THE MOLECULAR MECHANISMS OF ANTIFUNGAL DRUG RESISTANCE IN PATHOGENIC FUNGI

A DISSERTATION IN
Cell Biology and Biophysics
And
Molecular Biology and Biochemistry

Presented to the Faculty of the University
of Missouri- Kansas City in partial fulfillment
of the requirements for the degree

DOCTOR OF PHILOSOPHY

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2016

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University of Missouri-Kansas City, 2016

ABSTRACT

Pathogenic fungi including *Candida albicans*, cause oral, systemic, and vaginal infections, mostly in immune-compromised individuals. Azoles are the most common antifungals used in treating these infections. The most significant mechanisms of azole resistance include alterations or overexpression of the target enzyme, and overexpression of at least three distinct efflux pumps. Recently, azole-resistant vaginal *C. albicans* isolates have been detected in patients with recurring and refractory vaginal infections. However, the mechanisms of resistance in vaginal *C. albicans* have not been studied in detail. In oral and systemic resistant isolates, over-expression of ABC transporters Cdr1p and Cdr2p and the major facilitator transporter Mdr1p is associated with resistance. This is consistent with the molecular mechanisms of drug resistance observed in this study for the vaginal *C. albicans* clinical isolates.

Another important mechanism of azole resistance in *C. albicans* is overexpression and/or point mutation of *ERG11*, which is a rate-limiting enzyme in the ergosterol biosynthetic pathway. Other antifungals also target the ergosterol pathway, including fenpropimorph,(morpholine) which targets Erg24p, and terbinafine (allylamines), which targets Erg1p enzymes. Aberrant levels of ergosterol may affect many cellular processes. Recent studies have used deletion mutants of the ergosterol genes to analyze their cellular phenotypes. Some of these mutants showed severe growth defects, compromised respiration, weak cell wall, and lower tolerance to osmotic stress. However, only seven of total 25 ergosterol biosynthetic genes (*ERG* genes) can be deleted as they are non-essential. This thesis studies the ergosterol biosynthetic pathway in detail. All 25 *S. cerevisiae ERG* genes were overexpressed under a galactose inducible promoter. Nine of the 25 strains overexpressing the *ERG* genes showed severe growth defects. Furthermore, phenotypic changes in these strains were compared to wild-type under various stress agents. These agents affect several cellular processes that include cell wall biosynthesis, respiration, protein synthesis, osmotic stress, iron and calcium metabolism. A majority of the overexpressed strains were affected by high salt or by a calcium chelator. Two of the nine slow growing strains were affected by all the stress agents used. This project increases our understanding of the ergosterol pathway, and may identify potential targets for future drug design.

APPROVAL PAGE

The faculty listed below, appointed by the Dean of the School of Graduate Studies, have examined a dissertation titled “The Molecular Mechanisms of Antifungal Drug Resistance In Pathogenic Fungi”, presented by Somanon Bhattacharya, a candidate for the Doctor of Philosophy degree, and certify that in their opinion it is worthy of acceptance.

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TABLE OF CONTENTS

LIST OF ILLUSTRATIONS

Figure Page

LIST OF TABLES

LIST OF ABBREVIATIONS

AA	amino acids
ABC-T	ATP binding cassette transporter
AMB	amphotericin B
AMO	amorolfine
CLT	clotrimazole
ERG	ergosterol pathway genes
FD	facillitative diffusion
FEN	fenpropimorph
FLC	fluconazole
ITC	itraconazole
LOV	lovastatin
MFS-T	major facilitator transporter
NYS	nystatin
TRB	terbinafine
TRI	tridemorph

ACKNOWLEDGEMENTS

I hereby acknowledge my committee members, Dr. Michael D. Plamann, Dr. Michael O'Connor, Dr. Jeffery L. Price, and Dr. Alexander Idnurm for their advice on the work presented in this thesis. I would especially like to thank my mentor and committee chair, Dr. Theodore C. White, for being an outstanding teacher and advisor. I would also like to acknowledge the past and the present members of White Laboratory, and School of Biological Sciences for funding the projects discussed in this thesis.

DEDICATION

This thesis is dedicated to my father Subhash Ranjan Bhattacharya, mother Indira Bhattacharya, and brother Sayantan Bhattacharya for their support and inspiration throughout.

CHAPTER 1

THE MOLECULAR MECHANISMS OF ANTIFUNGAL DRUG RESISTANCE IN PATHOGENIC FUNGI

1.1 Abstract

Pathogenic fungi cause oral, systemic, and vaginal infections, mostly in immune-compromised individuals. These infections are treated with antifungals that target cell wall biosynthesis (echinocandins), ergosterol biosynthesis (statin, allylamines, azoles, and morpholines), and nucleic acid biosynthesis (5-Flucytosine). Azoles are the most common antifungals used in treating these infections. The most significant mechanisms of azole resistance include alterations or overexpression of the target enzyme, and overexpression of at least three distinct efflux pumps. These mechanisms of resistance are summarized in this review.

1.2 Introduction

The immune system protects individuals from various foreign molecules that include pathogenic bacteria, fungi, and viruses. However, over the years, the extensive use of drugs for immune suppression and chemotherapy has caused a significant increase in patients with immune dysfunctions. A compromised immune system increases the probability of infections by pathogenic organisms. Some of these organisms are part of the normal flora in a healthy individual. For example, fungi like *Candida albicans* are present in gut, mouth, and vagina. This organism causes oral, systemic, and vaginal infections in immune-compromised individuals (1, 2).

In the United States, 75% of women suffer from vulvovaginal candidiasis (VVC) caused by *C. albicans* at least once in their life-time (1). Besides *C. albicans*, VVC is caused by other *Candida* sp. including *C. glabrata*, *C. dubliniensis*, and *C. krusei*. These organisms can also cause oral, and systemic infections (3). Apart from *Candida* sp., other pathogenic fungi include *Aspergillus* sp. (pulmonary and systemic infections), and *Cryptococcus* sp. (pulmonary, systemic and cerebral infections) (4). Several antifungals are used to treat these fungal infections including azoles, polyenes, statins, allylamines, echinocandins, and 5-flucytosine. Together, antifungal drugs and the host immune system may control infections with pathogenic fungi.

1.3 Antifungal Drugs

Antifungal drugs target important biochemical processes within a cell. These processes include the biosynthesis of cell walls, cell membranes, and nucleic acids. Each biochemical process involves a series of enzymes, and each of these enzymes is a potential drug target. Antifungal drugs can either kill the fungi (fungicidal) or inhibit fungal growth (fungistatic). These drugs and their targets are explained below:

Inhibitors of Ergosterol Biosynthesis

Ergosterol is the major sterol component of the plasma and mitochondrial membranes of fungi. It is important in the structure and function of these membranes. Sterols and sphingolipids together form lipid rafts in the cell membrane. These lipid rafts contain many biologically important proteins involved in signaling, response to stress, mating, and nutrient transport (5). Ergosterol biosynthesis is catalyzed by a cascade of 25 enzymes (Fig. 1.1). Mammmalian cells synthesize cholesterol, a related sterol, by a

similar mechanism. Since ergosterol is important in fungi and plants, and is not present in humans, several antifungals target its biosynthesis.

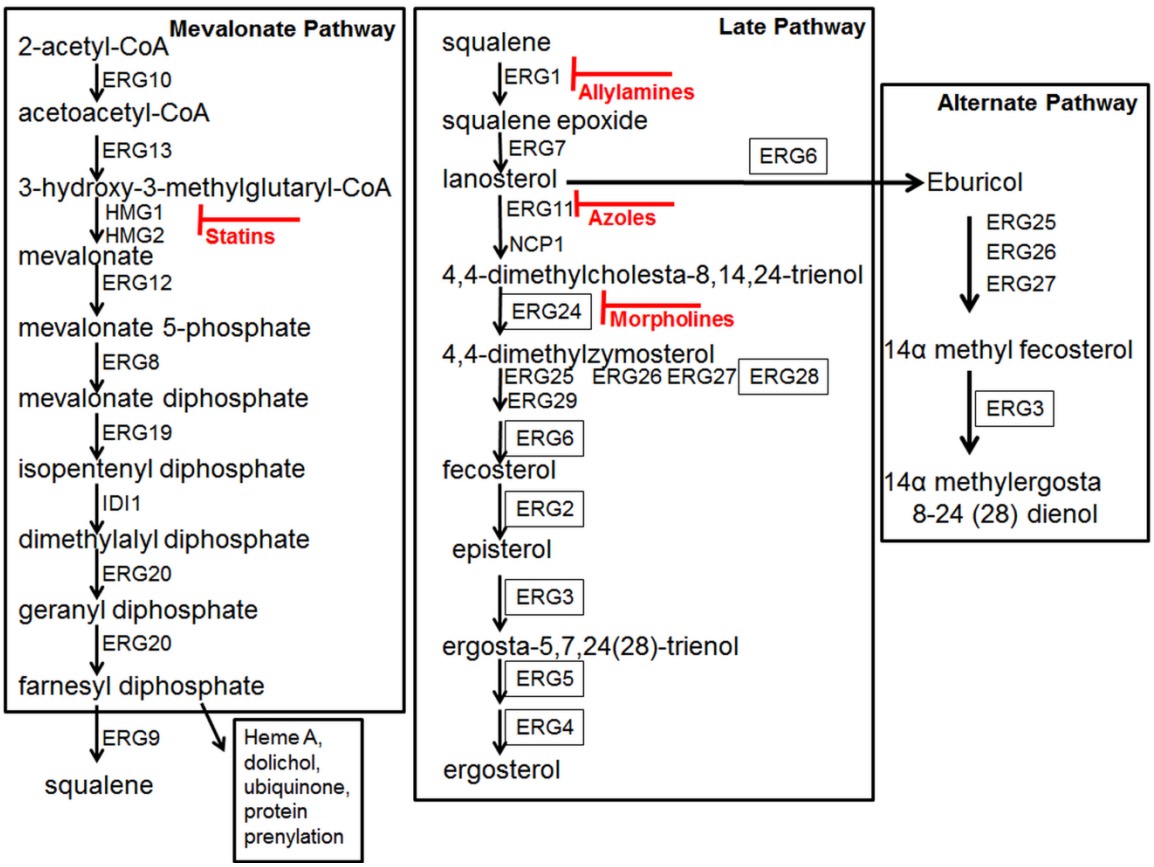

Fig. 1.1. The Ergosterol Biosynthetic Pathway.
The box on the left diagrams signifies the mevalonate pathway, which can channel into different biosynthetic pathways. The box in the middle is the late ergosterol pathway terminating in ergosterol. The box on the right is an alternate pathway leading to the toxic sterol (14α methyl ergosta 8-24-(28) dienol). Boxed gene names denote non-essential genes.

Azoles. These are the most common antifungal used for treating fungal infections. These drugs are fungistatic in nature, and include fluconazole, clotrimazole, itraconazole, voriconazole, and posaconazole. Fluconazole is the most common among all the azoles. Azoles target the enzyme 14α –demethylase (Erg11p), an enzyme in ergosterol biosynthesis (Fig. 1.1) (2). Erg11p is a Cytochrome P450 enzyme, and catalyzes a rate-limiting step in ergosterol biosynthesis (6). Azoles bind to Erg11p, and lower the ergosterol levels of the cell. When Erg11p is inhibited, other enzymes in the pathway (Erg6p, Erg25p, Erg26p, Erg27p, and Erg3p) synthesize a fungistatic sterol (14α methyl ergosta 8-24 (28) dienol, Fig. 1.1).

Statins. Statins are a class of drugs used in treating high cholesterol levels in humans. They target 3-Hydroxy-3-MethylGlutaryl-coenzyme A reductase (Hmg1p), which is a rate limiting step in the early part of the pathway (mevalonate pathway, Fig 1.1). (7). In vitro, statins are synergistic with fluconazole against pathogenic fungi (8), although they are not used in combination clinically.

