found flz-susceptible according to our MIC-based thresholding (**see Methods**). Note that two samples (marked with "?") were

found as susceptible but have rAUC values in the range of resistant samples. This mismatch is clarified in **Supplementary Figure 5.9 C, D (D)** The presence of *ERG3* non-synonymous mutations is correlated with discrete flz resistance in ANI samples. The number of ANI samples in each category, and the p-value of a Fisher test are shown.

5.4 Discussion

We have shown the suitability of in vitro approaches to study the evolutionary acquisition of resistance to antifungal drugs. Our results show that *C. glabrata* exhibits a remarkable capacity to acquire resistance to the tested drugs, independently of the phylogenetic background of the strain (Carreté et al. 2018). This is also true for the case of serial exposure to the two drugs, to which all strains and replicates adapted. However, the combined exposure to both drugs prevents adaptation in a significant fraction of the cases, with two strains from two different clades showing inability to develop resistance in this scenario. Altogether, our results show that neither phylogenetic clade, nor the presence of non-synonymous mutations in *MSH2* are good predictors of the ability to develop MDR, which is pervasive in *C. glabrata*. Whole genome sequencing revealed a relatively limited catalog of a few genes that are almost inevitably affected upon sustained adaptation to antifungal drugs. Apart from the common alterations in *FKS, PDR1* and *ERG11*, five other genes (*CDR1, CNE1, EPA13, ERG3,* and *ERG4*) were recurrently mutated in our experiments. This finding indicates that alternative mechanisms may be concomitantly used to achieve a stable resistance phenotype. Alterations in the promoter region of *CDR1* have been already reported in azole resistant strains (Tsai et al. 2006; Looi et al. 2005), and our results suggest that alterations of the protein product

may also contribute to flz adaptation. As discussed, *CNE1* is involved in quality control of misfolded proteins in the ER. *EPA13* is a sub-telomerically encoded lectin-like adhesin with a role in cell adhesion, whose potential role in drug resistance is unknown. *ERG4* is another gene involved in the ergosterol biosynthesis pathway which, similarly to *ERG3*, may influence resistance to fluconazole. Future experiments should help determine the order of appearance of these mutations, and their specific roles in drug resistance or adaptation. In addition, our results suggest that GRs and CNVs around these genes are related to drug resistance, as previously proposed in *C. albicans* (Todd and Selmecki 2020). This indicates that the traditional focus on SNPs is underpowered to understand the genomic drivers of drug resistance.

An important result from our experiment is the observation that adaptation to ani often results in cross-resistance to flz (but not the other way around). This result was unexpected, given the different modes of action of the two drugs, where ani affects the cell wall in a fungicidal manner and flz affects the cell membrane causing growth arrest. This observation is of high relevance given expanding MDR in *C. glabrata*, and also that some recent guidelines (e.g. from the Infectious Disease Society of America (Pappas et al. 2016)) recommend an echinocandin rather than an azole based initial therapy against most invasive *Candida* spp infections. The scarcity of sequenced genomes for MDR clinical strains and the lack of information of the treatment regime they were exposed to (see **Methods**), prevented us from assessing how commonly this cross-resistance mechanism occurs in the clinics, something which

deserves further investigation. We studied the possible molecular basis of such cross-resistance, and found compelling evidence of the involvement of *ERG3* mutations. In our experiment, alterations in this gene often appeared under ani exposure, and were retained in subsequent flz exposure, sometimes without any further mutation being acquired that would explain acquisition of resistance to flz. In addition, *ERG3* mutations were always present in ani-evolved strains that showed cross-resistance to flz, and we confirmed the causative association of flz resistance of the *ERG3* alteration by re-introducing it in an ani-resistant flz-sensitive background. This link, between *ERG3* and cross-resistance might not be restricted to *C. glabrata* as *ERG3* mutations leading to depletion of ergosterol and the accumulation of less toxic sterols when *ERG11* is inhibited have been implicated in cross-resistance between azoles and polyenes in *S. cerevisiae* and *C. albicans* (Cowen et al. 2014; Kelly et al. 1997; Martel et al. 2010; Morio et al. 2012) and between echinocandins and azoles in *C. parapsilosis* (Rybak et al. 2017; Papp et al. 2020). Why *ERG3* mutations are often acquired under exposure to ani, and how they contribute to resistance to flz remain unclear and need further attention. A speculative scenario is that certain *ERG3* mutations lead to alterations in the membrane composition in a way that partially compensates cell-wall alterations induced by ani exposure. In this regard, it has been reported that cell membrane modifications related to changes in ergosterol production affect the structure and composition of the cell wall (Lesage and Bussey 2006).

5.5 Materials and Methods

Experimental Design

The main objective of this study was assessing the evolutionary paths leading to antifungal drug resistance in *Candida glabrata* from the perspective of comparative genomics. More precisely, we wanted to find the mutational signatures of drug resistance and cross-resistance in drugs of clinical use. We also investigated the relationship between genomic changes, fitness and drug susceptibility. Our ultimate goal was to shed light on the cellular mechanisms and evolutionary constraints of antifungal drug resistance in this important pathogen.

To reach these objectives we carried out in vitro evolution for 12 *C. glabrata* strains in anidulafungin, fluconazole and the combination of both. We performed targeted and whole-genome sequencing of the evolved samples in order to find the mutational signatures of drug resistance and cross-resistance. In addition, we measured their fitness and drug susceptibility to assess the relationship between genomic changes and phenotypes. Finally, we used a CRISPR-Cas9 approach to validate how specific mutations confer the predicted phenotypes.

Strains

The 12 strains of *C. glabrata* used in this study are described in **Appendix 1: Data S1**. Eleven clinical strains had been previously analyzed for several phenotypic characteristics, including susceptibility to various drugs (Carreté et al. 2018). In addition, they have been shown to belong to seven genetically distinct clades. The remaining strain (SLL2_glab) was isolated from an oral wash of a

healthy individual from Spain, and can thus be considered commensal. SLL2 glab was sequenced within this project and assigned to clade 7.

In vitro evolution

We conducted experimental evolution experiments using a batch serial transfer approach (Bódi et al. 2017) (**Supplementary Figure 5.1**). Wild type (WT) strains were collected from glycerol stocks, plated, left to grow until single colonies could be detected and re-plated again for an overnight culture (YPD agar plate at 37ºC). A few colonies were suspended in sterile water and diluted to $2.5*10^5$ colony forming units per milliliter (CFU/mL). A 96 deep-well plate (2.2mL) with 450 µL of YPD – the master plate - was inoculated with 50 µL of the cell suspension in four replicates for each strain. To ensure lack of cross contamination the inoculations were organized using a checkerboard design (**Supplementary Figure 5.1**) and visually inspected for unwanted growth in non-inoculated wells. The master plate was covered with a sandwich cover (Enzyscreeen BV) to ensure optimal oxygenation and limit evaporation. It was then shaken at 300 rpm, and incubated at 37˚C for 72 h. Afterwards, 50 µL of each culture was transferred to a fresh 450 mL of YPD medium and left again to grow in the same conditions. Next, 50 µL of samples from the master plate were distributed into four independent 96-well plates containing 450 mL of YPD medium supplemented with the following: 1) an echinocandin: anidulafungin (drug: ani, outcome samples: ANI); 2) an azole: fluconazole (flz, FLZ); 3) anidulafungin and fluconazole (aniflz, ANIFLZ); or 4) no drug (YPD). Adaptation

to the drugs involved passages of the (50 µL) samples to a fresh (450 µL) medium every 3 days, and in every second passage the concentrations of flz and ani were gradually increased from 4 µg/mL and 0.016 µg/mL to 192 µg/mL and 4 µg/mL, respectively (**Appendix 1:Data S8**), except YPD where no change in the composition of the medium was applied. For each passage the medium with antifungals was freshly made on the same day using a frozen stock of the drugs. Before each increase in drug concentration, part of the culture was frozen and stored at -80˚C (100µL of the sample in 100 µL of 50% glycerol). All in all, the experiment involved 6 days of adaptation to the same conditions before increasing the stress, and further adaptation. Starting with 4 µg/mL flz and 0.016 µg/mL ani, the experiments finished after 54 days, 18 passages with drugs, and 9 increments in drug concentrations. We estimate this period to involve between 60 to 500 generations (assuming a minimum of three doublings per passage in a 1:10 dilution and a maximum of 5-10 generations/day based on earlier studies (Vale-Silva et al. 2017)). From the last passage we selected, stored and analyzed single colonies that were picked from agar plates and regrown on liquid medium supplemented with the last concentrations of the drugs used in each condition. In the second part of the experiment, we repeated the evolution experiment, this time evolving ANI isolates in flz (AinF), and FLZ isolates in ani (FinA), using the same regimes as explained above. Due to the inability to re-grow two samples (1 ANI and 1 FinA) from the glycerol stock, and several extinct populations in the simultaneous treatment with 2 drugs, the total number of analyzed samples was as follows: 48 FLZ,

47 FinA, 47 ANI, 48 AinF, 21 ANIFLZ and 48 YPD. The growth of the samples was visually assessed by their capacity to grow at the last drug concentration(s) after 4 x 3-day long passages in YPD medium without drugs.

Susceptibility tests

Susceptibility to flz and ani was studied in a high-throughput manner using a robot, and recording not only the endpoints but also the growth curves of all dilution assays over at least 18h. Susceptibility tests were performed in at least three replicates following the EUCAST E.DEF 7.3.1. protocol (Arendrup et al. 2012). Briefly, isolates were pinned on agar containing RPMI with 2% glucose buffered with MOPS (3-(N-morpholino) propanesulfonic acid) and grown at 37°C. Fresh overnight cultured strains were adjusted to 2-10*10^5 CFU/mL in distilled water. Next 50 µL of broth was then added to 150 µL antifungal solution (in RPMI /w MOPS) and incubated for around 18h at 37°C. OD600nm was measured every 60 - 90 min and growth was evaluated after around 18h. The range of concentrations tested was 16- 0.016 µg/mL for ani 256-0.25 µg/mL for flz, following EUCAST guidelines .

Fitness and susceptibility measurements

For each sample at each drug concentration, fitness was measured as the area under the time-vs-optical density curve (hereafter referred as fAUC, calculated with the qfa package (v0.0-44 http://qfa.r-forge.r-project.org/). Minimum Inhibitory Concentration 50 (MIC) values were calculated as the minimum concentration where the fAUC

relative to the no-drug control was below 50%. If 50% of the inhibition was not met within the tested concentration range, then MIC was set to twice the maximum assayed concentration for numerical analyses in **Figure 5.3, Supplementary Figure 5.2, Supplementary Figure 5.3.** We also define rAUC as the area under the drug concentration-vs-relative fitness curve (AUC), normalized by the maximum AUC_{MAX} where there is no change in fitness across the entire range of concentrations **(Figure 5.1 A)**. rAUC was used as a proxy for the quantitative levels of resistance for each sample. To filter out experimental artifacts, we kept the three technical replicates that were closest to the median for each sample and measure (fitness, relative fitness, MIC and rAUC).

To correct for intraspecific fitness differences (Carreté et al. 2018), we based our fitness analysis **(see Results)** on the log_2-ratio between the fAUC of each sample and the unevolved WT strain. This value was used as a proxy for fitness changes occurring during the experiment. Under the same reasoning, we defined strains with acquired resistance as those where the MIC was more than 2 times the WT MIC. This threshold separated our samples clearly into susceptible and resistant strains **(Supplementary Figure 5.3)**. Doubling rate per hour was inferred from the maximum slope in the time-vs-log_2OD data using bins of 3 timepoints for the analysis of EF1620_7B_ANI (see **Supplementary text**).

DNA extraction

A modified protocol from the MasterPure™ Yeast DNA Purification Kit was used to extract DNA. In brief, samples were grown overnight

in liquid YPD at 37˚C. Cells were pelleted and lysed with RNAse treatment at 65˚C for 15 min. After 5min of cooling down on ice, samples were purified by the kit reagent by mixing, centrifugation and removal of the debris as described in the kit protocol. Further, samples were left at -20˚C with absolute ethanol for at least 2 h after which the DNA was precipitated for 30 min at 4˚C. The pellet was washed in 70% ethanol and left to dry. TE buffer was used to resuspend the DNA. The Genomic DNA Clean & Concentrator kit (Zymo Research) was used for the final purification.

Target *FKS* and *ERG3* sequencing

All ani-exposed samples (ANI, ANIFLZ and FinA) were examined for mutations in 1 region of *FKS1* and 2 regions of *FKS2* encompassing echinocandin resistance mutational HSs (Ksiezopolska and Gabaldón 2018). Three samples without mutations in the above-mentioned HSs were also inspected in the HS2 of *FKS1*. We used PCR primers described earlier (Thompson et al. 2008) (**Appendix 1: Data S9**). ANI samples not subjected to WGS were also analyzed by 2 PCRs with 2 sets of primers to investigate *ERG3* mutations (**Appendix 1: Data S9**). PCRs were carried out by using Taq DNA polymerase from DongShengBio. The reaction mixture included primers of concentration of 0.4 μM, 20 μL Taq DNA polymerase, 1μL liquid sample grown for 24-48 h in YPD and water up to a final volume of 40 μL. Optimase ProtocolWriter™ was used to develop conditions for each primer set.

We tested for the possible trajectories of final *FKS* and *ERG3* mutations in the 10 ANI samples subjected to WGS and presenting

ERG3 alterations to infer which might have appeared first in the evolution. We selected and analyzed single colonies from our glycerols stocks of stored populations after the 2nd passage at 0.032, 0.064, 0.128 and 0.256 ug/ml ani (beginning of the adaptation). PCRs were carried out as described above.

Petite phenotype in ani adapted mutants

10 ANI samples that underwent WGS and show changes in *ERG3* gene, CBS138 WT and *S. cerevisiae* petite control were inspected for presenting a petite phenotype. Samples were grown on YPD (1% yeast extract, 2% bactopeptone, 2% glucose) and YPG (1% yeast extract, 2% bactopeptone, 2% glycerol) for 24h-48h.

Whole genome sequencing

Genome sequences were obtained at the Ultra-sequencing core facility of the CRG, using Illumina HiSeq 2500 sequencing machines, and as previously described (Carreté et al. 2018). In brief, libraries of paired-end, 125 bases-long reads were prepared. The DNA was fragmented by nebulization or in Covaris to a final size of ~600 bp. After shearing, the ends of the DNA fragments were blunted with T4 DNA polymerase and the Klenow fragment (New England Biolabs). DNA was purified using QIAquick PCR purification kit (Qiagen). 3′-adenylation was done by incubation with dATP and the 3′-5′-exo-Klenow fragment (New England Biolabs). DNA was purified using MinElute spin columns (Qiagen) and double-stranded Illumina paired-end adapters were ligated to the DNA using rapid T4 DNA ligase (New England Biolabs). After

another purification step, adapter-ligated fragments were enriched, and adapters were extended by selective amplification in an 18-cycle PCR reaction using Phusion DNA polymerase (Finnzymes). Libraries were quantified and loaded into Illumina flow-cells at concentrations of 7–20 pM. Cluster generation was done in an Illumina cluster station. Sequence runs of 2 × 100 cycles were performed on the sequencing instrument. Base calling was performed using Illumina pipeline software. In multiplexed libraries, we used 4 bp internal indexes (5 indexed sequences). De-convolution was done using the CASAVA software (Illumina). Sequence data of the genomes have been deposited in the Short Read Archive (SRA) database, with accession number PRJNA635652.

Small variant calling and interpretation

For each library, we first performed quality control of the reads with *fastqc* (v0.11.8, https://www.bioinformatics.babraham.ac.uk/projects/fastqc) and trimming with *trimmomatic* (v0.38 (Bolger, Lohse, and Usadel 2014)). The trimmed reads were aligned against the reference *C. glabrata* genome (the latest version by 12/03/2019, which is v_s02-m07-r35 from the Candida Genome Database (Skrzypek et al. 2017)) using *Burrows-Wheeler Alignment* (bwa v0.7.17) *mem* (http://bio-bwa.sourceforge.net/bwa.shtml). In addition, indexing of the genome and construction of a sequence dictionary was performed with *samtools* (v1.9 (Li et al. 2009)) and *picard* (v2.18.26 http://broadinstitute.github.io/picard/), respectively.

We next used three different algorithms (*GATK Haplotype Caller* (HC) (v4.1.2 (Poplin et al., n.d.)), freebayes (FB) (v1.3.1 https://arxiv.org/abs/1207.3907) and bcftools (BT) (v1.9, https://github.com/samtools/bcftools) to call and filter Single Nucleotide Polymorphisms (SNP) and small insertions/deletions (IN/DEL) in both haploid and diploid configurations. We defined as high-confidence (PASS) variants those with read depth above 20, with extra filters for HC and FB. For HC, we kept as PASS variants those where 1) there were less than four additional variants within 20 bases; 2) the mapping quality was above 40; 3) the confidence based on depth was above 2; 4) the phred-scaled p-value was below 60; 5) the MQRankSum was above -12.5 and 6) the ReadPosRankSum was above -8. For FB, we kept as PASS variants those where 1) quality was above 1 or alternate allele observation count was above 10; 2) strand balance probability of the alternate allele was above 0; 3) number of observations in the reverse strand was above 0; and 4) number of reads placed to the right/left of the allele were above 1. We further used *vcfallelicprimitives* from *vcflib* (v1.0.0 https://github.com/vcflib/vcflib) to uniformize the called variants across the three algorithms, and the *ensembl Variant Effect Predictor* (v96.3 (McLaren et al. 2016)) to annotate the potential functional effect of each variant in both coding and non-coding regions. In addition, we developed a tool to visualize (and better interpret) the genomic location of each variant across multiple samples using the *python plotly* package (v2.7). This pipeline is ready to use for any paired-end short-read sequencing library at

https://github.com/Gabaldonlab/projects/tree/master/mschikora/scripts/VarCall_CNV_ReadProcessing.

We considered PASS variants to be those SNPs that passed the filtering of the three algorithms and those INDELs that passed both HC/FB filters (which were shown to have highest overlap)**.** For each sample evolved in drug conditions, we defined variants newly-acquired during the experiment to be those that were not called in any of the corresponding WT and YPD samples. We ran this variant calling pipeline in both haploid and diploid configurations for all samples. Diploid variants may have appeared in regions that are under whole-chromosome duplications. We keep only as true "heterozygous" or "homozygous" diploid variants as those that appear to be like this by all the programs tested and within a duplicated chromosome **(see below)**.

Identification of large aneuploidies, segmental duplications and deletions

To detect genes affected by CNV we calculated the read depth for each gene relative to the median read depth per gene across all nuclear chromosomes that did not have signs of large duplications **(see Results)** (hereafter referred to as relative coverage). The read depth was calculated using *mosdepth* (v0.2.6 (Pedersen and Quinlan 2018)). We then defined deleted genes as those with >50% of their length not covered by reads. To keep only gene deletions appearing during the experiment we further filtered out genes that were also lost in the corresponding WT or with a relative coverage below 0.1 in YPD-evolved sample (which may suggest a loss also in the WT or in

the YPD). We manually curated the deletion list to find regions potentially deleted in a previous sample of the evolution experiment, which was the case of a small region in chromosome D (including *CNE1*, with a relative coverage below 0.1 in EF1620_7B_ANI) and the *S. cerevisiae GPB2* ortholog (with a relative coverage below 0.1 in EF1620_7B_ANI). Importantly, these two genes were lost in a single genomic rearrangement **(Supplementary text, Supplementary Figure 5.5 F).**

CNV was defined by calculating the log_2 ratio between the relative coverage of each sample against the matching YPD (log_2cov_vsYPD). Copy-number (CN) increase refers to log_2cov_vsYPD above 1 and a relative coverage above 1.8, while CN decrease refers to log_2cov_vsYPD below -1 and a relative coverage of the corresponding YPD above 1.8. The rationale of this filtering was to detect genes lost and under CNV during drug exposure, correcting for intrinsic biases in per-gene coverage. As noted in other studies, we found that relative coverage was correlated with the distance to the telomere (hereafter referred as "smiley-pattern"), which may be an artifact of library preparation and/or sequencing, with this effect varying across samples. We hypothesize that this is partially why most of the CNV was found in subtelomeric regions (defined here as the first and last 50 genes of a chromosome). We thus filtered out any CNV call that was not supported by equivalent genomic rearrangements (see below). In addition, chromosomes with large aneuploidies were defined as those where we consistently observe genes with increased CN and relative coverage around 2x

across a region spanning at least 10% of the non-subtelomeric chromosome (**Supplementary Figure 5.7)**.

Analysis of genomic rearrangements

To identify GR we implemented an algorithm that uses split-reads, discordantly aligned read-pairs and de novo assembly evidence to call genomic breakpoints and interpret the resulting GRs and CNVs. Breakpoints were called using gridss (v2.8.1 (Cameron et al. 2017)) and integrated into complex structural variation with clove (v0.17 (Schröder et al. 2017)). The straightforward implementation of this pipeline was challenging because of the lack of established parameters for yeast genomes, and the "smiley-pattern" bias (see above) impeding the use of a single read-depth threshold for filtering deletions and tandem duplications (used by clove). We thus chose the running and filtering parameters from a simulation-based optimization implemented in the *perSVade* pipeline (v0, https://github.com/Gabaldonlab/perSVade).

GR appearing during the experiment were defined as those where none of the breakends (each of the ends of a breakpoint) matched a breakend in any of the parents (with an overlap of less 200 bp), in a way that resembles the small variant calling (see above). This is an extremely conservative approach (as most called breakends in the parents may be false positives) to ensure high confidence in our final set of variants. In addition, we defined "haploid breakends" as those with an allele frequency (AF) above 0.75 and "heterozygous breakends" as those with an AF>0.25. We also filtered out tandem duplications, inversions and deletions where any of the breakends

was not haploid, as these variants cannot yield heterozygous breakends in haploid chromosomes. Note that we did not detect any such heterozygous events in the aneuploid chromosomes. Furthermore, we manually curated the results to identify errors in the summarization of breakpoints into complex rearrangements. This approach yielded one sample (P35_10E_FinA) with two reciprocal inverted interchromosomal breakpoints between close positions (less than 200 bp apart) of ChrG (breaking the CDS of *FKS1*) and ChrM. These were called as two independent unbalanced translocations, but we interpret them as an inverted balanced translocation between the two chromosomes. The coverage "smiley-pattern" was also consistent with this model.

Presence of all the GRs discussed in the text was confirmed with PCR using primers specifically designed to provide amplicons only in the presence of the GR (translocations) or with a different size (deletions). Results are presented in **Figure 5.7 F**. All events were positively confirmed. Primers used for each GR validation are presented in the supp **Appendix 1: Data S9**. PCRs were performed using Taq DNA polymerase from DongShengBio. The reaction mixture included primers of concentration of 0.4 µM, 15 µL Taq DNA polymerase, 1µL liquid sample grown for 24 h in YPD and water up to a final volume of 30 µL. Optimase ProtocolWriter™ was used to develop conditions for each primer set.

Statistical Analysis

The association between acquired mutations and experimental conditions, clades, or strains was calculated using chi-square test, implemented in R. All the Fisher, Kolmogorov-Smirnov and Spearman correlation tests were performed using the python scipy.stats package.

Analysis of clinical isolates' sequencing datasets

We obtained all the variant calling files for publicly available whole genome sequences of *Candida* clinical isolates from the CandidaMine database (v1, http://candidamine.org, publication in progress). The MIC values for each sample were obtained by manual curation of the associated literature, when available.

In *Candida glabrata* we could find these data in 126/393 clinical isolates, including resistance to fluconazole (flz 126/126), posaconazole (pos 84/126), voriconazole (vrz 91/126), isavuconazole (ivz 37/126), micafungin (mif 42/126), anidulafungin (ani 9/126) and caspofungin (cas 91/126). Some of these drugs lack established clinical resistance breakpoints, which did not allow a direct identification of resistant isolates. We thus defined the resistance breakpoint for each drug as 2x the maximum MIC reported in a set of susceptible isolates (from (Carreté et al. 2018)). Ani susceptibility was not measured for these isolates, so that we took the standard EUCAST breakpoint to define ani resistance. This data is sparse, so that we do not always know the MIC values for all drugs in a given isolate. We thus focused our analysis on "azole" or "echinocandin"

resistance instead of splitting by individual drugs. In order to achieve this, we defined an isolate to be “resistant” to a given class of drugs if it was resistant to all the measured drugs of that class. This yielded 41/126 and 19/91 isolates resistant to all tested azoles or echinocandins, respectively. We could find two samples with resistance to both classes of drugs.

In *Candida albicans* we could find MIC data for 187/478 clinical isolates. We could define the resistance breakpoints according to EUCAST for all tested drugs but caspofungin (cas). We defined an isolate to be cas-resistant if the MIC was above the percentile 90. This yielded 39/186 and 9/150 isolates resistant to all tested azoles or echinocandins, respectively. We could find one sample with resistance to both classes of drugs.

Given the low numbers of samples with resistance to both drugs we conclude that the available data is insufficient to perform analysis of cross-resistance or multidrug resistance.

CRISPR-Cas9 based genetic modifications

CRISPR-Cas9 based genetic modifications were introduced using ribonucleoproteins (RNPs) and following a previously described method by Grahl (Grahl et al. 2017). In brief, RNPs were created using the Alt-R CRISPR-Cas9 system bought from Integrated DNA Technologies, Inc.). The CRISPR machinery included: purified Alt-R S.p. Cas9 Nuclease V3, and guide RNA containing universal transactivating Alt-R CRISPR-Cas9 tracrRNA and target specific

crRNA. All crRNAs and synthetic donor sequences are in the **Appendix 1: Data S9**.

Three synthetic DNA fragments were ordered from Integrated DNA Technologies, Inc.: donor_FKS1, donor_FKS2 and donor_ERG3. All three contained mutations, W681L (G2042T) in *FKS1*, A651T (G1951A) in *FKS2* and D122Y (G364T) in *ERG3*, as well as additional synonymous mutations in PAM region (short NGG sequence that follows the DNA region targeted for cleavage by the CRISPR system) to bypass recutting by the Cas9 once the donor DNA is integrated, and thereby increasing the number of positive transformations. A large donor DNA containing *ERG3* mutation (D122Y) was also amplified from the 3B_ANI sample by FWD: TCCTCGACCAACAGACCATC and REV: TGTTCGAGACTAGTAGCGGG primers.

Both of the *FKS* mutations were transformed into the CBS138 reference strain and the positive transformants were selected on YPD medium supplemented with 0.5µg/ml of anidulafungin and inspected for the presence of the mutation by sanger sequencing.

The synthetic *ERG3* containing fragment as well as the large donor DNA containing *ERG3* mutation were transformed into 3H_ANI sample and selected on 64ug/ml fluconazole. The same trials of transformations were performed on CBS138 WT strain but the selection of positive transformants was unsuccessful. Spot tests were performed to visualize changes that the transformations exert on antifungal drugs susceptibilities. Briefly, overnight cultures were set

to the OD=1, serially diluted 10fold and 10ul was spotted on YPD agar plates supplemented with antifungal drugs.

5.6 Supplementary Text

Extended description of results MIC and rAUC measures of antifungal drug resistance

As discussed in the main text, both MIC and rAUC measurements were correlated (**Supplementary Figure 5.2**). However, they presented several important differences that we discuss here in more detail. First of all, MIC values presented clearer increments and a bimodal distribution, making it easier to define thresholds for resistant versus susceptible samples as compared to rAUC (**Figure 5.1 B**, **Supplementary Figure 5.3 B**). Accordingly, we used MIC values to define resistant samples. However, rAUC values provided a continuous estimate of resistance, which is better suited for quantitative analyses (such as those of **Figure 5.1 E and Figure 5.5 C**). Importantly, rAUC was not affected by the trailing effect. This effect occurs when total growth inhibition is not achieved with increasing concentration of the drug, but rather cell densities are maintained. This effect has been reported with azoles and *Candida* species (Zomorodian et al. 2016; Rueda et al. 2017; Marcos-Zambrano et al. 2016). We observed this effect occurring in most (8/10) ANI samples with *ERG3* mutations, leading to high MIC values that were in the range of FLZ samples **(Supplementary Figure 5.9 C).** The rAUC values, however, were not affected by the trailing effect and these strains presented flz rAUC values

intermediate between flz non-resistant ANI and flz-resistant FLZ samples **(Figure 5.1 B, Figure 5.5 C)**. Conversely, there is one sample (BG2_11H_ANI) bearing an *ERG3* premature termination codon and presenting a mismatch between flz MIC and rAUC. Although MIC is in the WT range, visual inspection of the flz concentration-vs-fitness curve showed a trailing effect around 50% of growth **(Supplementary Figure 5.9 C)**, implying increased resistance. This is consistent with the observed high rAUC **(Figure 5.5 C, Supplementary Figure 5.9 A)**. Taken together, these examples suggest that rAUC captures better the quantitative landscape of drug resistance.

Finally, we found another sample (EF1620_7B_ANI) where neither MIC nor rAUC captured the true nature of flz resistance. This sample shows a non-monotonic relationship between flz concentration and relative fitness **(Supplementary Figure 5.9 C, D)**. This motivated us to analyze this sample under another fitness estimate, the doubling rate per hour (DR), in addition to fAUC. We found that this sample had low fitness (by both fAUC and DR) in the absence of the drug, with a small increase in the lower flz concentrations. This low level of basal fitness results in high relative fitness at low drug concentrations (as compared to other samples) **(Supplementary Figure 5.9 C, D)**. This analysis suggests that this non-monotonic relationship (if present) is very weak in terms of absolute fitness. This example illustrates how MIC and rAUC values can be misleading in strains with very low basal fitness.

Extended description of genome re-arrangements results

To focus on resistance-conferring events, we examined genes with ns mutations or nearby GR (within less than 2kb) appearing recurrently (at least twice) in our experiment. These included *ERG3*, *FKS1* and the ortholog of *S. cerevisiae CNE1*, mentioned in the main text (see **Results**). We confirmed all these rearrangements through PCR **(see Methods)**. Regarding *ERG3*, we found one ANIFLZ sample with a deletion at the beginning of the CDS and a FinA sample with a deletion in the 5' region (potentially spanning the promoter, and related to the loss of *CAGL0F01815g* (see **Results**). Both of these were associated with low relative coverage (<0.01) spanning the breakpoint, which further confirmed these deletions **(Supplementary Figure 5.5 A)**. These are additional *ERG3* mutations potentially related to ani exposure. We also found an inter-chromosomal breakpoint between chromosome (Chr) D and ChrL in EF1620_7B_ANI with the orientation of a deletion breakpoint. Importantly, the WT strain underwent a balanced translocation between these chromosomes (as compared to the reference genome), which means that the alteration appearing upon drug exposure was actually a deletion event (also confirmed by coverage). The deleted region included *CNE1*, which may be related to ani resistance (see **Results** and **Supplementary Figure 5.5 B)**. This also constitutes an example of how the rearrangements found in each strain modulate the interpretation of breakpoints appearing during the experiment. Finally, we found two FinA samples with GR breaking the *FKS1* coding region, including one deletion at the beginning of the coding sequence (with relative coverage < 0.01) and one balanced inverted

translocation between ChrG and ChrM **(Supplementary Figure 5.5 C)**. Both samples carried *FKS2* mutations (potentially conferring ani resistance), suggesting that these rearrangements are complementary *FKS1* alterations with a similar impact as the truncating small variants mentioned in the main text.

On another note, we attempted to infer the precise events leading to partial aneuploidies during the experiment **(Figure 5.3 Supplementary Figure 5.7)**. We found an unbalanced translocation explaining the partial duplication of ChrE in CBS138_9F_FLZ. Our GR-detection method predicted that the right arm of ChrE (matching the aneuploid region **(Supplementary Figure 5.7)** was duplicated and attached to ChrJ, replacing the left-end at the breakpoint. This region showed low coverage after the breakpoint (supporting the unbalanced translocation call), but not until the end of the chromosome (which would be expected from such an event). Interestingly, the deleted region was found between the unbalanced translocation breakpoint and a location with low WT coverage. We propose that this configuration is the result of a pre-existing rearrangement in the WT strain, which explains why the deleted region does not span the entire left-end of the chromosome. Accordingly, the ChrE breakend was called as heterozygous, while the ChrJ was haploid **(Supplementary Figure 5.5 D)**. Conversely, we could only find an inverted heterozygous breakpoint matching the start of the aneuploid region of ChrE in CST34_2A_AinF, which was not enough to explain the source of the duplication. Finally, we found that the (apparently) partial duplications of ChrI in the EB0911 samples are actually whole-chromosome aneuploidies. The WT

EB0911 depicted balanced translocations between chromosomes D, I and L, generating three (mixed) chromosomes from the successive fusions. We found one of these mixed chromosomes with 2x coverage in both samples with aneuploidies **(Supplementary Figure 5.5 E)**. Interestingly, this chromosome is much shorter than the reference ChrI, perhaps resulting in a lower fitness cost of this aneuploidy. We speculate that this is the reason why this aneuploidy is found only in this strain. Taken together, these results suggest that complex structural variation may contribute to drug resistance. They also show how breakpoint calling can explain the precise events leading to CNV and aneuploidies.

Extended description of the analysis of *Candida* clinical isolates

In order to assess whether the mechanisms driving drug resistance in vitro are clinically relevant we first analyzed the publicly available sequences of *Candida* clinical isolates. We obtained whole genome sequencing variant calls for 393 *C. glabrata* clinical isolates from the CandidaMine database (http://candidamine.org/candidamine/begin.do). We assessed how many of the drug resistance variants described in this work were also found in these clinical isolates, which yielded little or no overlap depending on the gene **(Supplementary Figure 5.8 A)**.

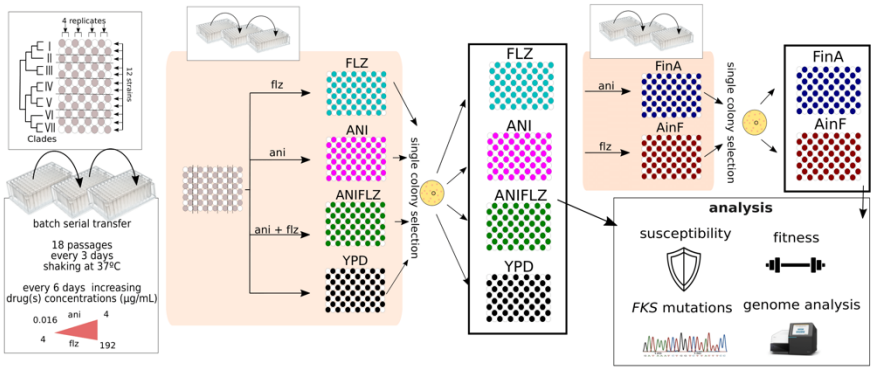
We hypothesized that the underlying reason is that several mutations in the same gene can explain drug resistance **(Figure 5.3)**. In order to test this we calculated the overlap between CandidaMine variants and two datasets of previously described drug resistance-mutations:

the SENTRY database (Pfaller et al. 2019) and a set of described *PDR1* mutations from the literature (Ferrari et al. 2009; Tsai et al. 2010; Spettel et al. 2019). This yielded low overlaps as well, comparable to those found in our work **(Supplementary Figure 5.8 A)**.

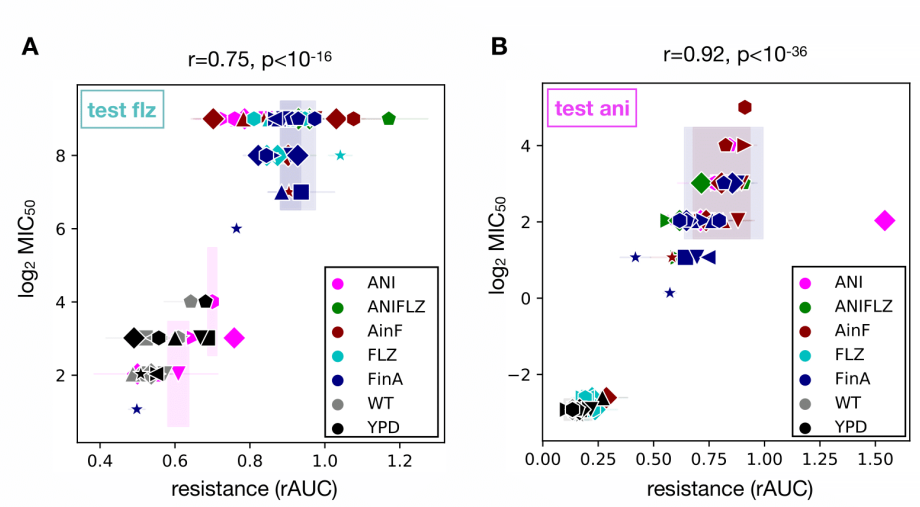
In addition, we inferred the expected overlap between different mutation datasets through a randomization strategy on our samples. We divided the samples carrying mutations in a given gene into two random subsets. For each subset we calculated the number of mutations only in the subset or also found in the other subset. This process was repeated 100 times, and the results **(Supplementary Figure 5.8 B)** show that the overlap is comparable to the observed between datasets of different works.

We conclude that it is difficult to measure the clinical impact of the mutations described here because most of them cannot be found in the currently available isolates. However, this low overlap is expected and comparable to other datasets of well-known resistance-conferring mutations.

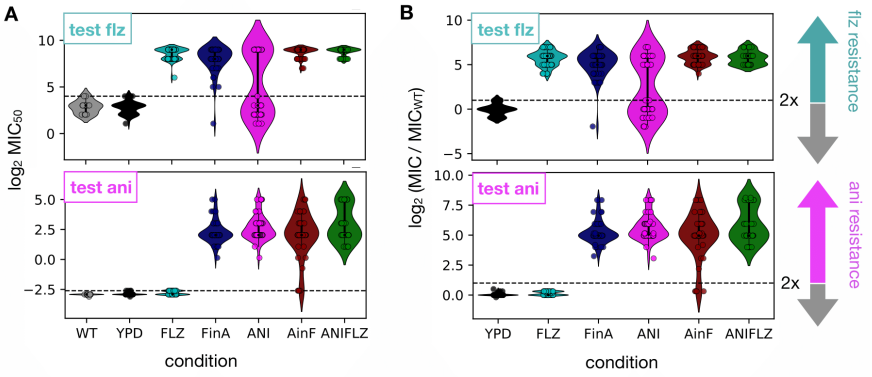
5.7 Supplementary Figures



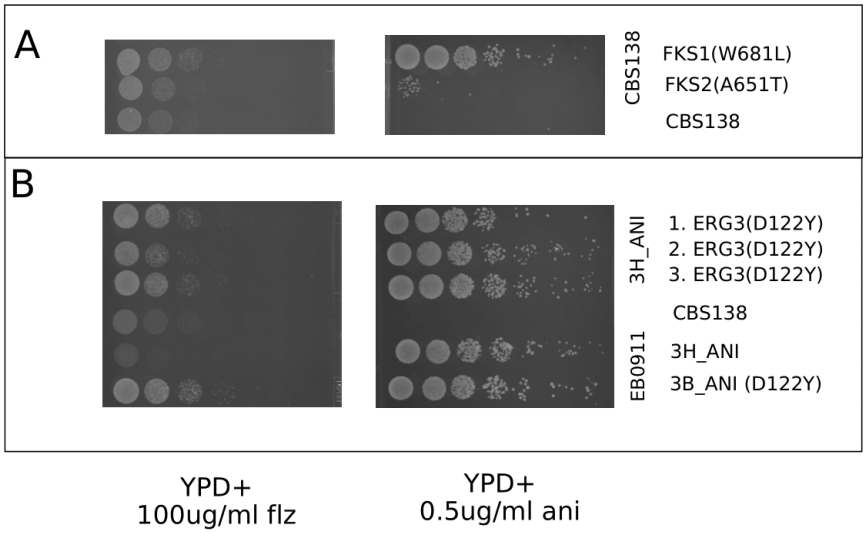
Supplementary Figure 5.1. Schematic representation of the in vitro evolution experiment. 48 populations -quadruplicates of each of the 12 strains- were grown with increasing concentrations of fluconazole (flz, FLZ samples); anidulafungin (ani, ANI); both drugs in combination (ANIFLZ) and no drug (YPD). Subsequently ANI samples were grown in flz (AinF), whereas FLZ samples in ani (FinA). The experiment involved batch serial transfer of the samples every 3 days, where every second passage involved an increase in drug concentrations up to 4 µg/mL and 196 µg/mL of ani and flz, respectively (see **Materials and Methods**). After the final passage, an aliquot was plated for single colony isolation and storage.