Allylamines. These antifungals target Squalene epoxidase (Erg1p) in the ergosterol biosynthesis pathways (Fig. 1.1). These drugs include terbinafine (Lamisil), flunarizine, and naftifine. Terbinafine (Lamisil) is commonly used in treating dermatophyte infections (7).

Morpholines. This class of drug includes fenpropimorph, tridemorph, and amorpholine. They target the ergosterol biosynthetic enzyme C-14 sterol reductase (Erg24p), (Fig 1.1). The morpholines are commonly used in agriculture, and have high toxicity in humans (7), although, amorolfine is used in treating nail dermatophyte infections (9).

Polyenes. This class of drugs target ergosterol in the plasma membrane, by binding to ergosterol, and forming pores that destabilize the membrane (10). Polyene drugs include amphotericin B and nystatin. These compounds are well known for severe side effects including nephrotoxicity (7).

Inhibitors of Cell Wall Biosynthesis

The cell wall is the rigid outermost layer of the cell. It is an important cellular component, which is present in plant, bacterial, and fungal cells. Cell walls are the first line of defense of fungal cells against the host immune response, and protect the cells from osmotic stress. The cell wall is absent in mammalian cells and hence cell wall biosynthesis can be an important target for antifungal drugs (11), including the echinocandins (caspofungin, anidulafungin, and micofungin) that target 1-3 glucan synthase.

Inhibitors of Nucleic Acid Biosynthesis

Drugs that target nucleic acid biosynthesis were originally designed in the 1950s, and are one of the oldest classes of antifungal drugs. 5-flucytosine (5-FC) is an antifungal that interferes with nucleic acid biosynthesis. The susceptible cells import 5-FC via the cytosine permease enzyme (12). 5-FC is converted to 5-Fluorouracil (5-FU) that gets metabolized to 5-Fluorouridine triphosphate (5-FUTP). 5-FUTP is incorporated in the fungal RNA instead of uridine triphosphate (UTP), thereby affecting protein translation. Alternatively, 5-FU can be converted to 5-Fuorodeoxyuridine monophosphate (5-FdUMP) that inhibits thymidylate synthase, an important enzyme in DNA biosynthesis (12).

5-FC was used in the past as monotherapy for treating candidiasis, aspergillosis and cryptococcosis. Side effects of this drug can include bone-marrow and liver toxicity. These drugs are now used in combination with azoles to treat fungal infections (12).

1.4 Contributing Factors for Drug Efficacy

Several factors contribute to the effectiveness of antifungal agents in treating fungal infections. First, the bioavailability of the drug can differ between different tissues (13). Second, antifungal drugs work with the immune system in treating the disease. Drugs may be less effective in patients with dysfunctional immune systems. Alternatively, different tissues display different immune responses. For example, the immune response to vaginal yeast infections is different from the immune response to systemic and oral infections (14). Third, fungi can develop biofilms on the surface of medical equipment such as catheters. These biofilms are resistant to various antimicrobial agents. Fourth, the pharmacokinetics of the drugs including drug metabolism, absorption, and distribution can alter drug effectiveness. Fifth, drug efficacy can depend on the severity of the infection, and the population size of the infecting organisms (15). Sixth, many antifungal drugs have severe side effects; although side effects are minimal with azoles especially fluconazole. Finally, prolonged use of a fungistatic azole drug may cause the pathogenic yeasts to develop resistance, making the drug less effective.

1.5 Azole Drug Resistance

Azole resistance in pathogenic fungi is an emerging problem (7). For example, azole resistance in *C. albicans* became a severe problem in 1990s, when 90% of AIDS patients had oral candidiasis, many received a long term azole therapy and certain patient

populations developed resistance (15). Azole resistance can develop at a clinical level as the result of the interactions between the host and the fungal cells; at a cellular level as the result of cell characteristics , and at a molecular level as the result of gene mutation and gene expression (15).

Azole Resistance at a Clinical Level

Fungal pathogens that do not respond to conventional azole therapy are considered clinically resistant. Several factors contribute to azole resistance at the clinical level. These include host-dependent factors and the drug-related factors.

Host Factors.

Immune Status. Successful azole therapy depends on the drug and on the host's immune response to the fungus. Without a functional immune system, azoles cannot control the fungal infections. For example, *Candida sp.* can colonize the mouth of 64% to 84% of HIV infected patients (15). Development of azole-resistance in these patients has many contributing factors. Typically, oral candidiasis regularly relapses in HIV infected patients with low $CD4^+$ cell counts ($<50/mm^3$) (16). These patients typically receive a long-term low dose azole therapy, which can select for azole-resistant *Candida sp.* In one case, a series of 17 *C. albicans* clinical isolates were obtained from a single HIV patient receiving azole therapy over two years. Azole-resistance increased steadily as these isolates were exposed to increasing amounts of drugs (2).

Site Of Infection. The site of infection can contribute to clinical azole resistance in at least three ways. First, the bioavailability of azoles varies from one site to another in the host. For example, the bioavailability of fluconazole is ~10-fold lower in vaginal tissues

than oral tissues, and normal antifungal dosage failed to treat 41% of vaginal candidiasis patients infected with azole-susceptible clinical isolates (13). Second, different sites in the host have different environmental conditions. For example, vaginal pH is 4.0-4.5, while oral and systemic pH is 7.0. Environmental pH can alter azole susceptibility in many pathogenic fungi including, *Aspergillus sp., C. glabrata,* and *C. albicans*.(17, 18). Third, different tissues in the host can have different immune responses. For example, the TH17 immune response is critical to oral candidiasis, while the innate immune response is critical for vaginal infections (14).

Medical Devices. Medical devices including, catheters, dentures, and prosthetic aortic valves, increase the risk of acquiring clinically resistant fungal infections. Pathogenic fungi produce biofilms that are less responsive to azoles on the surface of synthetic materials. For example, *Candida sp.* can produce biofilms on catheters, prosthetic valves, and dentures that aid in adhesion of the organism, and is resistant to standard azole therapy. To control the infections, removal of catheter and other devices is necessary. At least half of nosocomial infections are associated with medical devices (19).

Drug Related Factors.

Factors such as the fungistatic nature of the drug, long term therapy, cumulative dose, drug compliance, prior history of azole therapy, drug-drug interactions, and drug pharmacokinetics can select for azole resistant cells. (16, 20, 21). In addition, drug-drug interactions and pharmacokinetics can alter the bioavailability of the drug in specific tissues, causing therapeutic failure.

Clinical resistance can also develop from the environmental use of antifungal azoles in treating agricultural plant pathogens. For example, azole-resistant *Aspergillus*

fumigatus was isolated from aspergillosis patients with no prior azole exposure. These isolates were cross resistant to environmental azoles and clinical azoles including itraconazole, voriconazole, and posaconazole (22).

Azole Resistance at a Cellular Level

In clinical isolates, azole resistance can be classified as primary or secondary resistance. An isolate with primary or intrinsic azole resistance is defined as an isolate that is inherently resistant by chance in a random population of clinical isolates. An isolate with secondary resistance is defined as an isolate acquired from other patients or the environment. Secondary resistance can also be the result of an endogenous isolate that develops resistance in response to azole pressure in the patient. Both primary and secondary resistance are observed in oral, systemic, and vaginal clinical isolates of *C. albicans*. (3, 15, 23). Intrinsic resistance is observed in select fungal species such as *C. krusei* and *C. glabrata*. Acquired resistance has been found in most *Candida sp.,* Aspergillus sp., *and Cryptococcus neoformans* (24).

Resistance at a cellular level is measured *in vitro* by the minimum inhibitory concentration assay (MIC). MICs are defined as the minimum concentration of drug that inhibits 80% of fungal growth (25). MICs are clinically interpreted using breakpoints to identify resistant versus susceptible isolates, and can vary according to the site and nature of infections. Clinical breakpoints are defined based on the correlation between *in vitro* MICs and *in vivo* drug response. For example, the fluconazole MIC breakpoint for oral and systemic *C. albicans* clinical isolates is ≥ 64 μg/ml, whereas the breakpoint for vaginal *C. albicans* isolates is ≥ 2 μg/ml (3, 13). In contrast, the itraconazole break point is 1 μg/ml for oral and systemic isolates.

While standard MIC analysis detects most azole resistant isolates, some susceptible cells exhibit resistance that cannot be detected by MIC analysis. Some isolates exhibit heterogeneous resistance, in which a sub-population of cells in a predominately susceptible microbial population exhibits transient resistance which is not heritable. Hetero-resistance has been observed in many pathogenic fungi including, *Candida sp.*, *Aspergillus sp.*, and *Cryptococcus sp.* (26-28).

Finally, susceptible cells form biofilms that are resistant when compared to planktonic cells (29). Biofilms can form on synthetic materials such as medical devices, and have been observed in most pathogenic fungi (19).

Azole Resistance at a Molecular Level

For decades, extensive research has analyzed the molecular mechanisms of azole resistance in pathogenic fungi. The known mechanisms are described below (Fig 1.2):

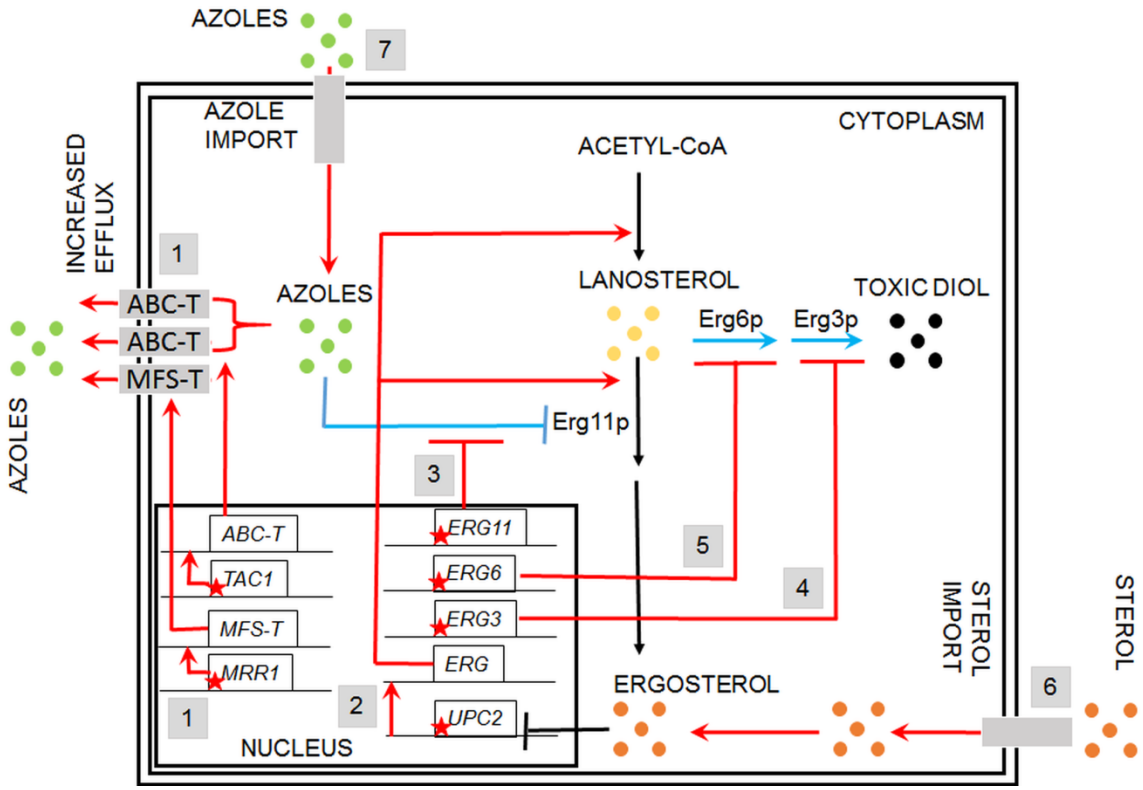

Fig. 1.2.The Molecular Mechanisms Of Azole Resistance.
A schematic representation of known molecular mechanisms of azole resistance. Black arrows represent the normal ergosterol pathway, including lanosterol (yellow circles), ergosterol (orange circles), and ergosterol inhibition of *UPC2.* Blue arrows represent the mechanism of action of azole drugs (green circles), including the inhibition of target Erg11p, and production of toxic diol (black circles) through several steps including Erg6p and Erg3p. The genes important for resistance are diagrammed within the nucleus as black rectangles in their chromosomal locations (black line). Red stars (mutations) and red arrows represent the different known molecular mechanisms of drug resistance in pathogenic fungi: Grey box 1 - Increased efflux of azoles due to point mutations in *MRR1* and *TAC1* (stars) resulting in over-expression of MFS-Ts and ABC-Ts respectively. Grey box 2 - Point mutations in *UPC2* causing increased expression throughout the *ERG* pathway. Grey box 3 - Point mutations in *ERG11* that prevents azole binding. Grey box 4 - Point mutations in *ERG3* that prevents the formation of toxic sterol. Grey box 5 - Point mutations in *ERG6* that prevents the formation of toxic sterol. Grey box - Sterol import that reduces the need for sterol biosynthesis. Grey box - Altered azole import that reduces intercellular azoles.

Azole Resistance via Over-expression of Membrane Transporters.

Membrane transporters are important in exporting molecules from the cells. Two types of membrane transporters have been identified that correlate to azole resistance in fungi. They are described above (Fig. 1.2):

ABC-transporters. ATP- binding cassette transporters (ABC-T) are active transporters requiring an energy source. Each ABC-T consists of two membrane-spanning domains (MSD), each containing six transmembrane segments, and two nucleotide binding domains (NBD). Each NBD consists of an ATP binding cassette (ABC) that binds ATP (30). Antifungal drugs may be substrates for the ABC-Ts that are listed in Table 1.1 (31).

MFS-transporters. Major facilitator transporters (MFS-T) require the proton gradient of the plasma membrane. MFS-Ts do not have the NBDs that are characteristic of ABC-Ts, and have 12 to 14 transmembrane segments (32). Antifungal drugs can also be substrates of the MFS-Ts listed in Table 1.1.

Table 1.1. Efflux Pumps Associated with Drug Resistance in Different Fungi

Efflux pump	Pump Type	Organism
AfuMdr3p	MFS-T	*Aspergillus fumigatus*
AfuMdr4p	ABC-T	*Aspergillus fumigatus*
AtrFp	ABC-T	*Aspergillus fumigatus*
Cdr1p, Cdr2p	ABC-T	*Candida albicans*
CgCdr1p	ABC-T	*Candida glabrata*
CgFlr1p	MFS-T	*Candida glabrata*
CgPdh1p	ABC-T	*Candida glabrata*
CgQdr2p	MFS-T	*Candida glabrata*
CgSnq2p	ABC-T	*Candida glabrata*
CkAbc1p	ABC-T	*Candida krusei*
CkAbc2p	ABC-T	*Candida krusei*
CneAfr1p	ABC-T	*Cryptococcus neoformans*
CneAfr2p	ABC-T	*Cryptococcus neoformans*

CneMdr1p	ABC-T	*Cryptococcus neoformans*
Mdr1p	MFS-T	*Candida albicans*
Pdr5p	ABC-T	*Saccharomyces cerevisiae*

In pathogenic fungi, increased expression of membrane transporters correlates with azole resistance (Fig. 1.2). For example, overexpression of *CaCDR1*, *CaCDR2*, and *CaMDR1* is commonly observed in azole resistant oral, systemic, and vaginal *C. albicans* clinical isolates (3, 23). *In vitro,* the disruption of genes encoding these transporters causes hyper-susceptibility to specific antifungals, including azoles (33).

While molecular mechanisms can be characterized in randomly collected resistant clinical isolates, matched isolates provide a more focused analysis of drug resistance. One matched set of 17 *C. albicans* clinical isolates were obtained from one individual over two-year period of antifungal therapy. Increased expression of *CaCDR1*, *CaCDR2*, and *CaMDR1* were observed in the matched resistant isolates when compared to their susceptible partners (2, 23). Analysis of random, non-matched resistant *C. albicans* isolates also shows increased efflux pump gene expression (3, 23). When these efflux pump genes were cloned into a *Saccharomyces cerevisiae* strain deleted for eight endogenous efflux pumps, the expression of *CaCDR1* and *CaCDR2* increased resistance to all azoles, while *CaMDR1* expression increased resistance to fluconazole and voriconazole alone (34).

Besides *CaCDR1*, *CaCDR2*, and *CaMDR1*, increased expression of *CaFLU1* (an MFS-T) and *CaPDR16* (an ABC-T) has been observed in azole resistant isolates (35). CaPdr16p is a phosphatidylinositol transfer protein, disruption of which causes increased susceptibilities to various antifungals (36). CaFlu1p is not considered a major azole transporter because *in vitro* overexpression does not confirm azole resistance (37). The *C.*

albicans genome contains many other membrane transporters. However, to date, none have been shown to affect azole resistance (38).

The efflux pumps *CaCDR1*, and *CaCDR2*, are regulated by transcription factor CaTac1p, and *CaMDR1* is regulated by transcription factor CaMrr1p (39). Both of these transcription factors are Zn_2-Cys_6 zinc-cluster transcription factors. Gain of function (GOF) mutations in *CaTAC1* and in *CaMRR1* result in constitutive overexpression of their respective pumps, which leads to azole resistance in many clinical isolates. For example, several GOF mutations in *CaTAC1* including T225A, V736A, N972D, N977D, G980E, and G980W have been correlated with resistance in clinical isolates (40). GOF mutations in *CaMRR1* including P683S and P683H have also been correlated with resistance in clinical isolates (41).

There are other transcription factors that regulate the expression of *CaCDR1* and *CaCDR2*, including the transcription factors *CaNDT80*, *CaFCR1*, and *CaFCR3* (42, 43). To date, no studies have associated *CaNDT80*, *CaFCR1*, or *CaFCR3* with resistance in clinical isolates. *CaMDR1* is transcriptionally regulated by *CaMRR1* as well as *CaCAP1* and *CaMCM1* (44). *In vitro*, a truncated C-terminus of CaCap1p correlated with increased azole resistance (45).

C. glabrata is intrinsically azole resistant. Overexpression of the ABC-T efflux pumps *CgCDR1*, *CgSNQ2*, and *CgPDH1* has been shown to contribute to azole resistance in clinical isolates (46, 47). Among the MFS-Ts, increased expression of *CgFLR1* and Cg*QDR2* is also observed in azole resistant clinical isolates (48, 49).

GOF mutations in the transcription factor CgPdr1p induce the ABC-T pumps *CgCDR1* and *CgPDH1* (47). In the presence of azoles, CgPdr1p is induced at the same time as the MFS-T pump CgQdr2p, suggesting that Cg*PDR1* may regulate Cg*QDR2* (48). Mitochondria also play an important role in efflux pump mediated azole resistance in *C. glabrata*. Loss of mitochondria leads to azole resistance, which correlated with upregulation of Cg*CDR1*, Cg*PDH1*, and other genes (47).

Membrane transporter overexpression can also effect intrinsically azole resistant *C. krusei*. In clinical isolates of *C. krusei*, overexpression of the ABC-T pumps CkAbc1p and CkAbc2p correlates with drug resistance; expression of both pumps can be induced by azoles; and expression of the pumps in a hyper-susceptible *S. cerevisiae* strain results in resistance (50). However, azole resistance was not affected by efflux pump inhibitors, suggesting that efflux mediated resistance is not due to efflux pumps alone (51). Increase efflux pump expression also correlates with increased azole resistance in *C. parapsilosis*, and *C. dubliniensis* (52, 53).

Besides *Candida,* efflux pump mediated drug resistance is also reported in the pathogenic fungi *Aspergillus* and *Cryptococcus*. *Aspergillus* is intrinsically resistant to fluconazole (54). Many *Aspergillus* transporters are hypothesized to play a role in azole resistance (55). To date, increased expression of *AfuMDR3* (MFS-T), *AfuMDR4* (ABC-T), and *AfuATRF* (ABC-T) has been observed in itraconazole resistant isolates (56). These genes are induced in the presence of itraconazole in some resistant isolates (56). Finally, in *Cryptococcus*, increased expression of the ABC-Ts *CnAFR1*, *CnAFR2*, and *CnMDR1* also correlate with azole resistance (57). To date, no MFS-T pumps have been linked to drug resistance in *Cryptococcus*.

Azole Resistance via Altered Ergosterol Biosynthesis.

Several known resistance mechanisms that alter ergosterol biosynthesis have been described, and are summarized below (Fig. 1.2).

ERG11/ CYP51: Erg11p is a cytochrome p450 enzyme that regulates a rate-limiting step in the ergosterol biosynthetic pathway (Fig. 1.1)(6). It is an essential enzyme in the pathway, and cellular ergosterol levels decrease when Erg11p is inhibited by azoles (Figs 1 and 2)(58). *ERG11* overexpression is linked with azole resistance in many fungi. Many azole resistant *C. albicans* clinical isolates show increased *CaERG11* expression (3, 23, 59). Induction of *ScERG11* by a regulatable promoter causes increased azole resistance in *S. cerevisiae* (60). *CgERG11* overexpression is observed in azole resistant *C. glabrata* isolates (61). Two *ERG11* homologs *CYP51A* and *CYP51B* are present in *Aspergillus* genomes (62). Both *AfuCYP51A* and *AfuCYP51B* are overexpressed in resistant *Aspergillus fumigatus* clinical isolates (62). Overexpression of *CnERG11* is also observed in azole resistant *Cryptococcus* clinical isolates (63).

Several point mutations in *ERG11* have been identified in azole resistant clinical isolates. For example, CaErg11p mutations A61V, A114S, Y132F, Y132H, K143Q, K143R, Y257H, S405F, G448E, F449S, G464S, R467K, and I471T are identified that contribute to azole resistance in *C. albicans* (64). Many of these point mutations lower azole binding in the active site of the enzyme. Point mutations in *ERG11* associated with resistance have been identified in other azole resistant *Candida* sp. These include, *C. glabrata* mutations C108G, C423T, and A1581G, and *C. krusei* mutations A497C and G1570A (65). Several point mutations in *CYP51A* in *Aspergillus*, including G54R, are correlated with resistance (66). In *Cryptococcus sp.,* Y145F causes increased azole

resistance (67). All these point mutations caused increased azole resistance without increasing *ERG11* gene expression (65-67).

ERG3: Besides *ERG11*, *ERG3* is also linked with azole resistance. Erg3p is C5 sterol desaturase enzyme, and converts episterol to ergosta-5,7,24 (28)- trienol during ergosterol biosynthesis. When Erg11p is inhibited, Erg3p and Erg6p synthesize a toxic sterol, 14α methyl ergosta 8,24 (28)-dien-3β, 6α-diol, in both *C. albicans* and *C. glabrata*, (Figs 1 and 2)(68-70). Disruption of *ERG3* results in increased azole resistance in *C. albicans* and *S. cerevisiae* (71). Q139A in Erg3p is reported in azole resistant *C. glabrata* isolates (72). Three *ERG3* homologs, (*ERG3A*, *ERG3B*, and *ERG3C*) are present in *Aspergillus fumigatus* genome (73). To date, *ERG3* has not been associated with azole-resistance in *Aspergillus* or *Cryptococcus* isolates.