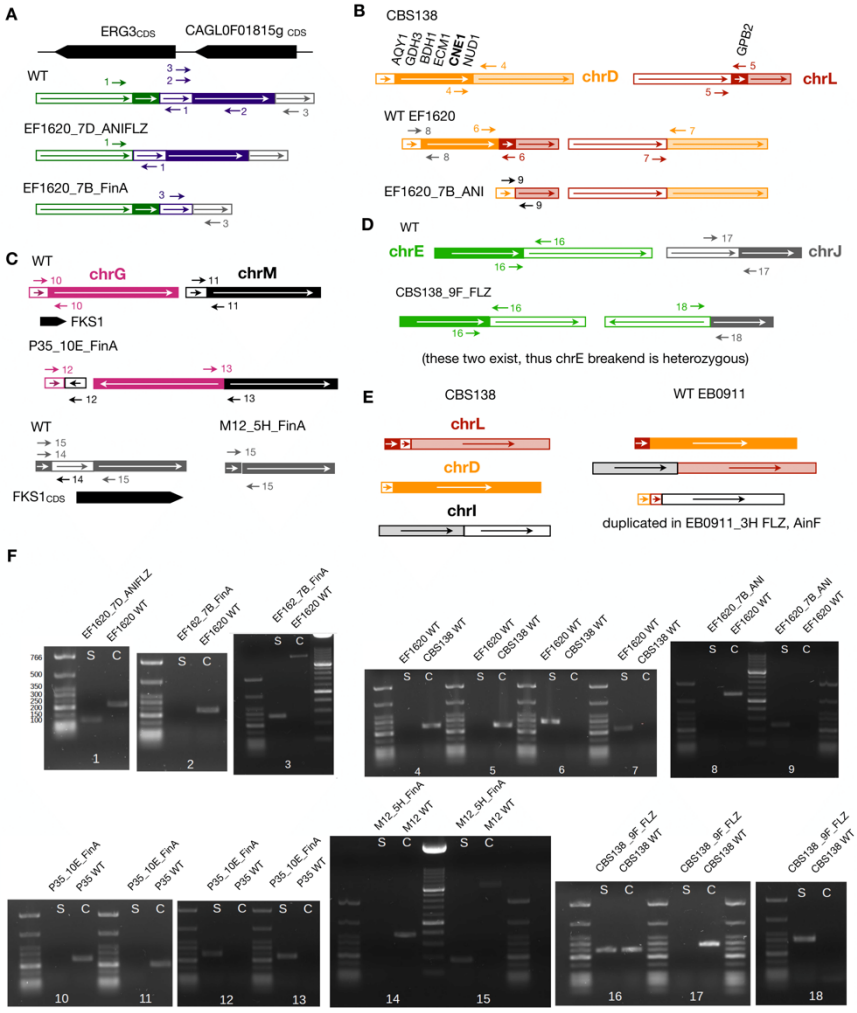
Supplementary Figure 5.2. Minimum Inhibitory Concentration (MIC) and rAUC are highly correlated. (A) We compared the flz resistance levels estimated from rAUC and MIC. The Spearman correlation coefficients and p-values are shown. Each point corresponds to a biological replicate and the error bars reflect the median absolute deviation across technical replicates. **(B)** The same as in **(A)** but for ani resistance.



Supplementary Figure 5.3. Minimum Inhibitory Concentration (MIC) values for all the samples analyzed in this study. (A) MIC for flz (top) and ani (bottom) was measured for all samples, presented here as single points. The dashed line indicates the maximum observed value in a YPD sample. **(B)** The increase in MIC relative to WT was calculated as the $\log_2$ ratio of MIC of the sample and MIC of WT. Resistant samples are defined as those having a MIC twice as high as the corresponding WT (dashed line).

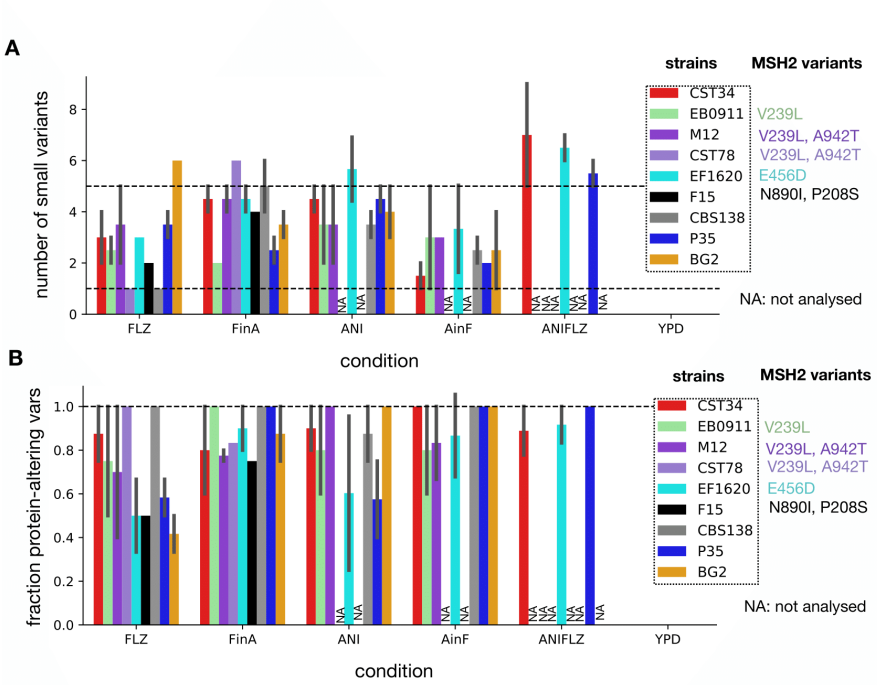


Supplementary Figure 5.4. Susceptibility of samples with introduced mutations in *FKS* and *ERG3* genes. Spot tests demonstrating the susceptibility changes (on a rich medium YPD supplemented with 100ug/ml fluconazole and 0.5ug/ml anidulafungin) in genetically modified transformants. **(A).** shows the two transformants carrying re-introduced point mutations in both *FKS* genes in a CBS138 WT background with the WT control. **(B).** shows three independent transformations of the D122Y mutation in *ERG3* gene in an anidulafungin resistant background (3H_ANI) - 1. transformed with a long fragment with *ERG3* and crRNA_ERG3_1 and 2 and 3 are 2 different colonies obtained from a transformation with synthetic *ERG3* fragment and crRNA_ERG3_2; and controls: CBS138, 3H_ANI strain that does not contain *ERG3* mutation, and of 3B_ANI, that contains *ERG3* mutation (D122Y) - both are progenies of EB0911 WT strain.

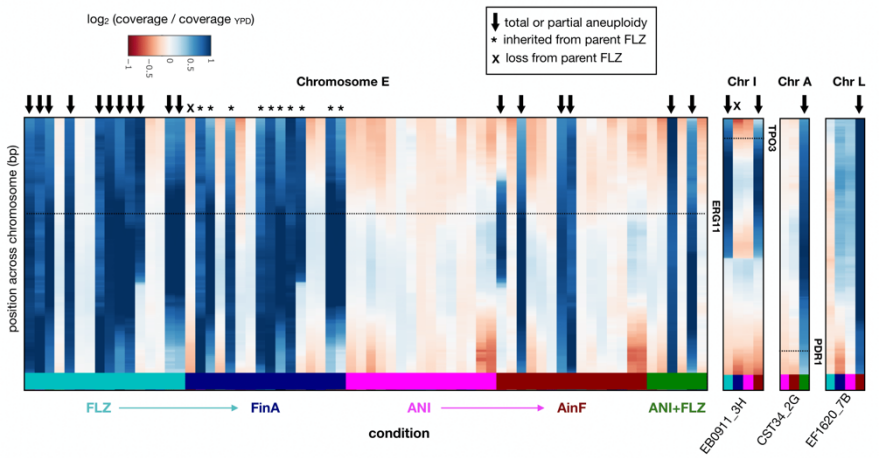


Supplementary Figure 5.5. Genomic rearrangements appear during evolution in antifungal drugs. (A) We found two samples with a deletion in the *ERG3* CDS (medium) and upstream region (bottom), respectively. The browser represents the genomic coordinates of *ERG3* and the upstream gene *CAGL0F01815g*. The boxes represent the WT regions that are rearranged in each sample. We confirmed these rearrangements with three PCRs on these samples (using primer pairs 1, 2 and 3). The results are shown in **(F)**, with the numbers matching the primer pairs of each PCR. **(B)** *CNE1* and *GBP2* were lost due to a single deletion rearrangement in EF1620_7B_ANI. The representation is analogous to (**A**), showing a EF1620 WT balanced translocation between chromosomes D and L which, in addition to the deletion-like breakpoint appearing in ANI, generates a loss of the region between the two breakpoints. **(C)** Two FinA samples carried rearrangements breaking the

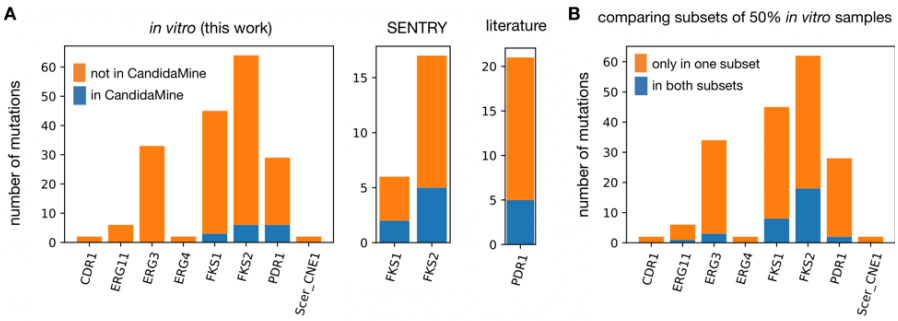
FKS1 CDS (black box). P35_10E_FinA had an inverted balanced translocation between chromosomes G and J (top), and M12_5H_FinA carried a partial deletion (bottom). **(D)** Genomic rearrangements can explain the partial chromosome E aneuploidy in CBS138_9F_FLZ **(Supplementary Figure 5.7)**. This sample carried an unbalanced translocation between chromosomes E and J. Both chromosome E breakends were heterozygous, while the chromosome J breakend was haploid. **(E)** The apparent partial duplication of chromosome I **(Supplementary Figure 5.7)** is actually a complete aneuploidy in two EB0911 samples. We found WT balanced translocations between these chromosomes that result in three mixed chromosomes in this strain (bottom). We found that two EB0911_3H samples had one of these mixed chromosomes duplicated (bottom), including mostly half of the reference chromosome I. **(F)** We performed PCRs using primer pairs around the rearrangements (1 to 18 in **(A)** - **(D)**) to confirm them. Each PCR was carried on a given sample and the corresponding control. We note that we could obtain bands with the expected sizes in all samples.



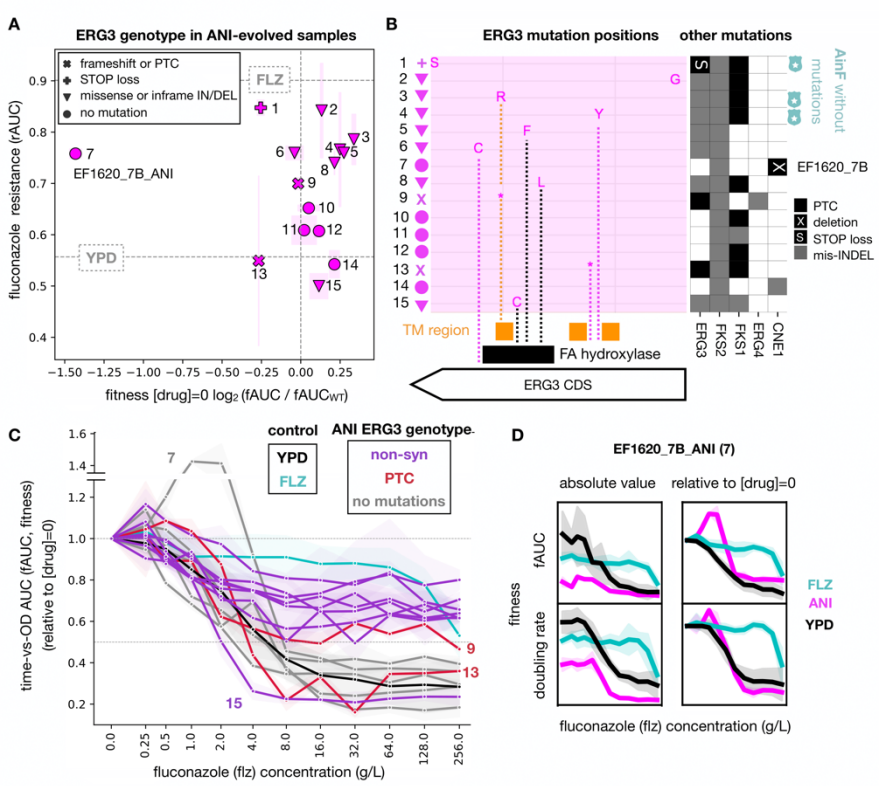
Supplementary Figure 5.6. The number of small variants (synonymous and non-synonymous) that appear during the experiment is variable across strains and replicates. (A) To select only newly-acquired mutations in each drug-evolved sample we subtracted from called variants those also called in the corresponding WT, YPD and the parental drug condition (ANI from AinF, and FLZ from FinA), while the corresponding variants called in WT, ANI, AinF, FinA and FLZ samples were subtracted from those found in the YPD sample. The dashed lines, from bottom to top, correspond to 1 and 5 mutations, respectively. We also represent the presence of one or more ns variants in the *MSH2* gene in the WT strain. The bars represent the mean number of mutations across biological replicates and the error bars the standard deviation. **(B)** The same as in (**A**), but showing the fraction of protein-altering mutations.



Supplementary Figure 5.7. Evolution in fluconazole often leads to chromosomal aneuploidies. (A) We calculated the median relative coverage per gene for all samples analyzed in this work. This parameter appeared to be correlated with the distance to the telomere (**see Materials and methods**), so that the $\log_2$ ratio to the YPD (of the corresponding strain) was used as a proxy for gene copy number. Shown is the rolling-median of this value for windows of 50 genes and chromosomes where large duplications were observed (chromosomes E, I, A and L). Data for chromosomes I, A and L are shown only for those strains where aneuploidies are observed. Each column corresponds to a sample (ordered as in **Figure 5.3**), and the "*" and "X" correspond to FinA samples where the parent (FLZ) aneuploidy was maintained or lost, respectively. *ERG11*, *PDR1* and *TPO3* are genes that we speculate could be driving the selective advantage of the aneuploidy **(see Results)**. All values were cutoff at 1.0 (2x coverage as compared to the YPD) for clarity.

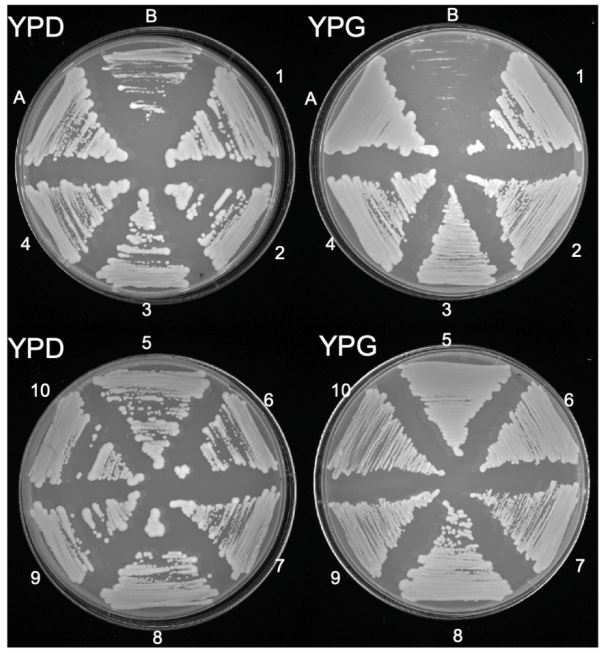


Supplementary Figure 5.8. The overlap between drug resistance-conferring mutations from different studies is (and is expected to be) low in *Candida glabrata*. (A) We compared the drug resistance variants described in this work (left), the SENTRY database (middle (Pfaller et al. 2019)) and a set of described *PDR1* mutations (right (Ferrari et al. 2009; Tsai et al. 2010; Spettel et al. 2019)) against those in clinical isolates with available whole genomes (393 in total) **(see Methods)**. Shown is the number of mutations that are found in each study and in some (blue) or no (orange) clinical isolates. **(B)** In order to estimate the expected overlap between drug resistance mutations among different samples we implemented a randomized strategy from our own experiments. We divided the samples carrying mutations in a given gene into two random subsets. For each subset we calculated the number of mutations only in the subset or also found in the other subset. This process was repeated 100 times, and shown is the median number of mutations not shared (orange) or shared (blue) across subsets.



Supplementary Figure 5.9. Acquisition of *ERG3* mutations in ANI samples often confers fluconazole cross-resistance. (**A**) Fitness (relative to the WT as in **Figure 5.1 D**) is high in most ANI-evolved samples (EF1620_7B_ANI is an exception), while flz-resistance (shown as rAUC) is variable. The symbols correspond to different types of *ERG3* protein-altering mutations. The dashed lines correspond to the median flz rAUC for all the FLZ and YPD samples. Each point represents the median across technical replicates for a given sample, while the boxes show the median absolute deviation. The numbers are related to the order of flz-resistance, used to show the relationship of each sample to panels (**B**), (**C**) and (**D**). (**B**) *ERG3* amino acid mutations are scattered throughout the coding region of the gene. The boxes in the bottom represent annotated protein domains (see **Materials and methods**), where the "catalytic domain" is the Fatty acid (FA) hydroxylase superfamily (PF04116) and TM are transmembrane regions. Three samples with no additional mutations nor increase in flz resistance in subsequent flz treatment (AinF) are marked with blue shields. PTC and '*' indicate Premature Termination and S indicates the loss of the STOP codon. (C) Growth of the ANI samples (with colored *ERG3* genotype) at increasing concentrations of flz shown as fAUC and compared to all FLZ (blue) and YPD (black) samples. Purple lines indicate samples with non-synonymous alterations, red - with protein termination codon (PTC) and gray - no *ERG3* changes. Samples 9 and 13 bear a PTC but the former showed improved growth at higher flz concentrations. Although assessed as susceptible based on MIC, sample 9 presented a curve more similar to that of resistant samples, and maintained a relative growth around ~50% across increasing concentrations

(see **Supplementary text**). Sample 15 bears the only ns mutation that did not result in increased resistance to flz by rAUC, MIC or shape and position of the curve. The points and error bars correspond to the median and median absolute deviation for each assayed concentration in each sample, respectively. The numbers (7, 15, 9, 13) correspond to those in (**A**) and (**B**). (**D**) EF1620_7B_ANI (number 7 in this figure) was found to be flz susceptible according to our MIC-based thresholding (**Supplementary Figure 5.3**) but depicted an rAUC in the range of resistant samples (**A**). To understand this mismatch, we studied the quantitative relationship between flz concentration and several fitness estimates (the doubling rate per hour (bottom) and fAUC (top)) in both absolute (left) and relative to no drug (right) representations. The median values across all FLZ and YPD EF1620 samples are shown for comparison.



Supplementary Figure 5.10. Petite phenotype assessment. Growth of ANI evolved mutants (1. 2G_ANI, 2. 3B_ANI, 3. 5F_ANI, 4. 7D_ANI, 5.7F_ANI, 6. 9F_ANI, 7. 9H_ANI, 8. 10G_ANI, 9. 11G_ANI, 10. 11H_ANI), CBS138 (A) and petite *Saccharomyces cerevisiae* mutant (B), on YP medium supplemented with glucose (YPD) and glycerol (YPG).

5.8 Supplementary Data

Appendix 1:
The supplementary material supporting the results of this thesis project is available at:
https://drive.google.com/drive/folders/1wBfzISbO9qRsLuZa-yH19me9Sr-EDqKU?usp=sharing

Supplementary data captions:

Data S1

***Candida glabrata* strains used in this study**. Columns indicate, in this order: strain identifier, isolation site, location, phylogenetic clade (Carreté et al. 2018). All strains are further described elsewhere (Carreté et al. 2018), except SLL2 glab, which is first described here and was isolated in 2018 from an oral wash sample of a healthy person in Spain, and thus it can be considered a commensal strain.