ERG6: In the presence of azoles, *ERG6* contributes to the formation of the toxic diol from lanosterol (Figs 1 and 2)(68-70). Erg6p is a Δ24 sterol C-methyl transferase, a non-essential enzyme in the ergosterol biosynthetic pathway (Fig. 1.1). Significant azole resistance is observed in the heterozygous *ERG6* deletion (*ERG6*/Δ*erg6*) in *C. albicans* (74). Similarly, Δ*erg6* in *S. cerevisiae* showed increased azole resistance (75), increased membrane permeability, and low Pdr5p efflux activity (76). This suggests that *ERG6* dependent azole resistance is the result of toxic sterol formation, and not due to efflux pump overexpression. To date, *ERG6* has not been identified in azole resistant clinical isolates of any pathogenic fungus.

UPC2: Upc2p is a transcription factor that regulates the majority of ergosterol biosynthetic genes. For example, it regulates the expression of *CaERG2* and *CaERG11* in *C. albicans* (77). Upc2p is auto-regulatory (78) , and is induced by azoles, anaerobic

growth, and low levels of ergosterol (79). Upc2p is a Zn_2-Cys_6 zinc cluster transcription factor, similar to *CaTAC1* and *CaMRR1* (77), and is well characterized in *C. albicans.* The N-terminus of Upc2p is the DNA binding domain, while the C-terminus is an activation/regulatory domain (80). Ergosterol binds to the C-terminus of Upc2p and negatively regulates the transcriptional activity of *UPC2* (78). Several mutations in CaUpc2p, including G648D, G648S, A643T, A643V, Y642F, G304R, A646V, and W478C correlate with azole resistance in clinical isolates (59). Seven of these mutations increased the expression of *CaERG11* (59). Besides *CaERG11*, GOF mutations in *CaUPC2* increase the expression of other ergosterol pathway genes. For example, A643V GOF mutation in *CaUPC2* induces the expression of *CaERG2*, *CaERG3*, *CaERG5*, *CaERG6*, *CaERG9*, and *CaERG10* (81). However, this mutation only causes a two-fold increase in azole resistance (81). *In vitro*, GOF mutations A643T and A648D in *CaUPC2* significantly increased azole resistance (82). Since *C. albicans* is diploid, GOF mutations in both *CaUPC2* alleles will cause more resistance than a GOF mutation in a single allele (82).

The *C. glabrata* genome consists of two homologs of *CaUPC2*, *CgUPC2A* and *CgUPC2B* (83). Cg*UPC2A* is an important regulator of the ergosterol pathway in *C. glabrata*, and is required for azole resistance (83). Deletion of *CgUPC2A* in *C. glabrata* leads to azole susceptibility, whereas no effect is observed in the *CgUPC2B* deletion strain (83).To date, no GOF mutations have been reported in *CgUPC2A* or *CgUPC2B. S. cerevisiae* contains *ScUPC2* and its paralog *ScECM22* (84). *In vitro* disruption of both Sc*UPC2* and Sc*ECM22* causes azole hyper-susceptibility (84). In *S. cerevisiae*, azole resistance is observed in *S. cerevisiae* carrying G392E mutation in *ScUPC2* (85).

UPC2 is absent in the pathogenic fungi *Aspergillus* and *Cryptococcus*. Instead of *UPC2*, these organisms have genes similar to the sterol regulatory-element binding proteins (SREBP) of higher eukaryotes. These genes include *AfuSRBA* in *Aspergillus* (86), and *CnSRE1* in *Cryptococcus* (87). Unlike CaUpc2p, both AfuSrbAp and CnSre1p are helix-loop-helix transcription factors (86, 87). Disruption of *AfuSRBA* in *Aspergillus* causes hyper-susceptibility to triazoles (86), a phenotype similar to Δupc2/Δupc2 in *C. albicans*. To date, no GOF mutations in AfuSrbAp have been reported. However, a recent study demonstrated the role of AfuSrbAp in azole resistance in *Aspergillus* through regulation of AfuErg11p (86).

Azole Resistance via Sterol Import.

Sterol import as a potential mechanism of azole resistance has been identified recently (Fig. 1.2). Azoles lower the ergosterol levels of the cell, which can be compensated by exogenous sterol import. Sterol import has been well characterized in *S. cerevisiae*, *C. albicans*, and *C. glabrata* (88). Both *S. cerevisiae* and *C. glabrata* import sterols under anaerobic or microaerophilic conditions using the sterol importers Aus1p and Pdr11p (89-91), and show increased azole resistance (88). Mutations in *UPC2*, as well as defects in the biosynthesis of ergosterol, and heme, can cause increased sterol import (92, 93). For example, *S. cerevisiae* strains with mutated *ScERG1* and *ScERG7*, have increased sterol import (94).

Unlike *C. glabrata* and *S. cerevisiae*, *C. albicans* imports sterols aerobically, increasing azole resistance in the presence of both serum and cholesterol (88). Thus, *C. albicans* may develop resistance by importing cholesterol and serum from the blood. Similarly, itraconazole and voriconazole MICs in *Aspergillus* are increased in the

presence of serum and cholesterol (95). Sterol import in *Aspergillus* has not been correlated with an importer gene, though the *Aspergillus* genome encodes homologs of *AUS1* and *PDR11.*

Azole resistance via Azole Import.

Several studies have hypothesized that defective azole import may contribute to drug resistance (Fig. 1.2). Azoles are imported by facilitative diffusion (FD) in *C. albicans*, *A. fumigatus, C. neoformans,* and *S. cerevisiae* (96, 97), and the import is energy independent. Clinical isolates of many pathogenic fungi vary in azole import (97). However, to date no correlation has been observed between azole import and resistance. Further studies are required to identify the azole importers, and characterize their roles in azole resistance.

Azole resistance via Genome Plasticity.

Genetic variations including loss of heterozygosity (LOH) and aneuploidy can correlate with azole resistance in fungi. If one allele of a gene is mutated, LOH can copy the mutation to the second allele. In clinical isolates of *C.albicans*, LOH has been observed in *CaTAC1*, *CaERG11*, and *CaMRR1*, correlating with increased resistance (98). Clinical isolates of *C. albicans* can also include segmental aneuploidy in which two copies of the left arm of chromosome 5 containing *CaERG11* and *CaTAC1* form an isochromosome that correlates with azole resistance (99). Similarly, in *Cryptococcus* isolates, disomy of chromosome 1 and chromosome 4 correlates with azole resistance (100). Chromosome 1 contains *CnERG11* and *CnAFR1*, while chromosome 4 maintains the integrity of the endoplasmic reticulum (100).

1.6 Resistance to Other Drugs

Polyene resistance is also linked to changes in both *ERG3* and *ERG6.* For example, *in vitro,* disruption of *ERG3* and *ERG6* causes decreased ergosterol levels, and amphotericin B resistance in *C. albicans*, *C. glabrata*, and *S. cerevisiae* (101). However, polyene resistance in clinical isolates has not been well characterized.

Echinocandins are the newest antifungal category, targeting β 1-3 glucan synthase, an enzyme important for cell wall biosynthesis localized to the plasma membrane (102). The enzyme is encoded by the homologs *CaFKS1* and *CaFKS2.* Mutations in *CaFKS1* are observed in echinocandin resistant clinical isolates of *C. albicans* (102), clustering in two regions (amino acid regions 637-654 and 1345-1365) (103, 104). Mutations in *CaFKS2* can cause echinocandin resistance in *C. albicans in vitro,* but not been observed in clinical isolates (105). *FKS1* point mutations have also been observed in *S. cerevisiae*, *C. glabrata*, and *C. krusei* resistant isolates (105). Echinocandin resistance mechanisms have also been studied in *Aspergillus sp*. An artificially generated point mutation S678P in AfuFks1p caused increased echinocandin resistance (106).

The other medically important drug 5-FC is metabolized in the pyrimidine salvage pathway (107), and is used in treating candidiasis and cryptococcosis in combination with other antifungals. 5-FC resistance is observed in both *Candida sp.,* and *Cryptococcus sp.* In *C. albicans.*, 5-FC is imported by cytosine permease, CaFcy2p, and is then deaminated to 5-FU by cytosine deaminase, CaFcy1p. 5-FU is then converted to 5-FUMP by phosphoribosyl transferase, CaFur1p. Inactivation of any of these enzymes can cause increased 5-FC resistance (108).

This review outlines the known clinical, cellular, and molecular mechanisms of antifungal resistance in pathogenic fungi. The ongoing developments in understanding drug resistance mechanisms should assist in detecting resistant clinical isolates, designing new therapeutic strategies, and developing strategies to inhibit antifungal drug resistance.

1.7 Research Summary

Azole resistance mechanisms have been well studied in oral and systemic *C. albicans* clinical isolates (2, 3, 59). However, no studies have reported the resistance mechanisms in vaginal clinical isolates. The vaginal tissues have different environmental niches than oral and systemic tissues. Vaginal pH is 4.0-4.5, whereas oral and systemic pH is 7.0 (1). Recent studies have shown the effects of pH on the bioavailability of drugs in vaginal tissues (17). The drug bioavailability is much lower in vaginal tissues than oral and systemic tissues (13). Furthermore, the immune response in vaginal tissues is different from oral and systemic tissues (98). Thus it is important to study the resistance mechanisms in vaginal *C. albicans* isolates. My first project analyzes the resistance mechanisms in matched and unmatched vaginal isolates. Overexpression of the membrane efflux pumps (Cdr1p, Cdr2p, and Mdr1p) is responsible for drug resistance in the majority of these isolates. A combination of two fluorescent dyes was used to analyze the efflux activities of these pumps. Like azoles, these dyes are substrates for efflux pumps. Increased efflux correlated with pump gene overexpression in a majority of the resistant isolates (23).

Another important mechanism of azole resistance in *C. albicans is* overexpression and/or point mutation of *ERG11*, which is a rate-limiting enzyme in the ergosterol biosynthetic pathway. Other antifungals also target the ergosterol pathway, including

fenpropimorph, which targets Erg24p, and terbinafine, which targets Erg1p enzymes (7). Aberrant levels of ergosterol may affect many cellular processes (see above). Recent studies have used deletion mutants of the ergosterol genes to analyze their cellular phenotypes (5). Some of these mutants showed severe growth defects, compromised respiration, weak cell wall, and lower tolerance to osmotic stress. However, only seven of total 25 ergosterol biosynthetic genes (*ERG* genes) can be deleted as they are non-essential. To date, no studies have reported the effects of overexpression of ergosterol genes on cell phenotypes. My second project studies the ergosterol biosynthetic pathway in detail. All 25 *S. cerevisiae* genes (*ERG* genes) were overexpressed under a galactose inducible promoter. Nine of the 25 strains overexpressing the ergosterol genes showed severe growth defects. Furthermore, phenotypic changes in these strains were compared to wild-type under various stress agents. These agents affect several cellular processes that include cell wall biosynthesis, respiration, protein synthesis, osmotic stress, and iron and calcium metabolism. A majority of the overexpressed strains were affected by high salt or by a calcium chelator. Two of the nine slow growing strains were affected by all the stress agents used. This project increases our understanding of the ergosterol pathway, and may identify potential targets for future drug design.

My third project studied the effects in *S. cerevisiae* of known ergosterol inhibitors on 25 *ERG* overexpression strains, and eight *ERG* deletion strains. These inhibitors include, fluconazole, lovastatin, fenpropimorph, nystatin, and amphotericin B. Fluconazole, fenpropimorph, and nystatin had an effect on three or more deletion strains, while fenpropimorph and terbinafine had an effect on three or more overexpression strains.

This project contributes to our understanding of how altered *ERG* expression can effect the susceptibilities of the cells to different antifungal agents.

The two most common molecular mechanisms of drug resistance in pathogenic fungi are a) overexpression of membrane transporters, and b) altered ergosterol biosynthesis. My thesis analyzes both mechanisms in *C. albicans* and *S. cerevisiae*. In *C. albicans*, the overexpression of membrane transporters is correlated with drug resistance in vaginal clinical isolates. In *S. cerevisiae*, overexpression of *ERG* genes is correlated with altered drug susceptibilities. Overexpression of *ERG* genes also has an effect on cellular processes including cell wall biosynthesis, respiration, the response to osmotic stress, and iron and calcium metabolism. This thesis provides a better understanding of the molecular mechanisms of drug resistance, and also identifies potential targets for future drug design.

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CHAPTER 2

A COMBINATION FLUORESCENT ASSAY DEMONSTRATES INCREASED EFFLUX PUMP ACTIVITY AS A RESISTANCE MECHANISM IN AZOLE-RESISTANT VAGINAL *CANDIDA ALBICANS*

2.1 Abstract

Candida albicans is a pathogenic fungus causing vulvovaginal candidiasis (VVC). Azole drugs such as fluconazole are the most common treatment for these infections. Recently, azole-resistant vaginal *C. albicans* isolates have been detected in patients with recurring and refractory vaginal infections. However, the mechanisms of resistance in vaginal *C. albicans* have not been studied in detail. In oral and systemic resistant isolates, over-expression of ABC transporters Cdr1p and Cdr2p and the major facilitator transporter Mdr1p is associated with resistance. Sixteen fluconazole susceptible and twenty-two fluconazole resistant vaginal *C. albicans* isolates were obtained, including six matched sets containing a susceptible and a resistant isolate from individual patients. Using qRT-PCR, 16 of 22 resistant isolates showed over-expression of at least one efflux pump gene, while only one of 16 susceptible isolates showed such over-expression. To evaluate pump activity associated with over-expression, an assay was developed that combined data from two separate fluorescent assays using Rhodamine 6G and Alanine β-napthylamide. The qRT-PCR results and activity assay results were in good agreement. This combination of two fluorescent assays can be used to study efflux pumps as resistance mechanisms in clinical isolates. These results demonstrate that efflux pumps are a significant resistance mechanism in vaginal *C. albicans* isolates.

2.2 Introduction

C. albicans is a pathogenic fungus that causes oral, systemic and vulvovaginal candidiasis (VVC) infections (1-3). VVC caused by *C. albicans* occurs in at least 75% of women once in a lifetime, and systemic recurrence of vaginitis caused by *Candida* species is common (2, 4-7). The most common drugs for treating this infection are azoles that target the ergosterol biosynthetic pathway.

Ergosterol is an important component of the cell membrane of *C. albicans*, supporting fungal membrane fluidity and proper functioning of the membrane. Many antifungal agents target different components of the ergosterol biosynthetic pathway. For example, terbinafine inhibits squalene epoxidase (Erg1p), fenpropimorph inhibits C14 sterol reductase (Erg24p), and azoles inhibit lanosterol-14α-demethylase (Erg11p). Erg11p is encoded by *ERG11* gene, an essential gene in the ergosterol biosynthesis (8-10).

Based on previous studies in oral and systemic isolates, long term azole treatment and exposure selects for azole-resistant *C. albicans* isolates (11-13). Several factors can contribute to drug resistance in this organism. *C. albicans* possesses two families of efflux pumps, ATP-binding cassette transporters (ABC-T) and major facilitator transporters (MFS-T). The genes *CDR1* and *CDR2* encode ABC-transporters that are over-expressed in the majority of resistant clinical isolates obtained from oral and systemic infections (14). These transporters are energy dependent; and deletion of these genes results in hyper-susceptibility to azoles (15, 16). The MFS- transporter Mdr1p depends on the proton gradient and transports azoles and other compounds across the plasma membrane. Over-expression of *MDR1* has also been identified as an azole resistance mechanism in oral and systemic clinical isolates (14, 16, 17). Apart from the

actions of the efflux pumps, *C albicans* can gain azole resistance by over-expression and point mutation of the *ERG11* target gene (9, 14, 18, 19).

Recent documentation of efflux pump over-expression in clinical isolates has used quantitative real time reverse transcriptase PCR (qRT-PCR) (20). In addition, the fluorescent dye Rhodamine 6G (R6G) has been used to quantitate the ABC-transporter activity of Cdr1p and Cdr2p (21). While azole efflux is restricted to Cdr1p and Cdr2p ABC-T, R6G is a substrate for many ABC-T (21). In general, the R6G assay has been used in experimental studies. One experimental use of R6G has been in the expression of *C. albicans* efflux pumps in *Saccharomyces cerevisiae* to study substrate specificity and to aid in drug development (22).

Recently a bacterial assay for generalized efflux that uses the fluorescence molecule Alanine β-napthylamide (Ala-Nap, (23)) has been applied to *Aspergillus fumigatus* and *C. albicans* (24, 25). No oral or systematic clinical isolates have been studied with Ala-Nap.

Azole-resistant vaginal *C. albicans* isolates have only recently been identified (26). While resistance mechanisms have been studied in oral and systemic isolates, no resistance mechanisms in vaginal isolates have been characterized. There are several possible factors that contribute to azole resistance in *C. albicans* isolates obtained from vaginal cavity. First, the bioavailability of fluconazole (FLC) in the vaginal cavity is 12 fold lower than bioavailability in other tissues (4, 27). This results in colonizing vaginal isolates that are exposed to lower concentrations of FLC after drug administration. Second, the vaginal pH is 4.0 compared to oral and systemic pH of 7.0, which is important as minimum inhibitory concentration (MIC) values are pH dependent (2, 28).

Understanding pH dependences has required adjusting the clinical breakpoints for vaginal MICs (4). Third, the altered pH of the vagina will have an effect on Mdr1p pump activity, which is dependent on the proton gradient. Finally, vaginal infections have been commonly associated with a hyper immune response rather than over-growth of yeast (29).

To characterize resistance mechanisms in vaginal isolates required a collection of susceptible and resistance vaginal isolates. The characterization of matched susceptible and resistant isolates from single patient, which are part of this collection, can contribute significantly to the analysis of resistance. This study describes two fluorescent assays which when used in combination measure increased efflux activity resulting from overexpression of *CDR1*, *CDR2* and *MDR1*. The study also is the first to describe the molecular mechanisms of azole drug resistance in *C. albicans* vaginal isolates.

2.3 Materials and Methods

Strains and Cell Growth Conditions

Thirty eighty vaginal clinical isolates of *C. albicans* were obtained from the Vaginitis Clinic of Wayne State University in Detroit. Isolates were collected from patients with informed consent and IRB approval. Of these 38 isolates, 12 isolates represent six matched pairs, i.e. two isolates were obtained from the same individual reflecting clinical response and failure after FLC therapy. The six matched sets are designated as S1/S2, S3/S4, S5/S6, S7/S8, S9/S10, and S11/S12. The matched pairs were verified using Multi Locus Sequence Typing (MLST) on four variable housekeeping genes, *VPS13*, *ADP13*, *ACC1*, and *AATa,* as previously described (30). In brief, genomic DNA was isolated from single colony of each strain as described previously (31). Defined oligonucleotides

were used to amplify each of the four genes using PCR on genomic DNA. While isolates from each of five pairs represented the same strains, isolates S9 and S10 are not same strain of *C. albicans* (Table 2.1). To verify the isolates were of *C. albicans* they were streaked on ChromAgar plates and development of green colonies signified that they were of *C. albicans*. For further identification, the strains were grown in YAD media (1.7 g of yeast nitrogen base without amino acids and ammonium sulfate, 5.0 g of ammonium sulfate per liter) with 5% Xylose (32) to distinguish between *C. albicans* and *C. dubliniensis*. *C. albicans* grows on YAD + Xylose while *C. dubliniensis* does not (33). Finally, the isolates that had reduced growth in xylose were verified as *C. albicans* by PCR amplification of the cell wall protein-coding gene *CRR*. The size of the PCR product distinguishes between *C. albicans* and *C. dubliniensis*. All isolates were confirmed to be *C. albicans*. PCR primers used were CRR-f 5′-GTTTTTGCAACTTCTCTTTGTA-3′ and CRR-r 5′-ACAGTTGTATCATGTTCAGT-3′ as described (34). PCR conditions used were 30 s at 98° C followed by 30 cycles of 10 s at 98° C, 30 s at 60° C, 5 m at 72° C and a final extension of 10 m at 72° C. PCR reactions were verified on 0.8% agarose gel. All oligonucleotides were ordered from Sigma-Aldrich.

Table 2.1: Multilocus Sequence Locus Typing to Verify the Matched Isolates

Strains	2-76	S1	S3	S5	S7	S9	S11
12-99	0	5[a]	1[a]	2[b]	3[a]	5[a]	3[b]
S2	5[a]	0	4[a]	7[a]	8[a]	6[a]	5[a]
S4	1[a]	4[a]	0	3[a]	4[a]	4[a]	2[a]
S6	2[b]	7[a]	3[a]	0	2[a]	3[a]	3[b]
S8	3[a]	8[a]	4[a]	2[a]	0	5[a]	3[a]
S10	3[a]	4[a]	2[a]	1[a]	3[a]	2[a]	1[a]
S12	3[b]	5[a]	2[a]	3[b]	3[a]	3[a]	0

a- Numbers represent the total number of single nucleotide changes when compared between the listed isolates for two housekeeping genes AATa and ACC1. Matched isolates showed no change in nucleotides

b- Numbers represent the total number of single nucleotide changes when compared between the listed matched sets for four housekeeping genes AATa, ACC1, VPS13 and ADP1

Growth curves were performed to determine the growth rates of the isolates in YAD media. Altered cell growth in these isolates might affect the susceptibilities to the drugs. Single colonies were suspended in YAD media plus glucose. After 24 h, 0.1 OD of cells were used as inoculum and cells were grown at 30° C for 48 h in a BioTek Take 3 plate reader (BioTek Instrument Inc.). The growth rates of all 38 isolates and control SC5314 were similar in YAD + glucose (data not shown)

Minimum Inhibitory Concentration (MIC)

The 38 clinical isolates were tested for susceptibility to the azoles FLC, itraconazole (ITC), and clotrimazole (CLT). FLC, ITC, and CLT were obtained from Sigma-Aldrich. The MICs of the isolates were determined using the CLSI approved microbroth microdilution protocol that determines MICs as the concentration of drug that inhibits 80% growth of the organism (35). A single colony was suspended in YAD media with 2% glucose and grown overnight at 30° C. After 24 h, 0.1 OD of cells were used as an inoculum. Cells were grown in 96 well plates containing a gradient of drug in two- fold serial dilution. The plates were incubated for 48 h at 35° C. YAD media with 2% glucose was used for the cell growth. YAD media was used instead of standard RPMI media, as some of the clinical isolates did not grow well in RPMI media. A positive growth control lacking drug, and a negative growth control lacking cells were included. Cell growth in the wells containing drug was measured by the BioTek plate reader and standardized to the positive growth control.

Assessment of Efflux Activity

Two types of fluorescent assays were used to determine the efflux activity in an isolate. First, alanine- β- napthylamide (Ala-Nap) was used to detect efflux activities of

both types of efflux pumps (ABC-T and MFS-T). Second, rhodamine 6G (R6G) was used to specifically detect the efflux activities of the ABC-T.

In preparation for the assay, a single colony of each isolate was suspended in 5 ml YAD with 2% glucose and incubated at 30° C with shaking at 180 rpm. After 24 h, 0.1 OD of cells were inoculated in YAD with 2% glucose and grown at 30° C with shaking at 180 rpm to exponential phase (approximately 6-7 h). Equal ODs of cells for each isolate were the washed three times with 1X PBS phosphate buffer saline (pH 7), and starved for 2 h in 1X PBS and incubated at 30° C.