Data S2

List of shared polymorphisms found in CST109 (clade 1) and M12 (clade 3) that were not found in other representatives of their respective clades - CST34 and CST78 for clade 1 and 3, respectively. We highlight the ortholog of *Saccharomyces cerevisiae MAD1* for which polymorphisms in CST109 and M12 were found to affect nearby residues in the protein sequence (390 and 387, respectively). Dysfunction of this gene has been previously related to chromosome instability in *S. cerevisiae* (Zhu et al. 2012)**.** Thus, these polymorphisms might be associated with higher chromosome instability resulting in lower capacity to preserve long-term drug resistance.

Data S3

Results of analysis of growth curves in microdilution antifungal susceptibility tests. Columns indicate, in this order: mutant name, condition, clade, strain, replicate, rAUC (median, absolute deviation, technical replicates), MIC (median, absolute deviation, technical replicates) for anidulafungin and fluconazole, respectively and Fitness: fAUC with absolute deviation and as fitness $\log_2$ fold-change vs WT and absolute deviation.

Data S4

***FKS* mutations.** Columns indicate, in this order: mutant name, evolution media, clade, strain, replicate, mutations in *FKS1* and *FKS2* genes. The variants are encoded as "type of mutation" / "molecule affected" . "position" | "reference allele" / "alternative allele". The "type of mutation" can be: mis - missense variant, del - inframe deletion, PTC – Premature Termination Codon, FS - frameshift, ins – inframe insertion, lostSTOP – lost STOP codon, lostATG - lost START codon. The "molecule affected" can be "p" for protein and "c" for cDNA. The "reference" and "alternative" alleles correspond to amino acids or codons for proteins or cDNA alterations, respectively.

Data S5

Small variants (SV) obtained from whole genome sequencing analysis. Columns indicate, in this order: mutant name, experimental condition, clade, strain, replicate and mutated genes. Additional

information on genes are at the bottom of each column and include: systematic name, chromosome, start position and function obtained from CandidaGenome server (Skrzypek et al. 2017). The variants are encoded as in **S4.**

Data S6

***ERG3* mutations.** Columns indicate, in this order: mutant name, evolution media, clade, strain, replicate, mutations in *ERG3* gene from genome and sanger sequencing. The variants are encoded as in **S4.**

Data S7

Trajectory of final *FKS* and *ERG3* mutations. Rows indicate, in this order: evolution media, clade, strain, replicate, tested gene/fragment, final mutation, and concentrations of anidulafungin (μg/ml) corresponding to intermediate glycerol stocks (isolated single colonies) of tested trajectories. Mutations that were not found at the finalization of the evolution experiment are marked as 'new'.

Data S8

Information on drugs concentrations used in the evolution experiments. Columns indicate, in this order: number of passages, number of drug increases and corresponding fluconazole and anidulafungin concentrations (μg/mL).

Data S9

Information about all the oligos used in the study. The table includes primers used to confirm the GR, investigate *ERG3* gene and *FKS1* and *FKS2* fragments as well as primers to amplify fragments containing *ERG3*, sequences of donor DNAs and crRNAs used in CRISPR-Cas9 transformations.

6 Implications of mutational signatures associated to anidulafungin treatment

6.1 Abstract

Echinocandins are antifungal drugs that impair the fungal cell wall by inhibiting the synthesis of beta-glucans. Mutations in the genes encoding the drug target (*FKS*) drive resistance to echinocandins. However, in response to the drug, various stress responses are activated that are necessary to maintain cell wall integrity. In an in vitro evolution experiment to induce echinocandin resistance in *Candida glabrata*, we observed that several genes other than *FKS* were recurrently mutated, suggesting they were acquired in response to the treatment. These included genes involved in glycoprotein maturation and protein folding (*CNE1*) and in ergosterol biosynthesis pathway (*ERG*). Here we investigated in detail these detected mutations and analyzed additional strains carrying them. We observed that adaptation to echinocandin treatment triggers complex genetic and phenotypic changes, some of them affecting susceptibility to other classes of antifungal drugs, such as azoles and polyenes.

6.2 Introduction

Echinocandins are one of the few drug classes used to treat fungal infections in humans. Drugs of this class block the biosynthesis of β-1,3-glucan, a major structural component of the fungal cell wall. Due

to the efficiency and safety of the echinocandin therapy, echinocandins are also recommended to treat candidiasis in high-risk patients (Pappas et al. 2016). Mechanisms of resistance to echinocandins include modifications of the *FKS* genes encoding for the catalytic subunits of β-1,3-glucan synthase (Park et al. 2005). In most *Candida* species resistance driving mutations occur in highly conserved "hot-spot" regions of *FKS1*, however in *Candida glabrata* there are two functionally redundant *FKS* genes that are under selective pressure when echinocandin treatment is applied (Katiyar et al. 2012).

Echinocandins are fungicidal to *Candida* species. Therefore survival under drug treatment has been attributed not only to the appearance of *FKS* mutations conferring resistance but also to earlier changes allowing drug tolerance and adaptation (Healey and Perlin 2018). Upon damage to the cell wall, fungi upregulate various stress responses and cell wall integrity pathways that help the cells tolerate the stress and survive (Cowen and Steinbach 2008). Such responses are also crucial for survival to echinocandin exposure (Robbins et al. 2017). Responses to echinocandin-induced stress involve those regulated by the protein phosphatase calcineurin or protein kinase C (PKC), which together increase chitin synthesis to maintain cell wall integrity (Munro et al. 2007; Wiederhold et al. 2005).

As a result of these necessary responses, adaptation to echinocandins may be associated with the appearance of genetic alterations related to adaptation to cell wall stress (Arastehfar et al. 2020). Considering that echinocandins are used as a first-line treatment, it is relevant to

understand its various effects on fungal cells, including the genetic changes that it may induce. The previous chapter has provided an overview of the main genetic changes observed after exposure to anidulafungin (ani). Here, we report more detailed analysis of the most interesting alterations, and include follow up experiments and analyses performed to further characterize them.

6.3 Results and conclusions

6.3.1 Resistance driving mutations in *FKS* genes

Numerous surveys report that *C. glabrata* isolates with lower susceptibility to echinocandins often carry mutations in specific regions – called mutational hotspots (HS) – of *FKS1* or *FKS2* genes (Arendrup and Perlin 2014). As described above (**Chapter 5**), we sequenced *FKS1* HS 1 and *FKS2* HS 1 and 2 in the 121 evolved samples that acquired resistance to echinocandins (47 ANI, 47 FinA and 21 ANIFLZ samples). This analysis revealed that all examined samples presented non-synonymous mutations in at least one of the *FKS* genes, with 61.98% samples bearing multiple changes (total 223 non-synonymous mutations including indels, average 1.83 mutations/sample). More specifically, 42.15%, 19.01% and 0.83% of the samples have 2, 3 and 4 mutations, respectively. Importantly, many (62.78%) of the identified mutations had been previously described in clinical isolates, underscoring the suitability of our experiment to recover mutational processes that also occur in the clinics.

As already mentioned, 63 samples (52.07%) carried a mutation in the F659 codon position of *FKS2*. The most common mutation in this position was the deletion of the codon specifying phenylalanine 659 (F659del), which affected 42.98% (52 samples) of all the samples, and in 26 samples (21.48%) it was present as the unique mutation in the surveyed regions. This high prevalence suggests a high selective advantage for this mutation under ani treatment, which is in disagreement with the conclusion of a previous study claiming that F659del is a caspofungin specific mutation (Shields et al. 2019). Furthermore, 19 samples (15.07%) carried a mutation at the codon position 1,378 in *FKS2* with R1378S being the second most commonly observed mutation in our experiment (present in nine samples). The third most mutated position was 632 in *FKS1* (17 samples), with D632Y appearing six times. Then, 13 samples had a mutation at position 663 in *FKS1* with S663P appearing in six samples; 12 samples were mutated at codon 666 of *FKS2* with D666N in five samples and eight samples were mutated at 629 in *FKS1* with S629P in six of them. All the mutations and their distributions can be found in the supplementary data of the previous chapter (**Chapter 5, Appendix1: Data S4**).

Although most samples carried mutations within HS regions, 21.5% of the mutations fell outside of HS (**Figure 6.1**). We found three mutants whose resistance can only be related with *FKS* mutations outside of the HS, and the changes are, respectively: *FKS1*-R1422L and *FKS1*-F708S; *FKS1*-W681L and *FKS2*-K265*; and *FKS2*-A651T. Two of these changes, *FKS2*-A651 and *FKS1*-W681 were

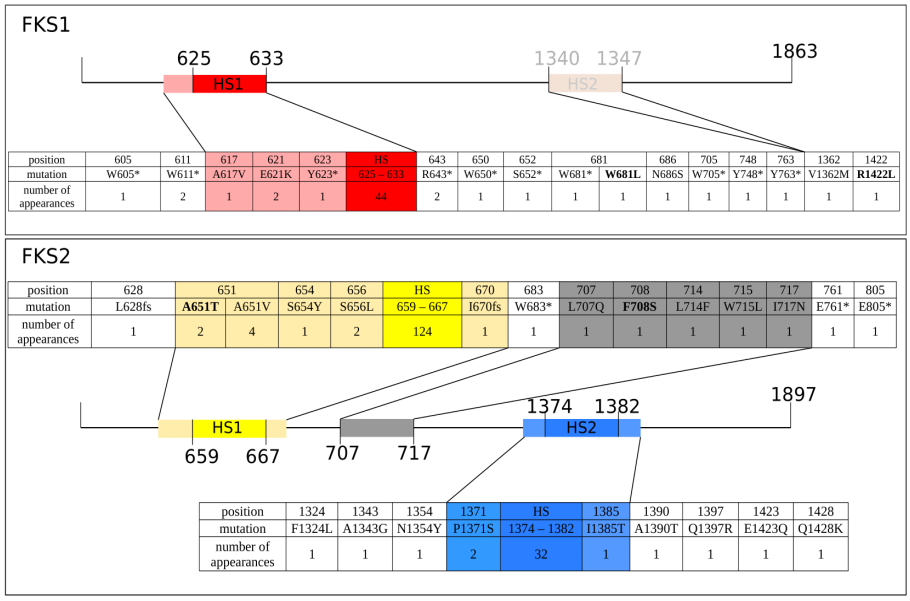
found in additional strains (in five and one other samples, respectively), and their contribution to ani resistance was confirmed by reintroducing the mutations in ani-susceptible strains, which resulted in the expected ani resistance phenotype (**Chapter 5 Supplementary Figure 5.4**).

Furthermore, our results suggest that the HS might be more extended than the established 8-9 codons and that an additional HS may exist (**Figure 6.1**). Firstly, we observed multiple changes in the proximity of the HS in 16 samples (13%). Even though these amino acids are mutated less frequently, they may still play a significant role in the acquisition of resistance. We hypothesize that mutations in these residues may affect the binding pocket either directly or indirectly by interacting epistatically with other mutated residues. Secondly, we observed an additional region with clustered mutations in *FKS2*. In this potential new HS, five samples present mutations in the non-HS amino acidic region 707-717, of which only L707S has been previously reported (Fernández-Silva et al. 2014). Additionally, this region aligns with a region of *FKS* gene in *C. auris* assigned as HS3 (Carolus et al. 2020). Although the incidence of these mutations is relatively low as compared to HS mutations, their recurrent appearance under ani exposure and their clustered nature suggests a relevant effect in Fks2p function.

Finally, whole genome sequencing of 37 ani-evolved strains identified no mutation in the presumed second HS region of *FKS1* spanning codon positions 1340-1347 (Arendrup and Perlin 2014). Although they are sometimes mentioned in the literature, samples

bearing changes in this region are uncommon, which suggests that these mutations play only a minor role in the adaptation to echinocandins in *C. glabrata*.

Figure 6.1. Scheme representing the distribution of the all HS regions in *FKS1* (upper) and *FKS2* (bottom) with all non-HS mutations in the three investigated regions of *FKS* genes. In lighter colors are marked regions of possible expansion of the HS regions and the potential extra HS is marked in grey. In bold letters are mutations in samples where no HS mutations were found.



position	605	611	617	621	623	HS	643	650	652	681		686	705	748	763	1362	1422
mutation	W605*	W611*	A617V	E621K	Y623*	625 – 633	R643*	W650*	S652*	W681*	**W681L**	N686S	W705*	Y748*	Y763*	V1362M	**R1422L**
number of appearances	1	2	1	2	1	44	2	1	1	1	1	1	1	1	1	1	1

position	628	651		654	656	HS	670	683	707	708	714	715	717	761	805
mutation	L628fs	**A651T**	A651V	S654Y	S656L	659 – 667	I670fs	W683*	L707Q	**F708S**	L714F	W715L	I717N	E761*	E805*
number of appearances	1	2	4	1	2	124	1	1	1	1	1	1	1	1	1

position	1324	1343	1354	1371	HS	1385	1390	1397	1423	1428
mutation	F1324L	A1343G	N1354Y	P1371S	1374 – 1382	I1385T	A1390T	Q1397R	E1423Q	Q1428K
number of appearances	1	1	1	2	32	1	1	1	1	1

One of our initial hypotheses driving the design of the experiment described in **Chapter 5** was that different genetic backgrounds may influence how *C. glabrata* adapts to drugs. To test this, we used R scripts to assess statistical associations between clades, strains, susceptibility to ani, and *FKS* mutations. We found that none of the experimental evolution conditions (i.e. ANI, AinF, FinA and ANIFLZ), clades, strains, mutated residues, nor particular mutations

presented statistically significant variations in susceptibility as measured by rAUC or MIC. Additionally, we found no significant correlation between the number of *FKS* mutations in a sample and the level of susceptibility to ani, indicating that the accumulation of a larger number of mutations does not result in a higher resistance. The only two statistically significant correlations we observed are that mutations in *FKS1*-P633 were found to be more likely to appear in ANIFLZ condition (Chi-squared test, $p = 0.001$), and *FKS1*-D632 mutation was significantly enriched in clade 4 ($p = 0.003$). With the data at hand, it is difficult to speculate whether specific epistatic effects could explain these associations, something that may deserve further exploration if they are confirmed in a larger dataset. The results indicate that susceptibility in *C. glabrata* is largely not influenced by any of the mentioned variables, at least to a level that can be detected with our sample size. Nevertheless, the reintroduction of two different non-HS mutations (*FKS1*-W681L and *FKS2*-A651T) into a WT strain showed that each of them results in different susceptibility levels to ani (**Chapter 5, Supplementary Figure 5.4**). This result is consistent with earlier reports describing that MIC values may vary depending on the position and substituted amino acid (Perlin 2015b; Arendrup and Perlin 2014), and suggest that our sample size may be too limited to detect statistical associations between specific mutations and level of susceptibility.

We next searched for patterns of co-occurring mutations which could reveal compensatory effects or other epistatic interactions between different residues in Fks proteins. To do so we calculated and plotted which residues and how many times each pair of residues were co-

mutated in the same sample (**Figure 6.2**). We observed that F659 in *FKS2* was altered multiple times (37) in combination with mutations at other positions and that F632 in *FKS1* appears 17 times in pairs of mutations and never as the sole *FKS* mutation. Considering that two amino acids that are seven residues apart from each other would face the same side of an alpha helix (seven residues are two turns of the helix) in the protein secondary structure, we stress the presence of such combinations: *FKS1* 625:632 – observed three times and *FKS2* 651:659 – two, 659:666 – five. These pairs may physically interact or face the same binding pocket so that alterations in both may be beneficial in the process of the acquisition of the resistance. Alternatively, one of the two mutations may compensate for some deleterious effect of the other one. Finally, mutations in the positions *FKS2*-659 and *FKS2*-663 were found to be significantly anti-correlated (fisher test p=0.012 after FDR correction).

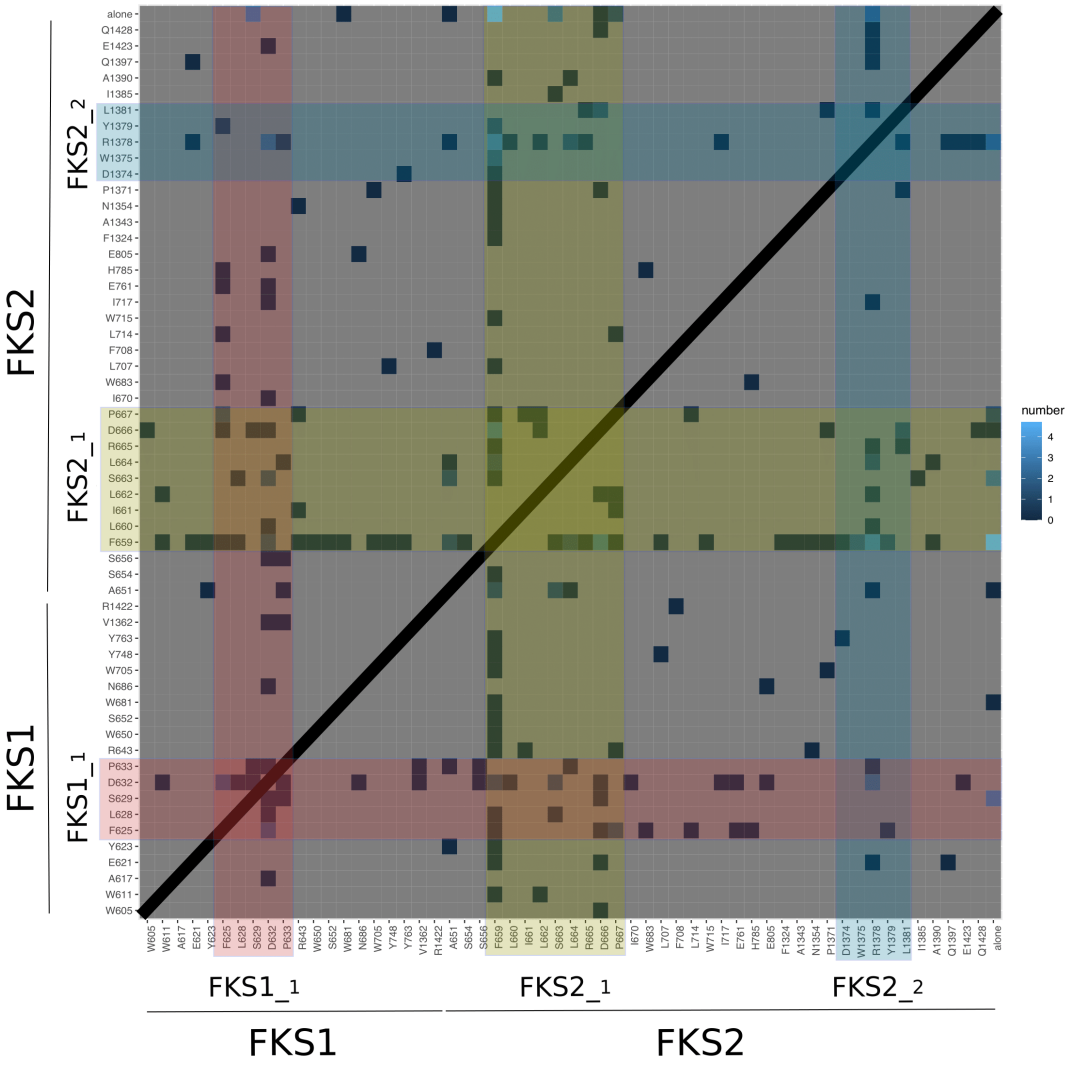


Figure 6.2 Heatmap representing pairs of mutated positions. Shown are amino acid positions in the investigated regions of Fks proteins and the number of times they appear co mutated with other positions. Colors: red, yellow and blue represent *FKS1* HS1, *FKS2* HS1 and *FKS2* HS2, respectively.

To gain insight on the relative timing of acquisition of *FKS* mutations, we investigated the evolutionary trajectory of *FKS* mutations (**Figure 6.3**). In other words, we intended to retrospectively determine when the mutation appeared during the

course of the evolution experiment (i.e. at which of the stored populations). In doing so we wanted to determine whether any particular mutation appeared earlier or later with respect to the other ones present in the final sample, and whether past or simultaneous treatment with fluconazole (flz) influenced the emergence of *FKS* mutations. To do so, we used the glycerol stocks preserving populations representing intermediate steps of the evolution experiments. We selected single colonies from populations grown at 0.032, 0.064, 0.126, 0.256 ug/ml of ani from 35 mutants of ANI, FinA and ANIFLZ samples and traced back the appearance of the mutations present after the finalization of the evolution experiment by PCR amplification coupled to Sanger sequencing (**Table 6.1**). Five of the tested evolved lines did not show any mutations in the investigated concentrations/regions, suggesting that the observed terminal mutations were acquired later in the evolution, while all the remaining samples presented mutations at 0.256 ug/ml (5^{th} ani concentration, 10^{th} passage) implying that these samples might have been resistant already at this point in the evolution. In four samples the final mutations were present as early as at 0.032 ug/ml (2^{nd} ani concentration used, 4^{th} passage with the drug), and in three of the mutants at 0.064 ug/ml (3^{rd} ani concentration, 6^{th} passage). Interestingly, none of the final premature stop codons (suggesting truncations) were detected, which indicates they appeared later during the evolution and only following the acquisition of other resistance driving mutations. This observation may additionally suggest that, under prolonged ani treatment, the absence of one of the two wild type glucan synthases may benefit survival. Additionally,

we observed a significant number of intermediate mutations that were absent at the end of the experiment, indicating fluctuations in allele frequency or successions of dominant genotypes during the evolution experiment. To investigate these dynamics, we started a follow up project where the genomes of whole populations will be analyzed so that the frequency of different alleles can be traced during the course of the evolution.

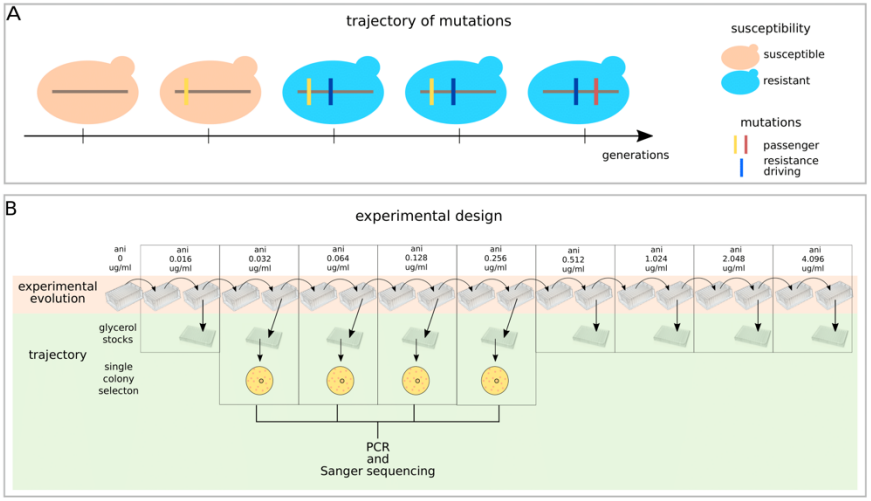


Figure 6.3. Trajectories of mutation signatures. (**A**) Schematic representation of an evolutionary trajectory. Five intermediate generations are represented, with relevant mutations and phenotypes indicated. (**B**) Scheme of the process followed to study the evolutionary trajectories. During the experimental evolution, glycerol stocks of the populations were made after the second growth with the same amount of ani. PCR and Sanger sequencing were performed on samples, following the isolation of single colonies as described in methods in the **Chapter 5**.

Table 6.1. Trajectory of *FKS* mutations. Columns indicate, in this order: evolution condition, clade, strain, replicate, MIC of ani and flz, tested region of *FKS* genes, mutation, and concentrations of ani (µg/ml) corresponding to intermediate glycerol stocks (isolated single colonies) of tested trajectories. Mutations found at particular concentration are marked with green boxes and the mutations that were not found at the finalization of the evolution experiment are marked in red

Condition	Clade	Strain	Replicate	MIC		FKS	mutation	ani (ug/ml)			
				ani	flz			0.032	0.064	0.126	0.256
ANI	1	CST 34	2A	8	8	FKS1 HS1	Y623*				
						FKS2 HS1	A651T				
							F659-				
							P667R				
			2G	8	>256	FKS2 HS1	F659-				
ANIFLZ			2A	4	256	FKS2 HS1	F659-				
			2G	2	256	FKS1 HS2	V1362M				
FinA			2A	4	>256	FKS2 HS1	F659-				
			2G	4	>256	FKS1 HS1	S629P				
ANI	2	EB0911	3B	4	>256	FKS1 HS1	S652*				
						FKS2 HS1	F659-				
			3H	4	4	FKS2 HS1	F659-				
FinA	2	EB0911	3B	4	>256	FKS2 HS1	S663P				
			3H	4	128	FKS2 HS1	F659-				
							F659L				
ANI	3	M12	5F	8	>256	FKS2 HS1	F659-				
			5H	4	4	FKS1 HS1	D632V				
						FKS2 HS1	F659-				
							S663P				
FinA	3	M12	5F	2	>256	FKS1 HS1	D632Y				
						FKS2 HS1	L660S				
			5H	8	256	FKS2 HS1	F659C				
							L659S				
ANI	4	EF1620	7B	8	8	FKS2 HS1	F659-				
			7D	4	>256	FKS1 HS1	W650*				
						FKS2 HS1	F659-				
			7F	4	4	FKS1 HS1	D632Y				
						FKS2 HS1	F659L				
ANIFLZ	4	EF1620	7D	4	>256	FKS2 HS1	F659-				
			7F	8	>256	FKS1 HS1	W681L				
FinA	4	EF1620	7D	4	256	FKS1 HS1	F625L				
							D632E				
						FKS2 HS1	E761*				
			7F	>16	>256	FKS1 HS1	F625Y				
							D632E				
ANI	5	CBS138	9F	4	4	FKS1 HS1	W611*				
						FKS2 HS1	L662W				
			9H	4	>256	FKS2 HS1	F659-				
							F659L				
FinA	5	CBS138	9F	1	64	FKS2 HS1	L662F				
							P667T				
			9H	2	2	FKS2 HS1	F659I				
							L662F				
ANI	6	P352	10E	4	8	FKS2 HS1	S663F				
			10G	16	>256	FKS2 HS1	F659-				
							R665G				
ANIFLZ	6	P352	10E	4	>256	FKS2 HS1	F659-				
			10G	8	>256	FKS1 HS1	E621K				
						FKS2 HS2	R1378S				
							Q1397R				
FinA	6	P352	10E	8	>256	FKS2 HS1	F659-				
							F659A				
			10G	4	>256	FKS2 HS1	F659-				
							F659L				
ANI	7	BG2	11B	8	>256	FKS2 HS1	A651V				
							S663P				
			11H	4	16	FKS2 HS2	R1378H				
FinA	7	BG2	11B	4	256	FKS2 HS1	S654Y				
							F659-				
			11H	4	>256	FKS2 HS1	L662W				

Our results indicate that treatment with ani always leads to mutations in the drug target and that these mutations might be a necessary mechanism for the acquisition of resistance to ani. The presented landscape of *FKS* mutations also includes many (37.22%) that, to our knowledge, have not been previously described.

6.3.2 *CNE1* mutations and loss of anidulafungin resistance

In the analysis described in **Chapter 5** we found that, despite the fact that most samples retained the acquired resistance, seven ANI samples that acquired ani resistance, experienced an increase in susceptibility to ani after subsequent exposure to flz (**Table 6.2**). Two of these ANI mutants and the corresponding AinF descendants were selected for whole genome sequencing. This analysis revealed that the two AinF descendants presented truncations in the *FKS* gene that was mutated in the ANI parental. As a consequence of this truncation, these AinF samples likely present a single functional Fks paralogous protein that can be targeted by ani, which would explain the high susceptibility to ani. Manual inspection of the genomic changes present in the ANI parentals that could influence the loss of resistance uncovered the presence of a singular mutation in only one of the *FKS* genes (codon F659del in *FKS2*) combined with the presence of a non-synonymous mutation or a deletion in CAGL0D00242g, the ortholog of *Saccharomyces cerevisiae CNE1* gene (from now on called *CNE1*).

Cne1p encodes an ER localized protein that functions as a molecular chaperone associated with nascent glycoprotein maturation and protein folding, and is a component of the ER quality control system that retains misfolded protein intermediates (Molinari et al. 2004; Williams 2006). It has been observed that disruption of Cne1p in *C. glabrata* induces ER stress and causes changes in the cell wall structure, specifically a decline in β-1,6-glucan content and accumulation of chitin (Tanaka et al. 2018). The same study claimed that ER stress modulates the fungal cell wall and regulates the cell wall integrity (CWI) pathway in the species, and connected malfunctioning of the quality control of glycoproteins in the ER with the induction of ER stress and cell wall modifications. In other words, the ER would serve as a platform for maintaining CWI under cell wall stress by sensing disturbances in the proper supply of nascent glycoproteins.

Considering the above mentioned possible connection between *CNE1* and CWI, we searched for *CNE1* mutations in the other ANI samples. We investigated the *CNE1* gene in five additional ANI samples that had only one of the *FKS* genes mutated and for which subsequent AinF samples presented a drop of ani resistance, to assess the connection of *CNE1* and the loss of the phenotype in a larger number of samples. Two of the five newly analyzed samples showed *CNE1* mutations in combination with F659del in *FKS2* (**Table 6.2**). Hence, all four ANI samples with *CNE1* alterations had AinF descendants that presented substantial decrease in MIC. Furthermore, from these four, three AinF descendants showed WT levels of susceptibility implying the complete loss of the phenotype.

Importantly, none of the other whole genome sequenced ANI samples, which retained the ani-resistance phenotype, presented *CNE1* alterations. Although the notion that *CNE1* mutations are connected with loss of the phenotype is promising, sequencing of additional strains will help to clarify this relationship. The three remaining samples that show decreased ani resistance but no *CNE1* alterations could carry alterations in other genes implicated in CWI.

Table 6.2. Mutations in *CNE1* and *FKS* genes in the 7 ANI samples where subsequent AinF offsprings show decrease in resistance to ani. The table presents mutant names, experimental condition, clade, strains, replicate and *CNE1* and *FKS* genes mutations, followed by information on susceptibility to the drug: MIC and rAUC in ANI and AinF progenies.

					Mutations			MIC		rAUC	
Mutant	**Condition**	**Clade**	**Strain**	**Replicate**	***CNE1***	***FKS1***	***FKS2***	**ANI**	**AinF**	**ANI**	**AinF**
TGL00052	ANI	1	CST109	1D	-		F659-A1390T L664Q	1	**0,064**	0,433	0,349
TGL00060	ANI	2	EB0911	3D	E281K		F659-	4	**0,064**	0,666	0,231
TGL00062	ANI	2	EB0911	3H	G261D		F659-	4	**0,064**	0,639	0,225
TGL00065	ANI	3	CST78	4E	-	L628I D632N		16	**2**	0,836	0,642
TGL00066	ANI	3	CST78	4G	-	S629Y		4	**1**	0,689	0,425
TGL00071	ANI	4	EF1237	6A	D157G		F659-	8	**0,5**	0,710	0,403
TGL00075	ANI	4	EF1620	7B	deletion		F659-	8	**0,064**	1,543	0,287

We further investigated whether *CNE1* mutations could have an impact on susceptibility to ani. Assuming that the mutation would confer ani resistance, we attempted to insert it in an ani susceptible strain using CRISPR-Cas9 transformation and using ani as a selective medium. The same approach was successfully used for the reintroduction of *FKS* mutations described in the **Chapter 5**. However, in the *CNE1* case, no transformant was obtained. If the transformation itself was successful and the mutation was inserted,

the absence of resistant transformants may imply that this *CNE1* mutation does not have a direct effect on the susceptibility to ani.

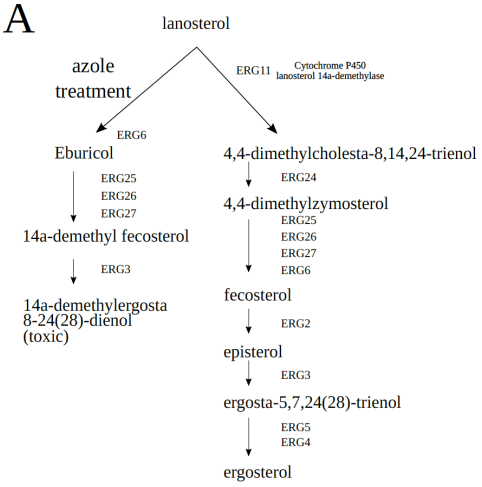
Although we report the presence of alterations in *CNE1* in 4 of the 7 ANI samples that increased ani susceptibility to ani when subsequently exposed to azoles, the basis of this association is still unknown. However, based on the notion that ER stress induces CWI changes (Tanaka et al. 2018), it can be speculated that ani treatment results in ER stress through its impacts on CWI. This may lead to the selection of genetic changes in the gene encoding the ER-localized Cne1p. Further, ani resistant cells carrying *CNE1* alterations and only one of the *FKS* genes mutated seem to benefit from the deactivation of the mutated *FKS* gene (here by a truncating mutations). It is unclear whether this inactivation, leaving a single active *FKS* gene, and in its wild type form, helps the cell return to normal levels of production of beta-glucans once ani is not present, or whether it helps to avoid the accumulation of some toxic intermediates, or any other effect. In any case, this deactivation renders the cells more susceptible to ani, as can be observed by the decrease in resistance to the drug.

6.3.3 Susceptibility to fluconazole and *ERG3* mutations

Although the topic of *ERG3* alterations and their contribution to flz resistance was already mentioned in **Chapter 5**, the broader issue of modifications in the ergosterol pathway as a response to ani exposure deserves further analysis.

ERG genes that acquired mutations during exposure to ani include not only *ERG3* but also *ERG4* and *ERG5*, all of which encode enzymes that catalyze the final steps of the ergosterol synthesis pathway (**Figure 6.4 A**). This suggests that the cell stress exerted by ani may impact cell membrane in a way that altering ergosterol biosynthesis pathway by such mutations may have a compensatory effect. Whole genome sequencing uncovered *ERG3* mutations in 10 ANI, 8 FinA and 1 ANIFLZ; *ERG4* in 1 ANI and 1 FinA (both combined with changes in *ERG3*); and *ERG5* in 1 FinA. Notably, the fact that we observed *ERG* genes modifications in FinA samples (that presented decreased susceptibility to flz before the evolution in ani) indicates that these mutations are the consequence of ani treatment and the possible acquisition of the resistance to flz in ANI mutants is only a secondary effect. This scenario is further reinforced by the observation that not all alterations in *ERG3* resulted in increased flz resistance.

A



B

species	type of alteration	response to fluconazole
Candida glabrata	PTC	susceptible
	missense mutation	resistant
	deletion	susceptible
Candida albicans	missense mutation	resistant
	defective	resistant
	deletion	resistant
Candida parapsilosis	missense mutation	resistant
Saccharomyces cerevisiae	defective	resistant

Figure 6.4. (**A)** Ergosterol biosynthesis pathway starting from lanosterol in the presence (left) and absence (right) of azoles. (**B**) table with the effects of alterations in *ERG3* on flz susceptibility in *Candida glabrata*, other *Candida* species and *Saccharomyces cerevisiae.*

As we observed a direct impact of *ERG3* mutations on flz susceptibility, a specific focus was put on this gene. Alterations of *ERG3* have been shown to contribute to decreased susceptibility to azoles in *C. albicans* (Martel et al. 2010; Sanglard et al. 2003), *C. parapsilosis* (Branco et al. 2017) and *Saccharomyces cerevisiae* (Kelly et al. 1995) (**Figure 6.4 B**). Inactivation of *ERG3* might be beneficial under azole treatment, as it causes the accumulation of 14α-methylfecosterol, an intermediate of the ergosterol pathway that is less damaging to the cell membrane than toxic products that accumulate due to Erg11p inhibition (14a-methylergosta-8,24-dien-3b,6a-diol), and further results in continued growth in the presence of azoles (**Figure 6.4 A**) (Branco et al. 2017). In contrast, *ERG3* deletion in *C. glabrata* has been shown to have no effect in azole susceptibility (Geber et al. 1995). In this context, to broaden our

knowledge about the changes contributing to flz resistance, we sequenced the *ERG3* gene in all ANI samples. As mentioned in the previous chapter, combining this information to that provided by WGS, we observe that 28/47 mutants present changes in this gene (**Figure 6.5**).

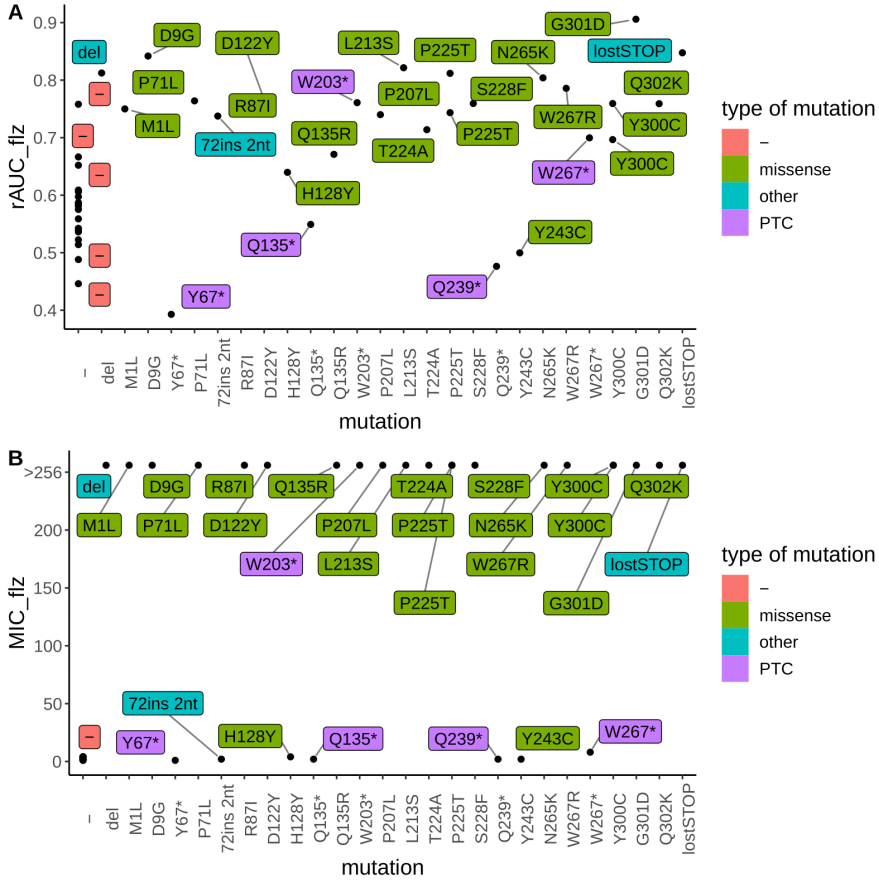


Figure 6.5. Susceptibility to flz in ANI samples. Presented are mutations in *ERG3* gene in ANI mutants with susceptibility to flz data **(A)** - rAUC and **(B)** - MIC. No alterations found are shown by "-" and PTC stands for premature termination codon. rAUC and MIC values were calculated as described in **Chapter 5**.

All the observed missense mutations correlate with higher levels of resistance to flz except two: H128Y and Y243C. We suspect these changes do not influence the function of the *ERG3* gene or evoke alterations similar to those caused by premature termination codons (PTC).

As expected, three of our ANI samples containing premature termination codons (PTC) remained susceptible to flz (Y67*, Q135* and Q239*) **(Figure 6.5)**. However, we observed other changes that similarly would imply inactivation of the gene but were associated to higher MIC/rAUC in flz. Nonetheless, since one resistant sample that presented a mutation in W267* (of note, analyzed in **Chapter 5** in a sample where low MIC was attributed to the trailing effect) had also an alteration in *ERG4* (D338E), we may hypothesize that other samples presenting: premature stop codon, deleted part of *ERG3* or two nucleotide insertion causing frameshift, may contain additional changes in other *ERG* genes influencing the low susceptibility to flz when *ERG3* is truncated. The recurrent acquisition of mutations in *ERG* genes in response to ani treatment suggests a contribution to the decreased susceptibility to ani. However, we observed that reintroduction of some of the *ERG3* mutations did not alter the level of ani resistance (**Chapter 5 Supplementary Figure 5.4**).