The Ala-Nap efflux assay was performed using a previously published protocol (24, 25). Briefly 100 µl of starved cells was added to a blank 96 well plate (Corning Costar 96 well plate black, Fisher Scientific). One row of the plate contained only cells while another row contained both cells and 2% glucose. A stock solution of 0.6 M Ala-Nap (Sigma-Aldrich) was dissolved in ethanol and added to both rows to a final concentration of 0.6 mM. The kinetics of fluorescence were measured for 1 h by using an excitation wavelength of 320 nm and an emission wavelength of 460 nm at 37° C in the BioTek Take 3 plate reader. A well-studied, matched set of oral isolates, 2-76 (#1, susceptible) and 12-99 (#17, resistant), were used as controls in the assay (36). First, the ratio of the slopes of the efflux of the individual strains in presence and absence of glucose were calculated for all strains and the control 2-76. Second, these ratios were compared to the control 2-76 and were plotted in the graph. All experiments were performed in biological triplicates. The slopes were calculated using linear regression on the efflux curves. Errors in the slopes were calculated with the help of LINEST function in Microsoft Excel.

The R6G fluorescent dye is specific for Cdr1p and Cdr2p efflux pumps. The R6G assay was performed as previously described (22, 37). R6G was obtained from Sigma-Aldrich. Briefly, a 5 µl of a 10 mM stock of R6G (in ethanol) was added to 5 ml of starved cell suspension to a final R6G concentration of 10 µM. The cells were incubated at 37º C for 30 m. After incubation, the cells were pelleted for 5 m at 3700 g and washed once with 1X PBS. The washed cells were re-suspended in 1X PBS plus 2% glucose. 200 µl samples were taken from the re-suspended cells at 0, 5, 10, 15 and 20 m. The samples were pelleted in the microcentrifuge for 30 s at top speed. The fluorescence of the supernatant was measured using excitation of 485 nm and emission of 525 nm. Strains 2-76 (susceptible) and 12-99 (resistant) were used as controls. The slopes of fluorescence of each strain, generated by linear regression on the efflux curves, were compared to the slope of 2-76 and the results were plotted as the ratio of the slopes. All experiments were performed in biological triplicates. Errors in slopes were calculated with the help of LINEST function in Microsoft Excel.

Efflux assays were performed on the *S. cerevisiae* strains in which *C albicans* genes were expressed (AD mutants, Table 2.2). Both Ala-Nap and R6G assays were performed in CSM-complete media as previously described in the materials and methods.

Table 2.2: Strains Used In This Study

Strain Name	Organism	Remarks/Genotype	TW No.	Ref.
2-76	*C. albicans*	Susceptible Clinical isolate	TW17205	(36)
12-99	*C. albicans*	Resistant Clinical isolate	TW17206	(36)
DSY1050	*C. albicans*	*cdr1::hisG/cdr1::hisG cdr2::hisG/cdr2::hisG mdr1::hisG-URA3-hisG/mdr1::hisG*	TW17207	(10)
SC5314	*C. albicans*	Wild Type	TW17209	(42)
S288C	*S. cerevisiae*	Wild Type	TW19001	(43)
ADΔ	*S. cerevisiae*	MATα *PDR1–3 ura3 his1Δyor1::hisGΔsnq2::hisGΔpdr10:: hisGΔpdr11::hisGΔycf1::hisGΔpdr3::hisG pdr5::hisG pdr15::hisG Δura3*	TW17238	(44)
AD-CDR1	*S. cerevisiae*	MATα *PDR1–3 ura3his1Δyor1::hisGΔsnq2::hisGΔpdr10::hisGΔpdr11::hisGΔycf1::hisGΔpd*	TW17239	(44)

		r3::hisG pdr5::hisG pdr15::hisG Δ*pdr5*::*pABC3-CaCDR1*		
AD-CDR2	*S. cerevisiae*	MATα *PDR1–3 ura3 his1*Δ*yor1::hisG*Δ*snq2::hisG*Δ*pdr10::hisG*Δ*pdr11::hisG*Δ*ycf1::hisG*Δ*pdr3::hisG pdr5::hisG pdr15::hisG* Δ*pdr5*::*pABC3-CaCDR2*	TW17240	(44)
AD-MDR1	*S. cerevisiae*	MATα *PDR1–3 ura3 his1*Δ*yor1::hisG*Δ*snq2::hisG*Δ*pdr10::hisG*Δ*pdr11::hisG*Δ*ycf1::hisG*Δ*pdr3::hisG pdr5::hisG pdr15::hisG* Δ*pdr5*::*pABC3-CaMDR1*	TW17241	(44)
S1-S38	*C. albicans*	Vaginal Clinical isolates	TW20301 - TW20338	This study

qRT-PCR Analysis

mRNA expression of the genes *CDR1, CDR2, MDR1* and *ERG11* were measured using qRT-PCR. Exponentially growing cultures of all 38 clinical isolates were prepared from single colonies. RNAs were isolated from the cells using Qiagen RNeasy mini purification kit following the manufacturer's protocol. The concentrations of RNAs were measured using a nanodrop plate reader in the BioTek Take 3. The A260/A280 ratio was assessed to determine the purity of RNAs. RNAs with a ratio above 2 were used in qRT-PCR. As an additional check, 1.2% agarose gels were run, and the appearance of two distinct ribosomal RNA bands confirmed the RNA quality. 1 µg of RNA from each strain was used to prepare cDNA by using Thermo-scientific cDNA kit following the manufacturer's protocol. qRT-PCR was performed in the Applied Biosystems 7500 Real-Time PCR System following the manufacturer's protocol using BIO-RAD iTaq^{TM} SYBR$^{®}$ GREEN supermix. Primers used to amplify the specified genes are listed in Supplementary Table 2.1. These primers were previously used to determine the mRNA expression levels of drug resistance genes in *C. albicans* (38). Wild-type strain SC5314 was also used as a control. After qRT-PCR was completed, a dissociation step was performed for each reaction to demonstrate that the reactions were specific (data not shown). qRT-PCR for each of the isolates was performed in triplicate. The formula $2^{-\Delta\Delta Ct}$ was used to determine the fold change. First, the Ct values were compared to the Ct values of the actin gene *ACT1*. Second, the relative values were compared to the values for SC5314. Error bars were calculated as previously described (20, 39). The elongation factor gene *CEF3* was used as an additional control. The expression of *CEF3* did not vary between the isolates (data not shown). Greater than 2-fold over-expression was defined as

significant; less than 0.5 fold of reduced expression was defined as significant when compared to SC5314.

Supplementary Table 2.1: qRT-PCR Oligonucleotides

Primer Name	Primer Sequence
CDR1_fwd	5'-AAGATGTCGTCGCAAGATGAAT-3'
CDR1_rev	5'-GAGTGAAAGTTCTGGCTAAATTCTGA-3'
CDR2_fwd	5'-TTGAGCCACATGTCCGACAT-3'
CDR2_rev	5'-GGAATCTGGGTCTAATTGTTCATGA-3'
MDR1_fwd	5'-ATCACCGGTAACGACAGAATCA-3'
MDR1_rev	5'-TCTAATGGTCTCCATAATGTATCAATGA-3'
ERG11_fwd	5'-CCCCTATTAATTTTGTTTTCCCTAATTTAC-3'
ERG11_rev	5'-CACGTTCTCTTCTCAGTTTAATTTCTTTC-3'
ACT1_fwd	5'-GAAGAAGTTGCTGCTTTAG-3'
ACT1_rev	5'-CGTCGTCACCGGCAAAA-3'
CEF3_fwd	5'- GCTCTCAAGATGGCAACTTTTTC-3'
CEF3_rev	5' – GCTGTCAAAGCCATCTTACCA -3'

Statistical Analysis

All data and statistical analysis were performed using Graph Pad Prism 6.0 and Microsoft Excel. The types of statistics used are mentioned in the figure legends.

2.4 Results

Characterization of Vaginal Isolates

There were 38 *C. albicans* vaginal isolates studied and obtained from 32 women seen at Wayne State University Vaginitis Clinic. These included 12 *C. albicans* isolates obtained from 12 women with acute *Candida* Vaginitis in whom no suspicion of clinical resistance existed and all 12 women responded promptly to conventional fluconazole therapy. 14 vaginal isolates of *C. albicans* were obtained from 14 women with clinically refractory vaginitis and considered to reflect clinical and *in vitro* resistance. Finally, twelve *C. albicans* isolates were studied and obtained from six women in whom two successive *C. albicans* isolates were available. All 20 women with resistant *C. albicans* strains provided a history of significant fluconazole exposure.

In the collection of 38 isolates, a pair of matched isolates was collected from six different patients. To determine if the two isolates in the pair are the same strain, MLST was used with four house-keeping genes (Table 2.1). In the table, the numbers in each box represent the single nucleotide differences between the initial isolates from the patients (top row) versus the later isolates (left column). Five of the six matched pairs, and the known matched set 2-76 and 12-99 (36) had no sequence differences between the

initial and the subsequent isolates, indicating both isolates are the same strain. In the sixth matched set (S9/S10) two nucleotide differences were observed.

MIC Susceptibility Testing

MICs were tested on all 38 vaginal clinical isolates for azole drugs FLC, itraconazole (ITC), and clotrimazole (CLT) (Table 2.3). Interpretation of MICs as susceptible or resistant requires clinical break points. In the present study the cut off value for *in vitro* susceptibility for FLC was $\leq$ 1.0 µg/mL as described previously (4). All isolates with MIC's of $\geq$ 2.0 µg/mL were considered fluconazole resistant. This is in contrast to CLSI standards which consider a value of 2.0 µg/mL as susceptible to fluconazole (40). However, the CLSI breakpoint is based upon oral and systemic clinical predictions and not vaginal isolates where the vaginal pH is 4.0 and not a pH of 7.0. Azole activity is dramatically affected by reduction in pH and clinical experience indicates a cut off of 1 µg/mL is more appropriate (2). Of the 38 vaginal isolates of *C. albicans* studied MIC's of $\geq$2 µg/mL were seen in 22; and 19/38 had MIC's of $\geq$4 µg/mL. For ITC and CLT, clinical break points have been determined for oral and systemic isolates to be 1µg/mL respectively (14). These break points were used for these vaginal isolates. Using these break points 22 isolates showed resistance to FLC (MIC $\geq$ 2 µg/mL), 18 isolates were resistant to CLT (MIC $\geq$ 1µg/mL) and three isolates were resistant to ITC (MIC $\geq$ 1µg/mL, Table 2.3). The three isolates (S36, S37 and S38) that are resistant to ITC are cross-resistant to both FLC and CLT, while 14 isolates are resistant to both FLC and CLT.

Table 2.3. MICs of Vaginal Clinical Isolates to FLC, CLT, and ITC.