In summary, samples evolved in ani benefit from alterations in *ERG* genes, which in turn may in some cases result in resistance to flz, an unfortunate byproduct. The observed phenotypic differences between strains carrying either non-synonymous SNPs or PTC in *ERG3*, as well as why ani treatment results in mutations in the

ergosterol biosynthesis pathway are two open questions that need further investigation. Similar to the case of mutations appearing in the *CNE1* gene, it is possible that CWI signaling stimulated by cell wall stressors is activating stress responses, also in the ER, where ergosterol is synthesized. These could trigger changes in ergosterol biosynthesis genes that compensate for alterations in the cell wall composition, maybe by contributing to the production of alternative sterols, a feature that may indirectly cause resistance to drugs targeting the sterol biosynthesis pathway, such as azoles.

6.3.4 Susceptibility to amphotericinB and *ERG3* mutations

Amphotericin B is a polyene drug that binds directly to ergosterol, thereby triggering cell death (Gray et al. 2012). Resistance to polyenes has been attributed to point mutations in genes involved in ergosterol biosynthesis leading to decreased content of ergosterol and to cross resistance to azoles in *Candida albicans* (Ksiezopolska and Gabaldón 2018). Considering this, we decided to investigate the susceptibility to amphotericin B in a selected set of ANI, FinA, and ANIFLZ, in total 22, samples for which whole genome sequencing had been performed and in all WT strains used in the evolution experiment (**Figure 6.6**). Only two of the analyzed samples (10G_ANI with *ERG3* S228F and 11H_ANI with *ERG3* W267* but also *ERG4* D338E) show increased MIC compared to samples with no *ERG3* mutation. This indicates the presence of unaltered ergosterol in the majority of *ERG3* mutated samples regardless of these being a missense mutation or PTC. On the other hand, this

observation also shows the potential of ani to cause resistance to three antimycotics belonging to three different classes of antifungal.

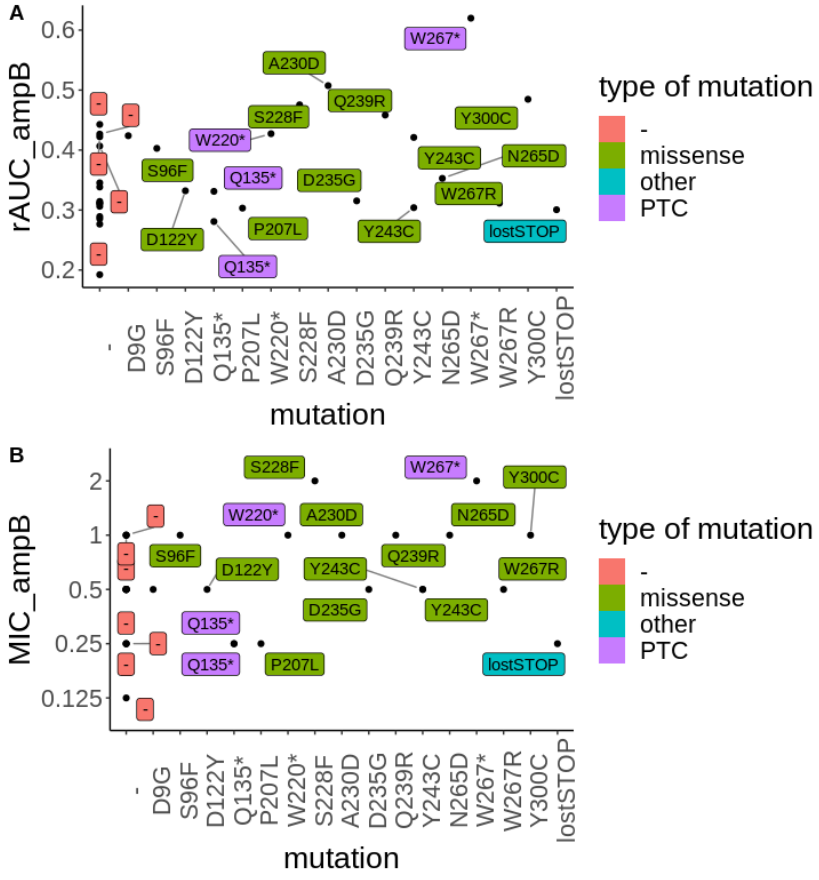


Figure 6.6. Susceptibility to amphotericin in selected ANI, FinA and ANIFLZ samples. Presented are mutations in *ERG3* gene with susceptibility to amphotericin B data **(A)** - rAUC and **(B)** - MIC. No alterations found are shown by "-" and "PTC" stands for premature termination codon. rAUC and MIC values were calculated as described in **Chapter 5**

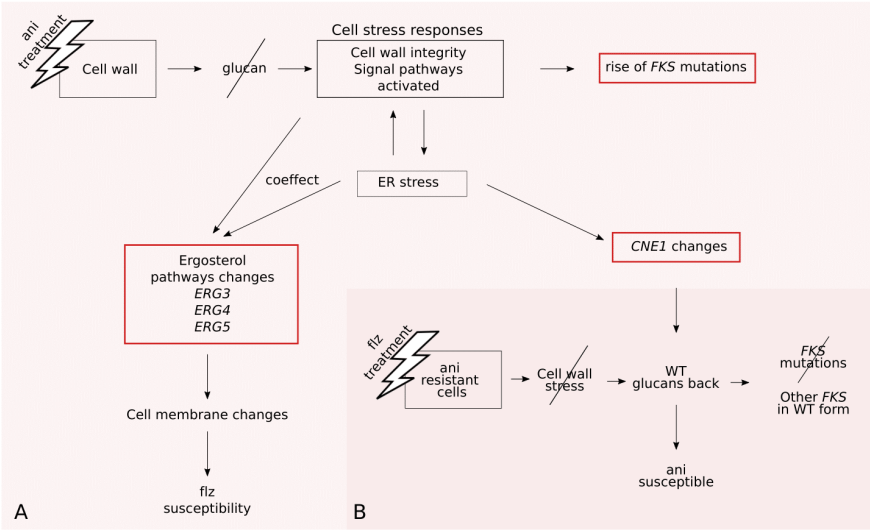


Figure 6.7 Scheme of discussed changes we observe after evolution in ani. (**A**). Ani treatment affects cell wall, which results in the decrease in glucan content and activation of cell wall responses. Cell wall responses can further affect ER stress response and these collectively lead to the emergence of *FKS* mutations, *CNE1* alterations and changes in genes involved in ergosterol synthesis pathway further influencing susceptibility to flz. (**B**) Loss of the ani susceptibility can be connected with *CNE1* changes that under elimination of cell wall stress could be involved in the cell transitions requiring the presence of wild type glucans and subsequently cause the decrease in ani resistance.

7 Persistence of genetically-acquired azole and echinocandin resistance in *Candida glabrata*

Ksiezopolska, E.; Schikora-Tamarit,M; Gabaldón T. "Persistence of genetically-acquired azole and echinocandin resistance in *Candida glabrata". (submitted)*

7.1 Abstract

The limited number of available antifungal drugs and the increasing number of fungal isolates that show drug or multidrug resistance pose a serious medical threat. Several yeast pathogens, such as *Candida glabrata*, show a remarkable ability to develop drug resistance through the acquisition of genetic changes in response to treatment. However, how stable the resistance phenotype and the underlying mutations are in non-selective conditions remains poorly characterized. The stability of previously-acquired drug resistance has fundamental implications for our understanding of the appearance and spread of drug resistant outbreaks. Here, we used an in vitro evolution approach to assess the stability under optimal growth conditions of resistance-conferring mutations previously acquired under exposure to anidulafungin and fluconazole. Our results reveal a remarkable persistence of the resistance phenotype

and the underlying mutations for at least two months. We found a higher conservation of anidulafungin resistance and of resistance-conferring point mutations as compared to fluconazole resistance or aneuploidies, respectively. We conclude that acquired resistance, particularly to anidulafungin, is a long lasting phenotype, which suggests a low fitness cost for the resistance phenotype.

7.2 Introduction

Although fungi can be part of the natural microbiome of healthy individuals (Hallen-Adams and Suhr 2017), they can be the source of invasive infections in immunocompromised patients (Silva 2010). Changes related to advances in medical progress such as the extensive use of antibiotics, the aging of the population, or the increased survival of immunocompromised patients, have been linked to a higher incidence of fungal diseases (Mason et al. 2012; Gabaldón and Carreté 2016). Pathogenic yeasts belonging to the polyphyletic genus *Candida* are the most common cause of both life-threatening invasive infections as well as mucosal diseases, such as vulvovaginal candidiasis (Berman and Krysan 2020).

Antifungal therapy and prophylaxis are key for reducing the mortality and comorbidity associated to fungal infections, however, they are also primary factors driving progressive epidemiological shifts from the most common *Candida albicans* to non-albicans *Candida* species presenting higher levels of intrinsic and/or acquired resistance, such as *Candida glabrata* (Lamoth et al. 2018). In addition, recent reports show a growing prevalence of clinical

isolates that are resistant to multiple drugs, mostly belonging to non-albicans species, including *C. glabrata* (Pham et al. 2014b; Beyda et al. 2014), *Candida kefyr* (Fekkar et al. 2013), *Candida lusitaniae* (Asner et al. 2015) or *Candida auris* (Vallabhaneni et al. 2016). Emergence of drug and multidrug resistance in fungi is particularly worrying given the limited arsenal of antimycotic agents at our disposal, and with most of them belonging to one of three major families: azoles, echinocandins, and polyenes (Krysan 2017). Azoles impair ergosterol biosynthesis by binding to one of the enzymes in the pathway (Erg11p), thereby inhibiting growth of *Candida* species. Polyenes bind directly to ergosterol, which weakens the cell membrane and leads to cell death. Echinocandins block glucan synthase, encoded by *FKS* genes, thereby inhibiting the biosynthesis of β-1,3-d-glucan, a major component of the fungal cell wall (Ksiezopolska and Gabaldón 2018). Mechanisms of antifungal drug resistance involve alterations in the sequence or expression of the genes encoding the drug targets, overexpression of drug efflux pumps, as well as gross chromosomal changes (Cowen et al. 2014; Ksiezopolska and Gabaldón 2018).

Previous studies have shown that gene copy number variations, including whole chromosome (Chr) aneuploidies contribute to antifungal drug resistance (Sasse et al. 2012; Selmecki, et al. 2006; Anderson et al. 2017; Yang et al. 2017, 2013, 2019; Todd et al. 2019; Coste et al. 2006). For instance, in *C. albicans* azole resistance was associated to the presence of an isochromosome (5L) which resulted in two extra copies of the left arm of Chr5 (Selmecki et al. 2006), which carry *ERG11* and *TAC1* (encoding the transcription factor

regulating ABC transporter genes *CDR1* and *CDR2*) genes. Furthermore, aneuploidies of Chr3, bearing *CDR1* and *MRR1* (encoding a transcriptional activator of the major facilitator superfamily transporter *MDR1*), and trisomy of Chr7 were connected with increased efflux of the drug (Mount et al. 2018). *C. glabrata* presents a considerable karyotypic variability with many analyzed isolates presenting gross genomic rearrangements, which have been sometimes attributed to a response to antifungal drug treatments (Healey et al. 2016; Muller et al. 2009; Poláková et al. 2009; Shin et al. 2007).

Genomic rearrangements can be advantageous to fungal cells by contributing to rapid responses and adaptation to stress, and can represent intermediate evolutionary steps in the acquisition of resistance to unfavourable conditions (Ksiezopolska and Gabaldón 2018). Supporting this view is the fact that some gross genomic rearrangements, such as aneuploidies, occur at higher rates than specific point mutations, especially under stress conditions (Duesberg et al. 2001; Healey et al. 2016). Hence, they are likely to be the first resistance-conferring mutations that appear spontaneously in an evolving population. Chromosomal aneuploidies result in up- or downregulation of several genes at a time, which may be advantageous in specific conditions. However, as they involve the dysregulation of many genes, they are expected to have a fitness cost and, consequently, be evolutionarily unstable (Rustchenko 2007). Considering all this, changes in ploidy can be regarded as a rapidly-acquired temporary solution to cope with stress conditions that allows suboptimal survival of the cells and facilitates the emergence

of fitter, more stable point mutations (Berman 2016). However, how stable these alterations really are is still poorly investigated.

Persistence of resistance phenotype has been reported in clinical and in vitro studies (Borst et al. 2005; Imbert et al. 2016; Hatwig et al. 2019). Imbert et al observed loss of resistance to echinocandins (but not azoles) in multidrug resistant *C. glabrata* after one month of treatment discontinuation, and the loss was attributed to the disappearance of a previously acquired *FKS* mutation. Borst et al. reported the stability of resistance to flz after 122 days and Hatwig et al. the resistance to ani and flz after a month of propagation under no antifungal stress. However, the number of investigated strains was relatively low (one patient, and five and six in vitro evolved strains, respectively), and none of the studies included analysis of the genomic changes involved in the emergence or loss of the resistance phenotypes.

In an earlier study, we used in vitro evolution to broaden our knowledge on how distinct clades of *C. glabrata* may acquire resistance to fluconazole (flz, an azole) and anidulafungin (ani, an echinocandin) (**Chapter 5**). From these experiments we obtained strains that successfully adapted to different drug treatment regimes and acquired resistance to one or two of the drugs. Subsequent genome sequencing analysis identified mutations that appeared during the process of adaptation and that are likely to drive the resistance phenotype. Among other alterations, 18 resistant strains carried whole chromosome or large segmental duplications affecting one of four chromosomes, always in combination with point

mutations in relevant genes. The most abundant aneuploidy consisted of the duplication of *ERG11*-bearing ChrE, found in 17 strains. Two of these strains presented additional partial duplications in ChrI (bearing *TPO3* gene which encodes for a predicted polyamine transporter of the major facilitator superfamily involved in azole resistance) and one strain presented both ChrE and ChrL duplications. Finally, one sample presented a duplication of ChrA, containing the *PDR1* gene, a transcription factor involved in azole resistance in *C. glabrata* (Tsai et al. 2006). Interestingly, in that study we observed extraordinary stability of these chromosomal duplications after harvesting the strains under the antifungal drug stress, with 10/11 mutants carrying chromosomal duplications acquired during flz treatment retaining it after subsequent ani treatment. The aim of this study was to investigate the stability of the emerged genomic alterations and correlate it with the stability of the resistance phenotype after propagation under optimal growth conditions. We found that aneuploidies are less stable when antifungal stress is withdrawn as compared to point mutations. In addition, we found that ani resistance was more stable than flz resistance.

7.3 Results

7.3.1 Stability of the resistance phenotypes

In total we analyzed 23 strains: of which 19 presented a duplication of ChrE only, two harbored duplications in ChrE and ChrI, one in ChrE and ChrL, and one in ChrA (**Chapter 5**, **Material and methods**). This set includes ten strains with ChrE aneuploidy (one with additional ChrI alteration) resulting from azole adaptation, and their ten direct progenies that maintained this chromosomal rearrangement after subsequent ani treatment (of which one lost flz resistance). Apart from the chromosomal alterations, the resistance to flz was connected to the presence of *PDR1* mutations and resistance to ani in parental strains was linked to mutations in *FKS* genes in the parental strains (**Chapter 5**).

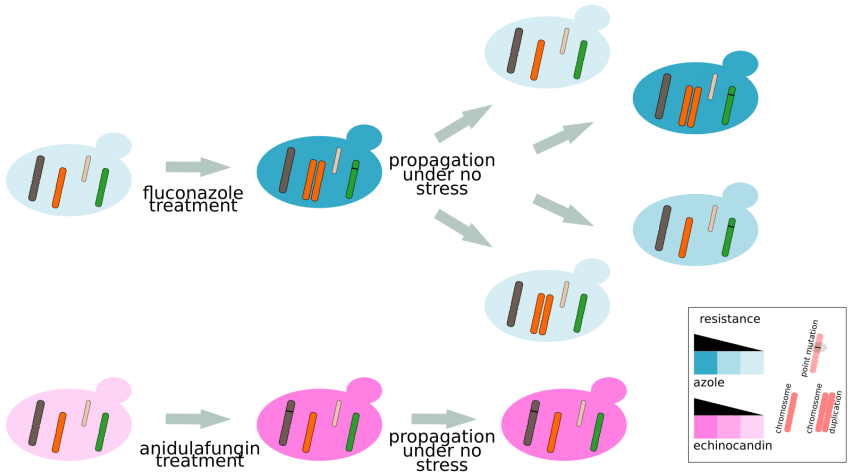


Figure 7.1. Scheme of the genomic changes appearing during and after the removal of antifungal drug treatment. Presented are chromosomal duplications and/or point mutations emerged under flz (blue) and ani (pink) stress. Upon finalization of the treatment, the changes and the resistance phenotype can be maintained, or they can be lost leading to total or partial loss of the resistance phenotype.

To assess the stability of the resistance phenotype in the absence of drug exposure, the strains were grown during eight weeks in rich medium (YPD) free of the antifungal agent. Changes in resistance phenotype after this period were evaluated by comparing the growth efficiency on the relevant drug of the parental and the evolved strains (**Figure 7.2** and **Materials and methods**).

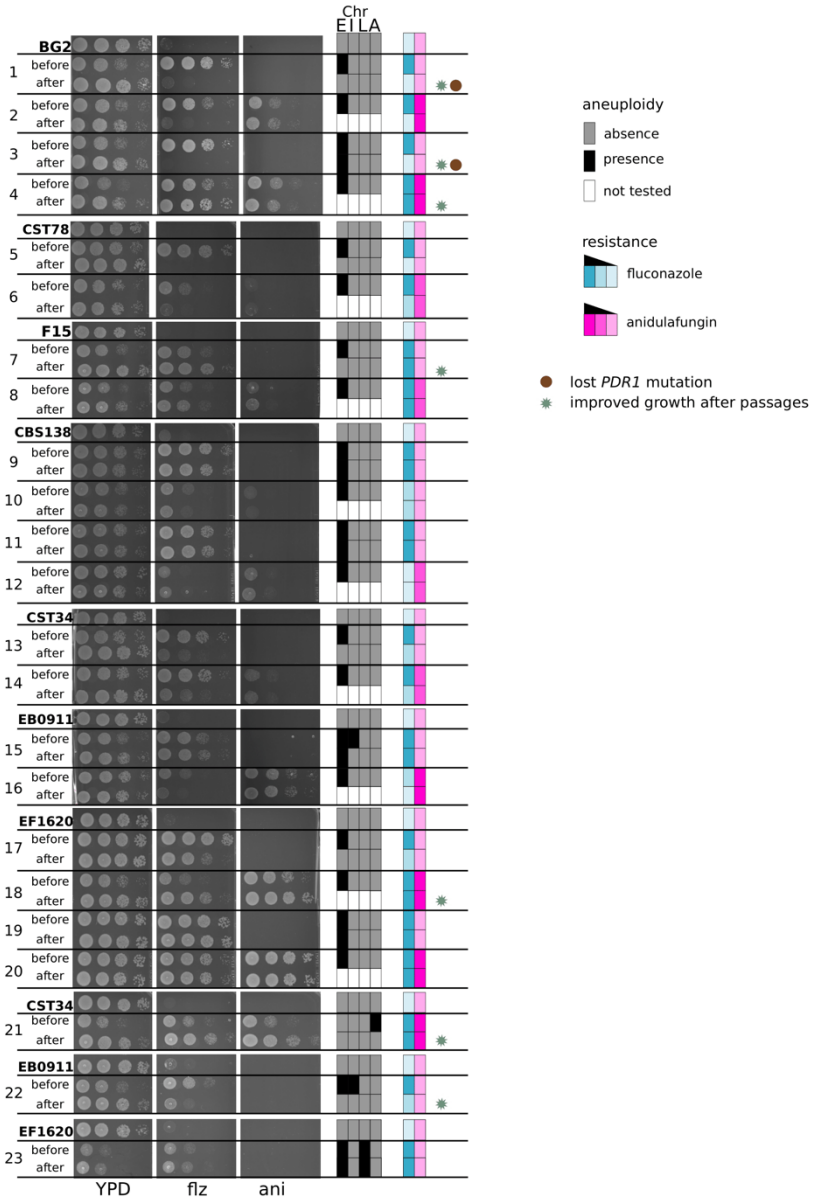


Figure 7.2. Summary of the results. Spot tests showing growth on rich medium (YPD) and on 100ug/ml fluconazole (flz) and 0.5ug/ml anidulafungin (ani) followed by information about the aneuploidy, susceptibility to flz and ani and additional comments. Samples are analyzed in pairs - before and after 8 weeks of regrowing without antifungal stress. The presence of aneuploidies was inferred from **Figure 7.3.**

Ten out of 22 (45.5%) evolved samples showed a decrease in flz resistance after propagation without stress. Resistance decline affected five of ten (5/10, 50%) strains that had been previously treated with flz, and three out of nine (3/9, 33%) strains corresponding to their progenies that additionally were exposed to ani treatment. Importantly, the decrease in flz resistance was total in five strains and partial in five of them, indicating that the strains retained part of the previously acquired resistance. None of the eleven samples that were initially resistant to ani showed a decrease in resistance to this drug.

Interestingly, seven YPD-evolved samples showed improved growth on a control plate containing YPD medium (indicating higher fitness) when compared to the parental resistant mutants, suggesting increased adaptation to this condition. In two of such cases (EF1620 strain 18 and CST34 strain 21, **Figure 7.2**) this might have additionally impacted growth in the presence of flz where an increase in resistance is observed. The spot test for these samples was repeated twice with the same results.

7.3.2 Stability of chromosomal aneuploidies

The stability of the acquired aneuploidies was investigated in 13 strains: nine that presented alterations in ChrE, two in ChrE and ChrI, one in ChrE and ChrL and one in ChrA. The rationale of this selection was to study the stability of ChrE in 10 flz resistant samples that had always maintained ChrE duplication when exposed to ani, and additionally we included samples presenting duplications of

chromosomes I, L, and A. The genomes of these strains were sequenced using a strategy that combined several strains belonging to different clades (therefore with identifiable SNP patterns) into a single sequencing library. The analysis of the relative coverage of strain-specific alleles in the different chromosomes enabled us to identify the aneuploidies present in the sample (see **Materials and methods**, **Figure 7.3**). We could calculate the coverage for at least 970 genomic positions in all aneuploid chromosomes **(Figure 7.3)**. We thus conclude that our strategy resolves the presence of aneuploidies in an accurate, cost-effective manner.

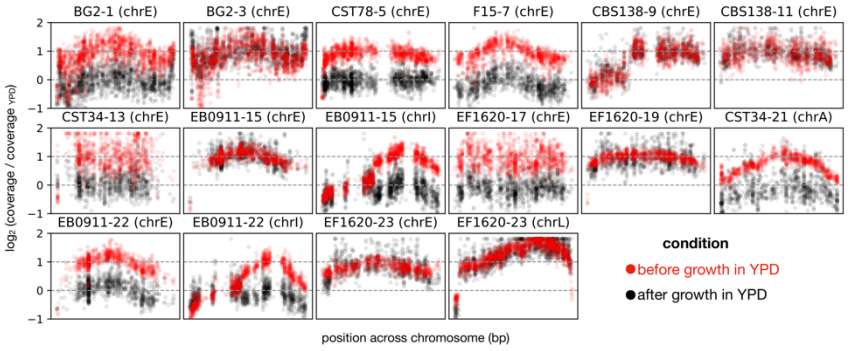


Figure 7.3. Aneuploidy assessment from depth of coverage of strain-specific SNPs coverage. Relative coverage (as compared to the median of chromosomes without aneuploidies) plots for several genomic positions before (red) and after (black) 8 weeks of growth in rich media. We $\log_2$-normalize the data by the coverage in the YPD-evolved sample (of the corresponding strain (see **Chapter 5**)) in order to correct strain-specific biases. Note that the coverage after growth in YPD (black) was measured from the sequencing of pooled samples (see **Materials and Methods**). This approach only allowed the calculation for some positions, which explains why there are gaps in these figures. Each panel corresponds to one aneuploid chromosome in a strain (named as in **Figure 7.2**).

ChrE was lost in six (6/12, 50%) of the investigated strains, and in five of these the loss of the aneuploidy was accompanied with a decrease in resistance to flz. Interestingly, in three stains (CST34

strain 13, EF1620 strain 17 and EB0911 strain 22) the resistance was not totally lost, strengthening the link between the aneuploidy and degree of resistance. However, we also observed the maintenance of the ChrE duplication in a strain that nevertheless showed a decrease in resistance (BG2 strain 3).

The single strain harboring ChrL aneuploidy maintained it, while aneuploidies affecting ChrI and ChrA were always lost. Loss of ChrI aneuploidy only resulted in increased susceptibility in a single strain (EB0911, strain 22) where ChrE was also lost. This suggests that alterations in chromosomes other than ChrE were either beneficial for the adaptation to the stress caused by the antifungal drugs or were passenger mutations, but do not necessarily influence the susceptibilities to the drugs.

7.3.3 Stability of point mutations

As the investigated strains also possessed resistance-related point mutations in *FKS*, *ERG11*, *ERG3*, *ERG4*, *PDR1, CDR1* and *CNE1* genes, we investigated their presence after the experiment by using the genome sequencing data, and confirmed putative reversions by targeted amplicon sequencing (see **Materials and Methods**). All samples, except two (21/23, 91.3%), retained the relevant mutations, indicating a higher stability of point mutations as compared to aneuploidies. The two samples that lost the resistance-conferring point mutation are progenies of the same WT strain (BG2). The lost mutations involved a missense mutation (L280F) and an insertion (V339/VE) in the *PDR1* gene. In both cases the mutated positions

reverted to the wild type configuration. Both of the samples showed a decrease in flz resistance, despite the retention of a ChrE aneuploidy in one of them, suggesting a larger effect on the phenotype of the *PDR1* mutation.

7.4 Conclusions and discussion

This study aimed to assess the stability of previously acquired resistance phenotypes and their genetic drivers in *C. glabrata* after cultivation under optimal growth conditions. Overall, we observed a higher stability of the ani resistance phenotype, which was always retained, as compared to the flz resistance phenotype lost in 5/22 (22.7%) of the strains. A higher stability of the ani resistance phenotype is consistent with our earlier observation that ani resistant isolates showed similar fitness values in antifungal free conditions as their wild type parentals whereas growth of flz resistant isolates was more impaired (**Chapter 5**). This may imply a higher fitness cost (in optimal growth conditions) of flz resistance as compared to ani resistance, which may explain in part the higher propensity to be lost. Additionally, in a previous experiment (**Chapter 5**) we observed that when ani resistant strains were exposed to flz, ani resistance was lost in four samples (8.4% of all tested), whereas flz resistance was more likely to be retained in cells exposed to ani (lost in one sample, 2.1%). This trend is opposite to what was observed here in optimal growth conditions, revealing the complex relationships between fitness, environment and phenotype.

We observed that chromosomal aneuploidies were more often lost than point mutations, reinforcing their temporary role. Nevertheless, half of the samples (50%) retained ChrE aneuploidy after two months of growth in non-selective conditions, which is not negligible and would suggest that aneuploidy-driven flz resistance may persist for long periods of time in *C. glabrata* populations in untreated patients or in the environment. Again, the relatively high level of aneuploidy loss under optimal growth conditions (6/12 ChrE, 2/2 ChrI, 0/1 ChrL and 1/1 ChrA), contrasts with our earlier results showing high retention of chromosomal aneuploidies when flz resistance samples were grown in ani (lost in 1/11). This suggests a higher stability of aneuploidies under stress conditions, even when the stress is different from the one that originated the aneuploidy. In clinical settings this would mean that the acquired duplications could be also stable after the change of drug regimens. Furthermore, aneuploidies of ChrE (bearing the azole drug target and being always accompanied by *PDR1* alterations) seem to enhance survival under flz treatment but not be the main factor. In all cases except one, loss of ChrE duplications was associated with a decrease in the resistance, albeit sometimes this was incomplete. Our ChrE duplications bearing strains also carried mutations in *PDR1*, and losing this mutation seems to be the determinant factor for the disappearance of the resistance phenotype, even when ChrE duplication is retained. This observation is in agreement with a previously reported observation (**Chapter 5**) where a strain hypersensitive to flz was found to carry both a truncation in the *PDR1* gene and ChrE aneuploidy.

In summary, our results indicate a relatively long lasting stability of acquired resistance which is also consistent with previously described low fitness costs of resistance (**Chapter 5**) and previous studies demonstrating the maintenance of the phenotype (Hatwig et al. 2019; Imbert et al. 2016; Borst et al. 2005). Importantly, apart from showing maintained resistance, we also observed strains with improved general fitness. The persistence of resistance is of clinical relevance as once cells become resistant, they may maintain the phenotype for extended periods of time. As a consequence, this can lead to treatment failure, or spread of resistant strains.

7.5 Materials and methods

Strains

Strains were obtained from previous experimental evolution of antifungal drug resistance study (**Chapter 5**). Detailed information about the strains can be found in **Table 7.1**.

Table 7.1. Strains used in this study. Table includes mutant names, antifungal treatment of the experimental evolution, clade, wild type susceptible parental strain, experimental evolution replicate, chromosomes that were altered and SNPs in the main genes. In the sequential drug treatment, SNPs that were obtained in the previous antifungal drug treatment are marked with 'ANI:'.

Sample	Mutant	Previous treatment	Clade	Strain	Replicate	Altered chromosome	PDR1	ERG3	ERG4	ERG5	FKS1	FKS2	Scer_CNE1
1	TGL00041	FLZ	7	BG2	11B	ChrE	L280F						
2	TGL00291	FinA	7	BG2	11B	ChrE		N265D				S654Y F659-	
3	TGL00044	FLZ	7	BG2	11H	ChrE	V339VE						
4	TGL00294	FinA	7	BG2	11H	ChrE					Q1230*	R1378C L662W	
5	TGL00016	FLZ	3	CST78	4G	ChrE	Y372H						
6	TGL00266	FinA	3	CST78	4G	ChrE		lostATG|c.1|Atg/Ttg				A651T	
7	TGL00032	FLZ	5	F15	8G	ChrE	V329F						
8	TGL00282	FinA	5	F15	8G	ChrE					R1422L	F708S	
9	TGL00035	FLZ	5	CBS138	9F	ChrE	G334W						
10	TGL00285	FinA	5	CBS138	9F	ChrE		A230D	Y397H			P667T L662F	
11	TGL00036	FLZ	5	CBS138	9H	ChrE	L935F						
12	TGL00286	FinA	5	CBS138	9H	ChrE	E340*	Y243C				F.659I R1378L	
13	TGL00006	FLZ	1	CST34	2C	ChrE	L946S						
14	TGL00256	FinA	1	CST34	2C	ChrE					D632G	D666Y F659L	P161L
15	TGL00012	FLZ	2	EB0911	3H	ChrE and ChrI	G1088E						
16	TGL00262	FinA	2	EB0911	3H	ChrE		D235G				F659-	
17	TGL00025	FLZ	4	EF1620	7B	ChrE	N768D						
18	TGL00275	FinA	4	EF1620	7B	ChrE					P1337S	L662F D666N	
19	TGL00026	FLZ	4	EF1620	7D	ChrE	L935F						
20	TGL00276	FinA	4	EF1620	7D	ChrE				L246*	D632E F625L	E761*	
21	TGL00108	ANIFLZ	1	CST34	2G	ChrA	W297C				D632N V1362M P633S	S201P	
22	TGL00212	AinF	2	EB0911	3H	ChrE and ChrI	R376Q					ANI: F659-; V532VX	ANI: G261D
23	TGL00225	AinF	4	EF1620	7B	ChrE and ChrL	L280F					ANI: F659-; TE365-366TX	

Propagation under no stress

The stability of the resistance in 23 samples that were previously in vitro evolved to be resistant to antifungal drugs were analyzed after regrowing the samples in rich media lacking any antifungal stress. A smear of biomass of each investigated sample was taken from the glycerol stock and inoculated in 500ul of YPD media. During eight weeks, every 1-3 days, 50ul of the sample was passed into a fresh 450ul of the media. After the finalization of the 35 passages, single colonies were selected and stored in glycerol until further analysis.

Susceptibility test - spot test

All the samples were grown in 500ul of YPD overnight. The cells were adjusted to an OD (Optical density) of 0.5 and serially diluted 10x 4 times. 5ul of the final dilution were spotted on YPD agar plates containing 0.5ug/ml anidulafungin, 100ug/ml fluconazole and on a control YPD-only plate.

DNA extraction

A modified protocol from the MasterPure™ Yeast DNA Purification Kit was used to extract DNA. In brief, samples were grown overnight in liquid YPD at 37˚C. Further, cells were pelleted and lysed with RNAse treatment at 65˚C during 15 min. After 5min of cooling down on ice, samples were purified by the kit reagent by mixing, centrifugation and removal of the debris as described in the kit protocol. Samples were left at -20˚C with absolute ethanol for at least 2 h and after that the DNA was precipitated for 30 min at 4˚C. The pellet was washed in 70% ethanol, left to dry and TE buffer was used to resuspend the DNA. The Genomic DNA Clean & Concentrator kit (Zymo Research) was used for the final purification.

Whole genome sequencing

We sequenced the samples in three pools each containing strains belonging to different phylogenetic clades (Carreté et al. 2018). First included 2C_FLZ (progeny of CST34), 3H_FLZ (progeny of EB0911), 7B_FLZ (one of the progenies of EF1620) and 8G_FLZ (progeny of F15) samples, second: 7D_FLZ (second progeny of EF1620), 9F_FLZ (one of the progenies of CBS139), 11B_FLZ (one

of the progenies of BG2) and 4G_FLZ (progeny of CST78), third: 9H_FLZ (the other progeny of CBS138), 3H_AinF (progeny of EB0911), 7B_AinF (progeny of EF1620), 2G_ANIFLZ (progeny of CST34) and 11H_FLZ (the other progeny of BG2).

Genome sequences were obtained at the sequencing core facility of the CNAG. The short-insert paired-end libraries for the whole genome sequencing were prepared with KAPA HyperPrep kit (Roche) with some modifications. In short, 1.0 microgram of genomic DNA was sheared on a Covaris™ LE220-Plus (Covaris). The fragmented DNA was further size-selected for the fragment size of 220-550bp with AMPure XP beads (Beckman Coulter). The size selected genomic DNA fragments were end-repaired, adenylated and Illumina platform compatible adaptors with unique dual indexes and unique molecular identifiers (Integrated DNA Technologies) were ligated. The libraries were quality controlled on an Agilent 2100 Bioanalyzer with the DNA 7500 assay for size and the concentration was estimated using quantitative PCR with the KAPA Library Quantification Kit Illumina Platforms (Roche).

The libraries were sequenced on NovaSeq 6000 (Illumina) with a paired-end read length of 2x151bp. Image analysis, base calling and quality scoring of the run were processed using the manufacturer's software Real Time Analysis (NovaSeq 6000 RTA 3.4.4).

Sequencing analysis

In order to evaluate the presence of aneuploidies in these samples we aligned the reads with *bwa mem* (v0.7.17, http://bio-bwa.sourceforge.net/bwa.shtml) and called small variants with the

"pool" mode from *freebayes* (v1.3.1 https://arxiv.org/abs/1207.3907). Our objective was to measure the coverage of each sample from its unique genomic features (i.e.: SNPs found only in that sample) as compared to the other members of the pool.

In order to achieve this, we first defined as "private SNPs" of a sample those that were not expected in any of the other samples of the pool. We defined as "expected SNPs" those that were called in the strains before the growth in YPD (see **Chapter 5**). We found that most samples (all non-CBS138 strains) had at least 15,098 such private SNPs. The read depth of each of these SNPs was taken as a proxy for the coverage of the corresponding sample. However, all CBS138 samples had less than three of these, suggesting that considering private SNPs is not enough to resolve the coverage of all samples. To overcome this, we identified "private no SNPs" in a sample as positions without SNPs where all the other members of the pool had some SNP. We calculated the coverage of each "no SNP" as the "total coverage in a position" - "sum of the coverage of each SNPs in the other samples". This yielded >16,755 "no SNPs" for all CBS138 samples, suggesting that considering "no SNPs" could be useful. In order to avoid errors derived from inaccurate variant calling we considered as "private SNPs" those that were "high-confidence" in a given sample (called by three algorithms in the parental and with at least 90% of reads of the position supporting that SNP, see **Chapter 5**) and not called in any of the other members of the pool. Importantly, we validated that most of these "expected" SNPs were also called in the pools (>98.47% in all samples). Similarly, we only

considered “private no SNPs” as those positions where the sample had no called SNPs and all the other members had “high-confidence” SNPs. We found that most positions that were expected to include some “no SNP” yielded the expected SNPs in the pooled sequencing (>99.04% in all pools). Taken together, these observations indicate that the combination of “private SNPs” and “private no SNPs” can be useful to measure the read depth of each sample from the pooled sequencing. We thus obtained the coverage of each position as the reads covering the “private SNPs” (or “no SNPs”) of a given sample. We also calculated a “relative coverage” measure by normalizing the coverage of each position by the median coverage across all positions of chromosomes not expected to have aneuploidies in any sample (chromosomes B, C, D, F, G, H, J, K, M). This “relative coverage” was expected to be proportional to the copy number in a given position.

In order to detect the loss of aneuploidies we measured the relative coverage in the initial sample (evolved in antifungal drugs, not sequenced in pools) with *mosdepth* for those positions with “SNPs” or “no SNPs”. We normalized the coverage before and after this 8-week growth by the read depth in the YPD-evolved samples of the same strain (see **Chapter 5**) to correct for strain-specific biases. We compared the normalized coverage of the two samples to determine if the aneuploidy was kept.

PCR and Sanger sequencing

The loss of *PDR1* mutations after the in vitro evolution was confirmed by PCR and Sanger sequencing. The PCR primers are:

FWD – TCAAAATGCACCCAGTTCGA and REV - TCTAACGGGTTGGCAATCGA. PCRs were carried out by using Taq DNA polymerase from DongShengBio. The reaction mixture included primers of concentration of 0.4 μM, 20 μL Taq DNA polymerase, 1μL liquid sample grown for 24 h in YPD and water up to a final volume of 50 μL. Optimase ProtocolWriter™ was used to develop conditions.

Part III

Discussion

8 Summarizing discussion

The overall objective of this thesis was to study the evolutionary processes underlying the acquisition of drug and multidrug resistance in *C. glabrata*. To this end, we used an experimental evolution approach coupled to gene and genome sequencing to trace the genomic changes that accompanied the acquisition of resistance to two important antifungal drug classes: azoles and echinocandins. Currently, there are few pharmaceutical options available to treat the increasingly occurring infections caused by *Candida* species (de Oliveira Santos et al. 2018). Azoles and echinocandins are used globally to treat *Candida* infections, however the rates of resistance to one or both of these drugs among clinical isolates is high, and is increasing, which poses a serious threat to human health (Pfaller et al. 2019; Alexander et al. 2013). Broadening our knowledge about the evolutionary mechanisms driving the adaptation to antifungals is important at various levels. First, it will enable the design of novel molecular tests that allow a rapid diagnosis of antifungal resistance. Second, it will help in the design of strategies directed to reduce the emergence of resistance and the spread of resistant strains. Third, it will guide the development of innovative therapeutic approaches against drug resistant organisms.

8.1 The experimental evolution approach

An experimental evolution approach provides remarkable advantages for the study the acquisition of drug resistance: it allows i) to observe the process of adaptation in real time, under controlled conditions that

can be modulated; ii) to assess the convergence and predictability of evolutionary outcomes by analyzing identical replicate populations; and iii) to store and resurrect ancestral populations in order to examine changes over evolutionary time, or to restart evolution from intermediate generations.

Our experimental design involved in vitro evolution of resistance by exposing susceptible strains to increasing concentrations of fluconazole (flz), anidulafungin (ani) separately, subsequently and/or concomitantly. We used a batch serial transfer method combined with deep well plates that enabled us to use small culture volumes and evolve a multitude of samples in parallel. By using this setup, we were able to investigate a total of 288 evolving populations (twelve strains in quadruplicates and in six evolution experiments) including also those with different starting susceptibilities (for example, ani and flz resistance evolved from flz and ani resistant samples, respectively). Moreover, our approach involved the phenotypic and genomic comparison of the evolved strains to their parental strains rather than to the reference strain, which enabled the specific identification of relevant changes: i.e. those appearing during the adaptation to the drug.