Isolates	FLC	CLT	ITC	Genes upregulated	Genes downregulated
S24	0.25	0.25	0.03	*CDR4*	
S29	0.25	0.25	0.03		
S31	0.25	1	0.02		*CDR1, ERG11*
S1	0.5	0.25	0.01		*CDR1*
S11	0.5	0.25	0.02		
S27	0.5	0.25	0.03		
S28	0.5	0.25	0.03		*CDR1, MDR1, ERG11*
S30	0.5	0.25	0.03		*CDR2, MDR1*
S33	0.5	0.25	0.03		*CDR1, MDR1*
WT	**0.5**	**0.25**	**0.03**		
S32	0.5	1	0.13		
S26	1	0.13	0.02		*ERG11*
S35	1	0.25	0.03		*CDR1, CDR2, MDR1, ERG11*
S25	1	0.5	0.06	*CDR4*	
S3	1	0.5	0.06	*CDR2*	*ERG11*
S34	1	0.5	0.06		
S7	1	1	0.06		*CDR2*
S18	2	0.13	0.03		*CDR1*
S17	2	0.25	0.03	*ERG11*	*CDR2*
S2	2	0.25	0.03		*CDR1, MDR1*
S16	4	0.25	0.13	*ERG11*	
S10	4	1	0.02	*MDR1*	
S9	4	1	0.03	*MDR1*	*CDR2, ERG11*
S12	8	0.5	0.02	*MDR1, ERG11*	
S8	8	1	0.06	*CDR1, MDR1*	
S19	8	4	0.25	*CDR1 ,CDR2, MDR1, ERG11*	
S20	8	4	0.25	*CDR1 ,CDR2, MDR1, ERG11*	
S36	8	>4	4	*CDR1 ,CDR2, MDR1, ERG11*	
S13	16	0.25	0.06	*ERG11*	
S21	16	>4	0.5	*CDR1 ,CDR2, MDR1, ERG11*	
S22	32	4	0.25	*CDR1 ,CDR2, MDR1, ERG11*	
S23	32	4	0.25	*CDR1 ,CDR2, MDR1, ERG11*	
S4	32	4	0.5	*CDR1,CDR2*	*MDR1*
S5	32	4	0.5	*CDR1,CDR2,*	
S6	32	4	0.5	*CDR2*	*MDR1, ERG11*
S14	>32	0.5	0.06	*CDR1, ERG11*	*CDR2*
S15	>32	1	0.13	*CDR1, MDR1, ERG11*	*CDR2*
S37	>32	>4	>4	*CDR1, CDR2, ERG11*	
S38	>32	>4	>4	*ERG11*	*CDR2*

Table 2.3. MICs of Vaginal Clinical Isolates to FLC, CLT, and ITC. Thirty-eight clinical vaginal isolates were tested for their MIC to FLC, CLT, and ITC. All MIC values are in μg/mL**.** The strains are arranged based on their MIC to FLC followed by CLT and then by ITC. MIC values are colored from susceptible (blue) to resistant (red). MIC clinical break points for resistance to FLC, CLT, and ITC were respectively ≥2 μg/mL, ≥1 μg/mL and ≥1 μg/mL. MICs colored yellow are at the clinical break points. All MICs were done in biological triplicate and the average value is listed in the table. The isolates were tested for their expression of genes *CDR1*, *CDR2*, *MDR1,* and *ERG11.* Genes that are up-regulated ≥2 fold relative to SC5314 are in red. Genes down-regulated ≥2 fold with respect to SC5314 are labeled with blue. Two susceptible strains S24 and S25 that showed high efflux were tested for over-expression of *CDR3* and *CDR4* and both showed over-expression of *CDR4*.

The matched isolates S2, S4, S8, and S12 showed an increase in FLC resistance (4-32 fold) compared to the initial isolates (S1, S3, S7, S11; Table 2.4) indicating that *C. albicans* vaginal strains can gain resistance upon exposure to FLC. S6 and S10 were also FLC resistant but their initial isolates S5 and S9 were resistant as well. Increased ITC resistance was seen in isolates S2 and S4 compared to their matched partners S1 and S3 respectively. CLT resistance was observed in S4 and S12, compared to their matched partners S3 and S11 (Table 2.4).

Table 2.4: Minimum Inhibitory Concentrations of Matched Vaginal Isolates in µg/mL

Isolates	FLC	ITC	CLT	Genes Over-expressed[a]
S1	0.5	0.01	0.25	
S2	2	0.03	0.25	
S3	1	0.06	0.5	
S4	32	0.5	4	*CDR1*, *CDR2*, *ERG11*
S5	32	0.5	4	
S6	32	0.5	4	
S7	0.5	0.06	1	
S8	8	0.06	1	*CDR2*, *MDR1*
S11	0.5	0.02	0.25	
S12	8	0.02	0.5	*CDR2*, *ERG11*

a- Genes over-expressions when compared to respective matched partners

mRNA Expression of Drug Resistance Genes

To determine the mechanism of azole resistance, qRT-PCR was used to access over-expression of genes associated with drug resistance. qRT-PCR for the four resistance genes *CDR1, CDR2, MDR1,* and *ERG11* was performed on all 38 isolates including the matched isolates (Fig. 2.1 and Fig. 2.2).

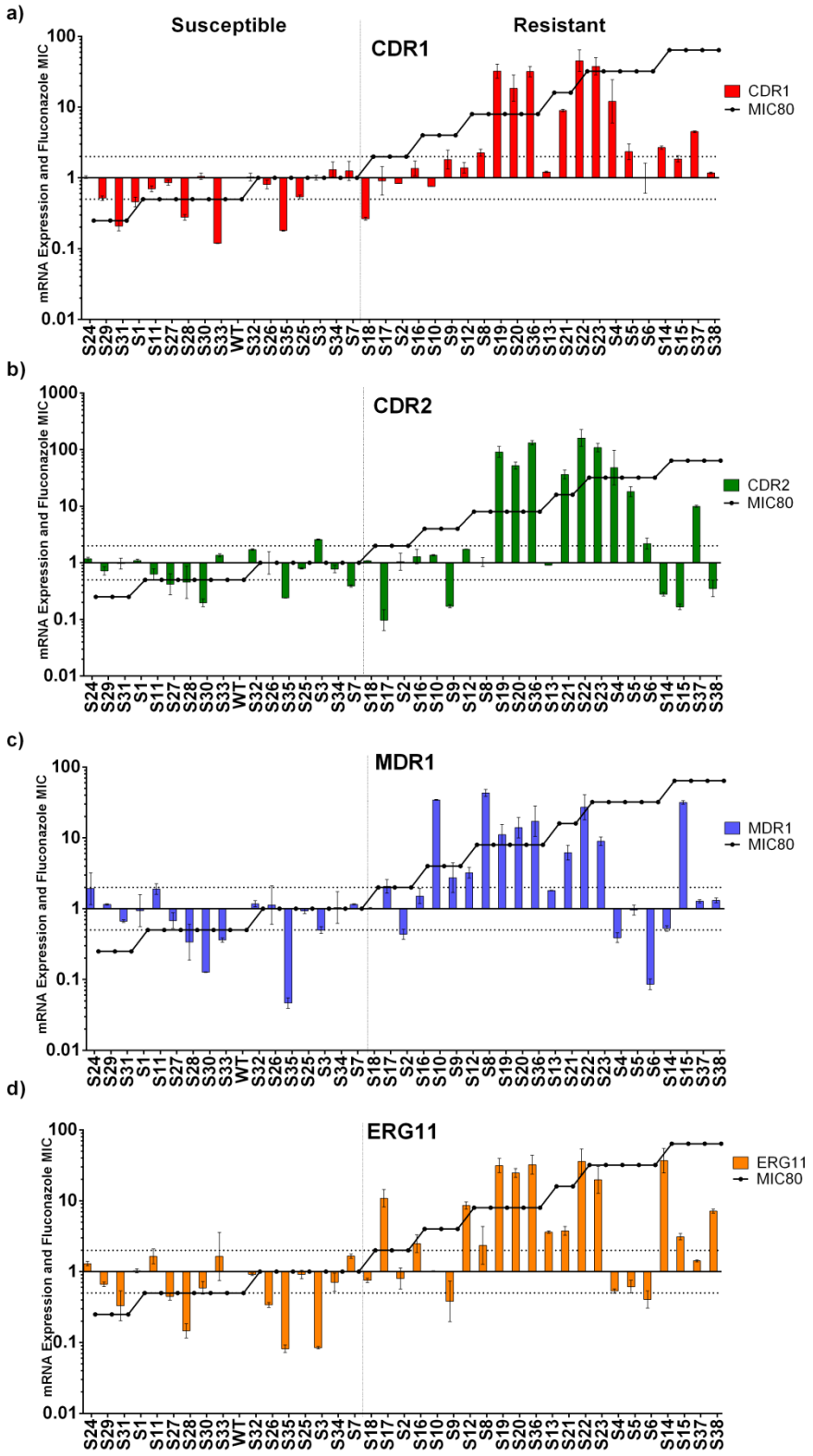

a)
Susceptible
Resistant
CDR1
mRNA Expression and Fluconazole MIC
CDR1
MIC80
b)
CDR2
CDR2
MIC80
c)
MDR1
MDR1
MIC80
d)
ERG11
ERG11
MIC80
S24 S29 S31 S1 S11 S27 S28 S30 S33 WT S32 S26 S35 S25 S3 S34 S7 S18 S17 S2 S16 S10 S9 S12 S8 S19 S20 S36 S13 S21 S22 S23 S4 S5 S6 S14 S15 S37 S38

Fig. 1.1. Gene Expression Levels of all 38 Vaginal Isolates.

Panels a-d show mRNA expression levels of genes *CDR1* (a), *CDR2* (b), *MDR1* (c), and *ERG11* (d). The data are normalized to wild-type SC5314 (WT). *ACT1* was used as an expression control. The gene expressions are plotted in log- scale as colored bars and the error bars for gene expression represent the standard errors for triplicate assays. The strains in the x-axis are arranged with increasing FLC MICs (line graph). Dotted horizontal lines in panels a-d signify 2-fold up or 2-fold down regulation in mRNA expression when compared to SC5314 (WT).

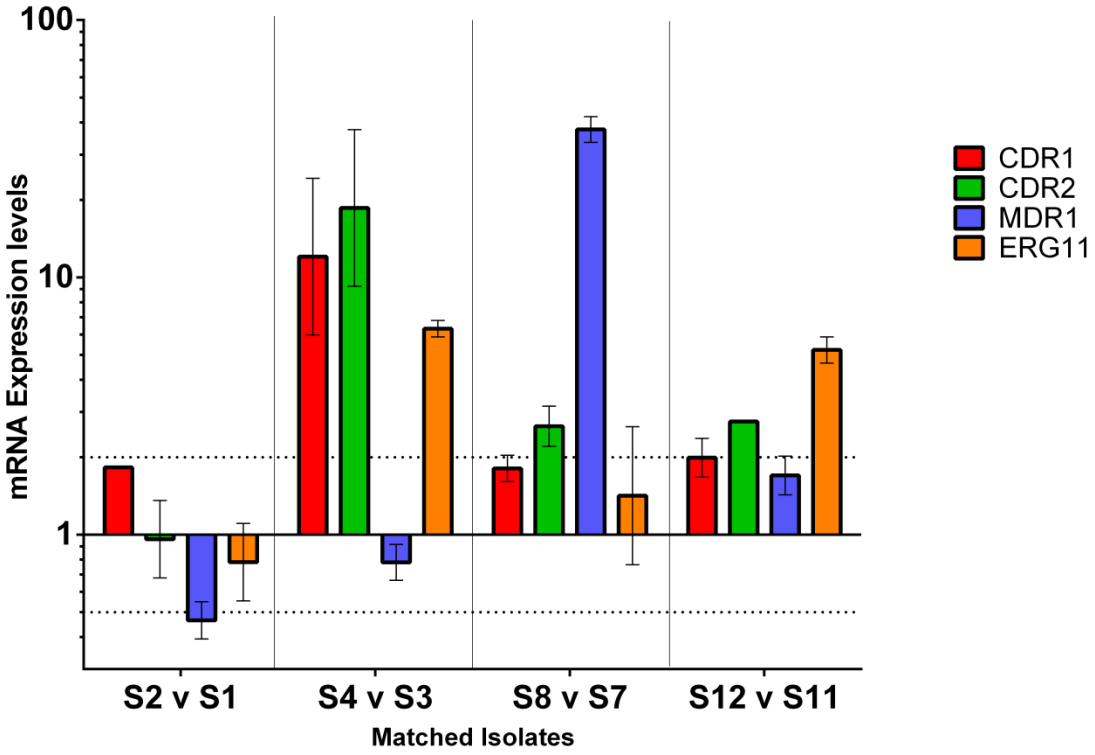


Fig. 2.2. **Gene Expression Levels between Matched Isolates.**

Gene expression of *CDR1*, *CDR2*, *MDR1*, and *ERG11* in matched resistant isolates is normalized to their susceptible partner (S2 to S1, S4 to S3, S8 to S7, and S12 to S11). The gene expressions are plotted in log- scale and error bars represent the standard errors of triplicate assays. Dotted horizontal lines signify 2-fold up or 2-fold down regulation in mRNA expression when compared to matched partner. S6 versus S5 is not shown because the MICs for these isolates do not change.