Our experiment resulted in a valuable collection of resistant mutants with their corresponding ancestors, and of populations stored at intermediate stages of evolution, which constitute a useful resource for future research. Our samples can be used in studies where a resistant strain and its parental background are required to circumvent the limitations of comparisons with a more genetically distant

reference strain, and also allow to consider the genetic and phenotypic diversity within the species. Part of our collection has been already proved useful in a study pointing out the utility of monitoring the echinocandin drug uptake as a simple assay for predicting susceptibility levels (Jaber et al. 2020). In addition, storing populations at the intermediate steps of evolution opens the possibility to track the order of occurrence of adaptive mutations — i.e., the evolutionary trajectory. This allows for the discovery of additional, intermediate changes which may be crucial in the phenotypic transition. Such alterations may play the role of a starting domino piece that initiates a series of changes, and as such could be useful as a marker predicting inevitable outcomes. In addition, the ability to trace the evolutionary trajectories leading to the acquisition of resistance could be translated into valuable information on the effects of the duration of the efficient antifungal therapy before the infecting strain acquires the resistance.

We are conducting a follow up project that involves sequencing whole populations, rather than individual isolates, from intermediate stages of our experimental evolution. Along with the discoveries of additional alterations present in the cell populations, we are going to investigate in depth the trajectories of all the mutations and explore, for example, i) the presence of any relevant alterations preceding the appearance of resistant driving mutations, ii) which of the final mutations appeared first in the evolution; iii) among genes presenting various mutations, which ones preceded the appearance of those that survived until finalization of the experiments; as well as iv) thoroughly inspect the alterations in samples with neither *PDR1* nor

ERG11 mutations (samples where flz resistance was connected with only *ERG3* mutations) or in the samples that lost ani resistance.

8.2 Susceptibility measurements

Some of the key insights from our experiment were obtained by measuring the changes in the relevant phenotype: the ability to grow in the presence of the drug. This was performed using standard broth microdilution antifungal susceptibility testing, however, we did so in a high throughput manner and with intermediate readouts of the growth. Although the test is standardized, the susceptibility results may vary across replicates, thus our results are a consensus of three separately conducted tests. Performing the test is a straightforward task, yet, the correct interpretation of the results may come with challenges. Our approach used two different scores as proxies for susceptibility: MIC and rAUC. This allowed us a more accurate interpretation of the results, which would have been impossible if only one of the measurements was used. As an example, let us consider samples A (11D_ANI) and B (12G_ANI). Both had a median MIC of 8ug/ml, which in a standard interpretation would be assigned as having equal susceptibilities. However, MIC ranges for the susceptibility test replicates were different: for A: [8,4,8] and B: [8,16,8], and rAUCs for A: [0,60; 0,65; 0,66], median – 0,65 and B:[0,77; 0,79; 0,99], median – 0,79, which indicates a higher level of resistance in sample B. Accordingly, MIC is represented as a range of discrete concentrations while rAUC presents continuous results. This allowed us to detect differences in susceptibility to flz between

flz resistant ANI and FLZ mutants that were only apparent in rAUC. Hence, we consider that rAUC should be preferred in the representation of levels of resistance. Furthermore, MIC values can be affected by the trailing effect. Along with the determination of MIC, comparisons of growth levels at different drug concentrations is essential. Such simple plots can drastically help the interpretation of the results and decrease the chances of presenting misleading results. However, due to small differences in rAUC levels in ANI samples in the flz susceptibility test, MIC appeared to perform better in assigning a threshold to discriminate between susceptible and resistance samples. Considering that it is not always feasible to measure the absorbance along time intervals in order to obtain growth curves, we emphasize the need to always perform the test in triplicate so that outlier measurements can be eliminated and the results can be represented as a more informative range of concentrations. Additionally, it has been suggested to visually examine the assay plates and raw optical density in order to properly interpret numerical MIC values (Gerstein and Berman 2020), and altogether draw more accurate conclusions regarding the susceptibility.

8.3 Genetic clades and acquisition of resistance

Among clinically relevant *Candida* species, *Candida glabrata* is the one that presents the highest incidences of azole, echinocandin, and multidrug adaptive resistance (Castanheira et al. 2014; Pfaller et al. 2019). Due to the observed considerable genetic diversity within the species, *C. glabrata* strains can be divided into at least seven distinct

clades (Carreté et al. 2018). In addition, genetic differences in this species have been correlated with phenotypic differences. One of the objectives of our experimental design was to test the hypothesis of whether distinct genetic backgrounds displayed different abilities to acquire drug resistance. Our results confirm that *C. glabrata* is capable of developing multidrug resistance in in vitro conditions. This ability was widespread among the different genetic backgrounds (clades) and among strains harboring or not *MSH2* mutations. *MSH2* is a mismatch DNA repair gene whose alterations were attributed to promote the acquisition of antifungal resistance in *C. albicans* and *C. glabrata* (Legrand et al. 2007; Healey, Zhao, et al. 2016). Hence, we consider that neither adscription to distinct clades (reflecting genetic diversity), population responses (i.e heteroresistance), nor the presence of naturally occurring variants in the *MSH2* gene are advantageous for the appearance of the conclusive alterations leading to adaptation to antifungal drugs. The main factors that seem to drive the process include alterations at the level of genomic nucleotide sequence and in chromosomal structures – attributes of plastic and easily mutable genome, as well as cellular responses to stressful conditions.

8.4 Multidrug resistance

Our results indicate that there is more than one route leading to multidrug resistance in *Candida glabrata*. The acquisition of multidrug resistance obtained in all three approaches (by means of simultaneous treatment with two drugs, of flz resistance in ani

resistant samples and of ani resistance in flz resistant samples) followed similar genetic patterns. We did not observe any particular genomic changes connected specifically with any of the evolutionary conditions indicating that resistance to one drug, resistance-driving mutations, and the affected genes are very much alike regardless of the susceptibility of the samples to the other drug. It is expected that samples that have been put under stress for a significant amount of time will show increased mutational rates (Shor et al. 2013; MacLean et al. 2013) and as a result could acquire resistance earlier than susceptible samples. Although we only analyzed the final generation of the evolution experiments, we did not observe elevated mutation rates in any of the mutants, even in the samples that bear non-synonymous variants in *MSH2* gene. Yet, further investigation is needed to assess a possible faster adaptation to ani in the samples containing *PDR1* mutations (FinA) as it has been suggested that alterations in *PDR1* increase the ability of *C. glabrata* to adapt to other stressors (Healey and Perlin 2018).

The list of genes recurrently altered in our evolved mutants is rather short (nine genes), and their relationship with the resistance phenotype is supported by the high number of investigated samples and the number of times the same genes were convergently mutated in independently evolved lines. Undoubtedly, mutations in *PDR1* are the most abundant changes in our collection of flz evolved mutants, making mutations in this gene the main drivers of resistance. However, even though these changes are expected to activate efflux pumps that eliminate azoles from the cell, prolonged flz treatment seemed to cause additional alterations affecting the direct drug target.

ERG11 alterations (including chromosomal duplications) are mostly accompanying *PDR1* mutations. This suggests that *ERG11* alterations could represent a secondary step and an aid in the acquisition of the resistance, rather than the initial or main cause of resistance. Nevertheless, these alterations should not be neglected. First, aneuploidies, which have been generally thought to be unstable and play only an intermediate role in the acquisition of resistance (Berman 2016), were still present after subsequent evolution with ani and secondly, we also found flz resistant mutants harboring only *ERG11* mutations. Additionally, based on the observation that most *ERG11* mutations involve changes at lysine 152, we suspect this might be a mutational hot spot implicated in the lower efficiency of the binding between azoles and the protein in *C. glabrata*, similar to Y132F *ERG11* mutation observed in *C. albicans* (Flowers et al. 2015), *C.auris* (Lockhart et al. 2017), *C. parapsilosis* (Berkow et al. 2015) or *C. tropicalis* (Tan et al. 2015). Since there is only one study associating *ERG11* alteration (G315D) with azole resistance in *C. glabrata* (Hull et al. 2012), it would be also beneficial to further investigate alterations caused by *ERG11* changes, for example, to check whether the expression of the gene is changed due to the mutations.

We hypothesize that gross genomic rearrangements, although important in the adaptation to drugs (Healey et al. 2016), may play a secondary role in the acquisition of resistance. We base this statement on the observation that *C. glabrata* naturally presents different genomic configurations (Carreté et al. 2018), they can appear spontaneously in optimal growth conditions (Bader et al. 2012), and

that half of our FLZ evolved mutants presenting chromosomal duplications lost them after being cultivated without stress for an extended period of time.

Although we hoped to discover alternative mechanisms, *FKS* mutations found in all of the ani evolved samples seem to be the main cause of resistance to ani. Importantly, and we cannot stress this enough, *FKS* mutations can also fall outside of the famous hot spots (HS). HS are commonly genotyped regions of *FKS* genes when echinocandin resistance is investigated (Zhao et al. 2016; Dudiuk et al. 2014). We have proved that mutations outside the HS region can also lead to the acquisition of resistance, thus we suggest investigating the whole gene if the susceptibility of a strain is to be assessed based on mutations.

An unexpected finding was that ani caused modifications in genes of the ergosterol biosynthesis pathway (*ERG3*, *ERG4* and *ERG5*), which further impacted the acquisition of co-resistance to flz. Acquisition of multidrug resistance due to ani action had been observed before (Hatwig et al. 2019) however, how it occurs remains to be investigated. Presence of *ERG3* changes in only a fraction of ANI samples mean that, although these mutations likely aid in the adaptation to ani, they are not indispensable for the acquisition of ani resistance. Introduction of *ERG3* alteration in ani resistant sample did not show growth improvement in ani susceptibilities, which is in agreement with other studies showing that in *C. parapsilosis* and *C. albicans* deletion of *ERG3* leads to small or negligible increases in echinocandin MICs (Rybak et al. 2017). However, we hypothesize

that acquisition of flz resistance caused by *ERG3* mutations requires also the alterations that ani exerts on the cell. It is a combination of cell wall stress responses, cell wall composition modifications (altered glucan contents resulting from *FKS* mutations) and ergosterol biosynthesis pathway alteration that produce the resistance phenotype. Additionally, susceptibility changes to flz evoked by *ERG3* alterations are not comparable to those involving *PDR1* or *ERG11*. Direct efflux of the drug or alterations in its target have a more advantageous impact on growth under the flz treatment than alterations in the sterol composition of the cell membrane. We propose that the determination of the sterol composition in the samples with the different *ERG* genes alterations should help in understanding the cellular impact of ani treatment. For example, changes associated with sterols may compensate for alterations in cell wall composition. Furthermore, a special emphasis should be put on the alterations involving premature termination codons and missense mutations in *ERG3*. It is important to understand why truncations have a different effect on flz susceptibility as compared to missense mutations. Finally, stressing the cell wall with a fungicidal drug and subsequent activation of stress responses could cause other alterations that await further investigations.

Altogether, multidrug resistance in *C. glabrata* is driven by alterations in drug target genes, *FKS1* and *FKS2*, conferring echinocandin resistance and by alterations in drug target gene, *ERG11*, in the transcription factor controlling the expression of major drug transporters, *PDR1*, and in genes involved in ergosterol biosynthesis pathway, *ERG* genes, conferring azole resistance.

8.5 Preventing the acquisition of resistance

There are two main approaches in the global combat to limit the emergence and spread of resistant fungal pathogens (McCarthy at al. 2017; Hamdy et al. 2017). The first one involves proper diagnosis of fungal pathogens which, in turn, enables the informed selection of a suitable antifungal regime adjusted to the infecting species. The second one deals with precise monitoring and directing the appropriate use of antimicrobial drugs (antimicrobial stewardship) in order to reduce adverse events, restrict selective pressure, and improve treatment outcomes. Our results can add another layer. Along with alterations driving the acquisition of resistance, we also reveal changes that resulted in the loss of the previously-acquired resistance phenotype. This could mean that either the stress coming from the subsequently used drug or, more likely, the interruption of the pressure exerted by the previously used drug resulted in changes leading to return to the more optimal for the cell WT- phenotype.

A possible strategy to prevent the emergence of resistance to azoles may include deactivation of Pdr1p. In our experiment truncation of this transcription factor resulted in hyper-susceptibility to flz even in the presence of other possible changes aiding the acquisition of resistance (ChrE aneuploidy). Coadjuvant reagents leading to the deactivation of this protein and of the overexpression of efflux pumps may be of clinical relevance, yet further thorough investigations should be conducted as *PDR1* may be additionally regulating other cell responses that could result in undesired side effects.

Prevention of the acquisition of echinocandin resistance can be more challenging. Firstly, *C. glabrata* is considered to be more prone to acquire resistance to echinocandins than other common *Candida* species (Pfaller et al. 2019). This probably relates to the fact that two *FKS* genes can be mutated as a result of echinocandin treatment in *C. glabrata* as opposed to a single gene in most other *Candida* pathogens. When exposed to echinocandins both genes are under selection which doubles the likelihood of the appearance of beneficial mutations. Secondly, one of the *FKS* genes has to be in the WT form while the other either in WT form as well (natural configuration) or deactivated. We observed that loss of ani resistance happened due to the appearance of a premature stop codon in the *FKS* gene that previously presented the resistance driving mutation and in strains where the other *FKS* gene was unmutated. Maintaining a WT form of the gene would allow for a correct binding of the drug and inhibition of the growth. However, the presence of a missense mutation or small indel, which causes alteration and not deactivation of the protein, in any of the genes, lowers the efficiency of the drug by preventing its binding regardless of the presence of the other gene in its innate form. Thirdly, *FKS* genes can accumulate mutations. With the futuristic vision of targeting and elimination of particular mutations, in case of the *FKS* genes, there is also a possibility that the resistant strain has multiple alterations. Hence, aiming only at a set of known resistance-conferring mutations will not be effective.

8.6 Clinical implications

Acquisition of resistance and the ease at which it appears in *Candida glabrata* is a serious threat in topical antifungal therapies as well as in clinical settings. Discoveries of mechanisms driving the adaptation to antifungal drugs and fast detection of these changes would allow to foresee the emergence of resistant populations and select treatment accordingly. Fungicidal echinocandins are being recommended over fungistatic azoles against infections caused by the species (Pappas et al. 2016) yet they are also not ideal.

Our results suggest that one of the possible methods to improve the efficiency of the antifungal therapy may involve the concomitant use of azoles and echinocandins. Synergistic antifungal activity of two representatives of the two drug classes has been observed in *C. albicans* (Cui et al. 2015). Synergistic drug combinations are claimed to potentially reduce the dose of single drug usage, increase the efficacy, and subsequently lower the drug toxicity (Cui et al. 2015). However, they need to be taken into consideration with caution due a possible appearance of adverse effects in the host. The reduced survival of the populations exposed to co-treatment in our experiment implies that the combined use of two drugs could not only be more effective in clearing out the infection but also such co-treatment could be less prone to lead to, or at least slow down, the acquisition of resistance. Furthermore, resistance developed in this manner may come with significant fitness costs at the end of the treatment, possibly preventing survival in the absence of the drugs. Both, elimination of the infecting microorganisms and preventing the

emergence of resistant strains are of great importance for both the patient and hospitals which face the problem of hospital transmitted infections and persistence of the microorganisms on medical devices.

The obtained catalogue of resistance associated mutations may be valuable in the development of clinical tests that are able to detect genetic predisposition to resistance (Consortium OPATHY and Gabaldón 2019). Reported changes in *FKS*, *PDR1, ERG11* or *ERG3* genes could become targets of molecular assays and act as indicators of infection resistant to ani or flz strongly suggesting alternative treatment. Although resistance driving mutations in these genes are not a novelty, our research complements this catalogue with many mutations reported here for the first time.

Finally, the possibility of emergence of multidrug resistance after ani treatment raises a grievous clinical concern. Our results show that this fungistatic drug is implicated in changes in both: targeted cell wall and cell membrane that further influence how the samples are responding to flz regimen. Clinically, this cross resistance may result in changes of therapy from echinocandins to azoles possibly also being ineffective. Adding the fact that the antifungal prophylaxis is not uncommon and that echinocandin and triazole use has increased significantly over the past years (Pfaller et al. 2012), medical staff should be aware of this risk while choosing appropriate medical care.

9 Conclusions

The main conclusions of this thesis are:

- The ability to genetically adapt to antifungal drugs is not clade- nor strain- specific in *Candida glabrata*. The majority of the strains are capable of acquiring resistance to assayed representatives of the two major antifungal drug classes and the acquisition of the phenotypes follow similar patterns, regardless of the drug combination regime and the prior susceptibility of the sample.
- Multidrug and fluconazole resistance are associated with greater fitness costs as compared to anidulafungin resistance. However, the acquisition of resistance to one or both drugs is often achieved at a moderate fitness cost.
- Selection for (multi)drug resistance in *Candida glabrata* is highly efficient and straightforward. Although there are various routes, the mutational path for the acquisition of resistance is short and only few genes are frequently and nearly unavoidably altered.
- Modifications of *PDR1* and *ERG11* drive the adaptation fluconazole in *Candida glabrata*. Additional chromosomal aneuploidies (especially of ChrE bearing the drug target) can occur, aid the adaptation and persist, at least in the stress conditions. They are, however, less stable when the antifungal stress is removed.

- All anidulafungin resistant samples present changes in *FKS* genes. The mutations can accumulate and not always are only present in the defined mutational hot spots.
- Multidrug resistance can be acquired not only as a result of combined or sequential exposure to the two drugs, but also of a cross-resistance effect of anidulafungin treatment, which can acquire resistance to fluconazole.
- Anidulafungin can have an impact on ergosterol biosynthesis genes. Alterations in *ERG3* play a role in adaptation to anidulafungin and cause resistance to fluconazole.
- Resistance to anidulafungin is more stable than resistance to fluconazole when cultivated under no antifungal stress.

Altogether, our results shed light on the evolutionary processes leading to the acquisition of resistance to one or two antifungal drugs. This information is valuable for the development of novel strategies for the rapid diagnosis of resistant isolates and for alternative treatments, which are urgently needed to overcome resistance to current drugs.

Appendix: List of publications

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1. **Ksiezopolska, Ewa***; Schikora-Tamarit, Miquel*; Reinhard Beyer; Christoph Schüller; and Toni Gabaldón.: "Narrow mutational signatures drive acquisition of multidrug resistance in *Candida glabrata". (under revision)*
   *equal contribution
2. **Ksiezopolska, Ewa**, Miquel Schikora-Tamarit, and Toni Gabaldón. "Persistence of genetically-acquired azole and echinocandin resistance in *Candida glabrata*" *(submitted)*
3. **Ksiezopolska, Ewa**, and Toni Gabaldón. 2018. "Evolutionary Emergence of Drug Resistance in Candida Opportunistic Pathogens." *Genes* 9 (9). https://doi.org/10.3390/genes9090461.
4. Carreté, Laia, **Ewa Ksiezopolska**, Cinta Pegueroles, Emilia Gómez-Molero, Ester Saus, Susana Iraola-Guzmán, Damian Loska, Oliver Bader, Cecile Fairhead, and Toni Gabaldón. 2018. "Patterns of Genomic Variation in the Opportunistic Pathogen Candida Glabrata Suggest the Existence of Mating and a Secondary Association with Humans." *Current Biology: CB* 28 (1): 15–27.e7.
5. Carreté, Laia, **Ewa Ksiezopolska**, Emilia Gómez-Molero, Adela Angoulvant, Oliver Bader, Cécile Fairhead, and Toni Gabaldón. 2019. "Genome Comparisons of Candida Glabrata Serial Clinical Isolates Reveal Patterns of Genetic Variation in Infecting Clonal Populations." *Frontiers in Microbiology*. https://doi.org/10.3389/fmicb.2019.00112.
6. Hovhannisyan, Hrant, Ester Saus, **Ewa Ksiezopolska**, and Toni Gabaldón. 2020. "The Transcriptional Aftermath in Two Independently Formed Hybrids of the Opportunistic Pathogen Candida Orthopsilosis." *mSphere* 5 (3). https://doi.org/10.1128/mSphere.00282-20.
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9. Pegueroles, Cinta, Susana Iraola-Guzmán, Uciel Chorostecki, **Ewa Ksiezopolska**, Ester Saus, and Toni Gabaldón. 2019. "Transcriptomic Analyses Reveal Groups of Co-Expressed, Syntenic lncRNAs in Four Species of the Genus Caenorhabditis." *RNA Biology* 16 (3): 320–29.
10. Willis, Jesse R., Pedro González-Torres, Alexandros A. Pittis, Luis A. Bejarano, Luca Cozzuto, Nuria Andreu-Somavilla, Miriam Alloza-Trabado, Antonia Valentín, **Ewa Ksiezopolska** et al. 2018. "Citizen Science Charts Two Major 'Stomatotypes' in the Oral Microbiome of Adolescents and Reveals Links with Habits and Drinking Water Composition." *Microbiome* 6 (1): 218.

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