12 of 22 fluconazole resistant isolates had over-expressed *CDR1*, 10 over-expressed *CDR2*, 11 showed increased *MDR1* expressions, and 14 over-expressed *ERG11* when compared to SC5314. In susceptible isolates, over-expression was only observed for *CDR2* in one isolate (S3). Over-expression of efflux pumps is seen in 72% of FLC resistant isolates, 77% of CLT resistant isolates and two out of three of ITC resistant isolates. *ERG11* over-expression is observed in 64% of FLC, 50% of CLT and in three of three ITC resistant isolates. Several susceptible isolates showed decrease in gene expressions of *CDR1*, *CDR2*, *MDR1* and *ERG11* (Table 2.3 and Fig. 2.1).

In the matched sets, gene expressions were compared between initial and the later isolates (Fig. 2.2), over-expression was observed in the later isolates for *CDR1* in S4, *CDR2* in S4, S8, and S12, *MDR1* in S8, and *ERG11* in S4 and S12. No gene over-expression was observed for S2. No over-expression of genes was observed in S6 compared to S5.

Efflux Assays

To measure efflux pump activity rather than gene expression, two efflux pump assays were employed. The R6G assay measures the activity of ABC-Transporters and the Ala-Nap assay measures efflux pump activity in general. A combination of the two assays was tested for their utility in measuring the efflux pump activity in resistant isolates. In the R6G assay, R6G is taken up by de-energized fungal cells. With the subsequent addition of glucose, the cells efflux R6G, which are measured in the supernatants. The efflux values are expressed as the slope of R6G efflux from the cells into the supernatant.

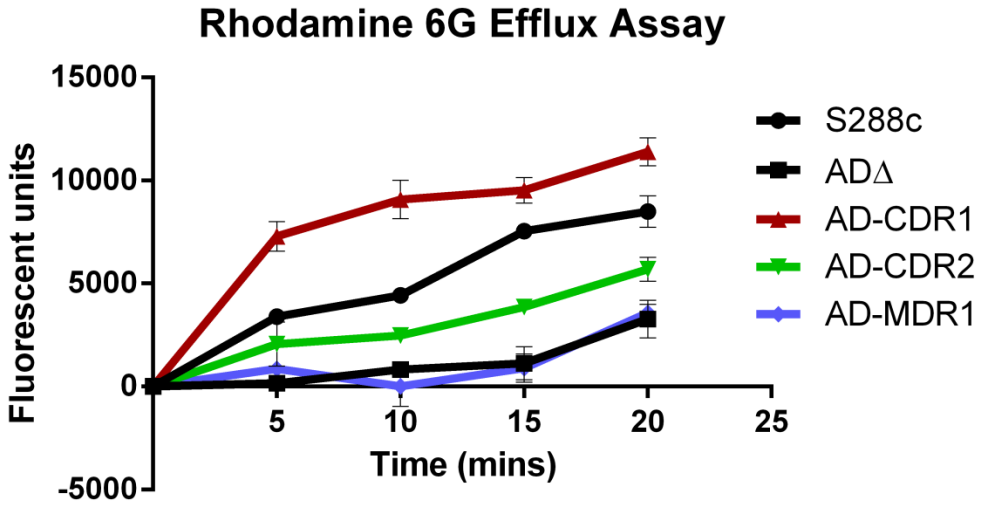
Ala-Nap is a dye that is cleaved by proteases within the cell to give fluorescent β-napthylamide. In the Ala-Nap assay, increased fluorescence indicates a reduction in

efflux activity of the pump (24). For each strain, Ala-Nap fluorescence over time is measured in the absence and presence of glucose. An Ala-Nap value is a comparison of the slope of the test strain compared to the slope of the negative control in presence and absence of glucose. A lower slope ratio means more efflux.

Five *S. cerevisiae* strains expressing *C. albicans* genes were used to evaluate the ability of the two assays to measure the efflux activity of the membrane transporters. AD-CDR1 is a *S. cerevisiae* strain expressing *CaCDR1* from the endogenous efflux pump Pdr5p promoter. Similarly, AD-CDR2 expresses *CaCDR2;* and AD-MDR1 expresses *CaMDR1*. These strains were constructed in ADΔ background, where all the efflux pumps of *S. cerevisiae* were deleted (22).

ADΔ strain showed very little efflux of R6G (Fig. S2.1a) and was used as a negative control for this assay. AD-CDR1 has a high rate of R6G efflux consistent with *CDR1* expression (Fig. 2.3 and Fig. S2.1a). Similarly, AD-CDR2 has a high rate of R6G efflux consistent with *CDR2* expression. AD-MDR1 has a similar R6G efflux to ADΔ consistent with the fact that *MDR1* does not efflux R6G. S288C shows high R6G efflux due to endogenous expression of PDR5, the *S. cerevisiae* homologue of *C. albicans CDR1*.

a)


Rhodamine 6G Efflux Assay
Fluorescent units
15000
10000
5000
0
-5000
5
10
15
20
25
Time (mins)
S288c
ADΔ
AD-CDR1
AD-CDR2
AD-MDR1

b)

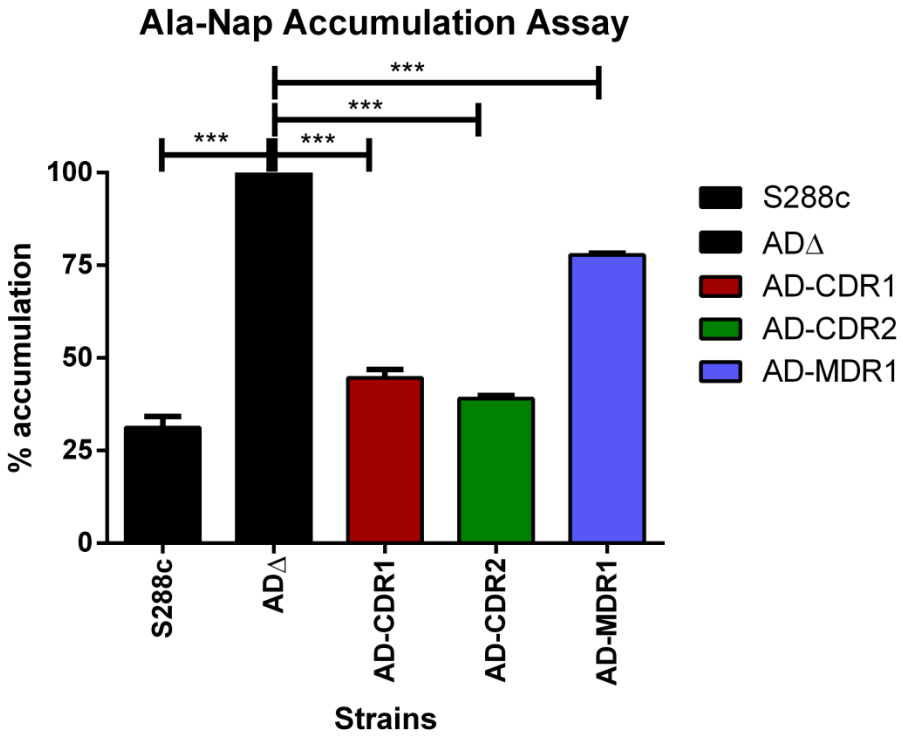

Ala-Nap Accumulation Assay
***
***
***
***
% accumulation
100
75
50
25
0
S288c
ADΔ
AD-CDR1
AD-CDR2
AD-MDR1
Strains
S288c
ADΔ
AD-CDR1
AD-CDR2
AD-MDR1

Fig. S2.1. **Efflux Assay for *S. cerevisiae* Controls**.
(a) Rhodamine 6G efflux assay in *S. cerevisiae* strains. ADΔ is used as a control. Data plotted is the R6G efflux curves. (b) Ala-Nap efflux assay in *S. cerevisiae* strains S288C, ADΔ, AD-CDR1, AD-CDR2, AD-MDR1. Data plotted as the ratio of the slope of Ala-Nap fluorescence curve in presence and absence of 2% glucose and normalized to the negative control ADΔ. For statistical analysis, a unpaired two-tailed student's t-test was performed and the data was found to be significant ($p<0.05$, ***)

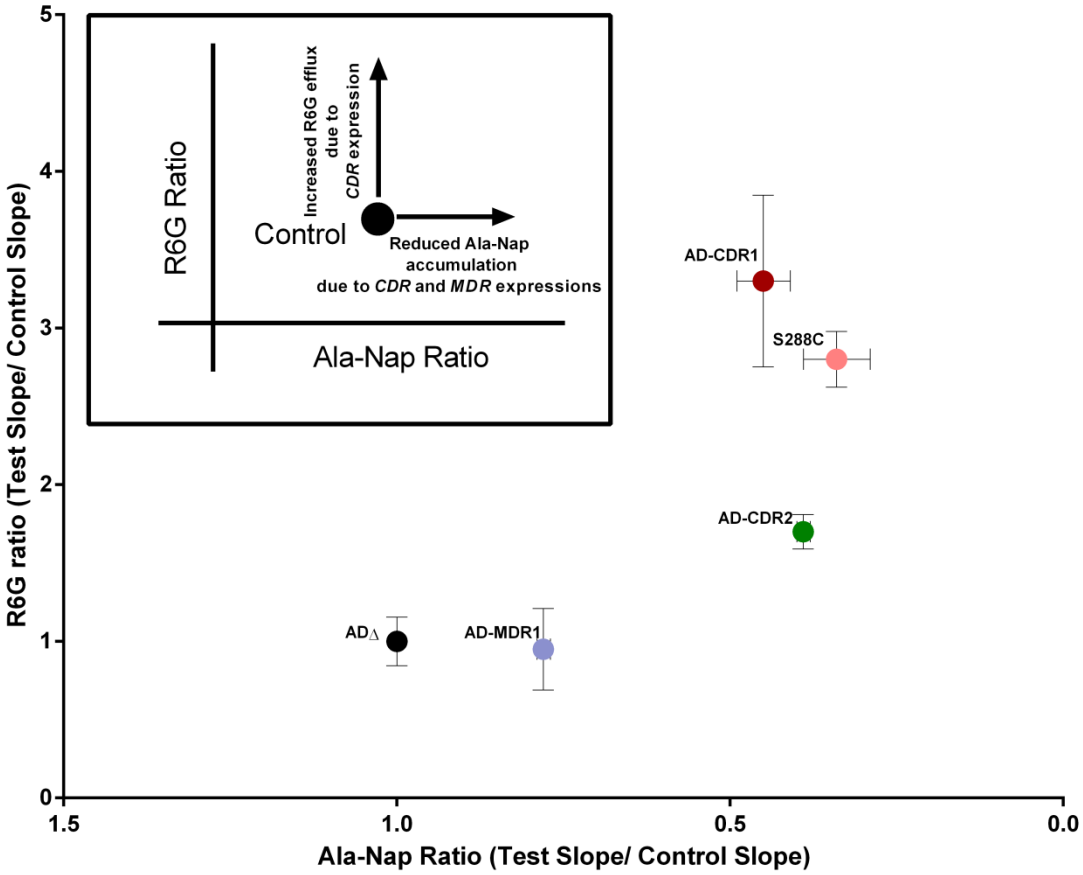


Fig. 2.3. Efflux map of *S. cerevisiae* Strains Expressing Efflux Pumps.

The insert panel illustrates how values are graphed. The R6G values (y-axis) represent the ratio of the R6G efflux slope in the test strain compared to the control (ADΔ). The Ala-Nap values (x axis) represent the ratio of Ala-Nap retention in the test strain compared to the control. Retention is measured as the difference in Ala-Nap retention in the presence of glucose compared to Ala-Nap in the absence of glucose. High Ala-Nap retention indicates low efflux and low retention represents high efflux. The negative control for Fig. 2.3 is ADΔ. Strains AD-CDR1, AD-CDR2 and AD-MDR1 express respectively *CDR1*, *CDR2* and *MDR1*. S288C express *PDR5,* a homolog of the CDRs. Error bars and slopes were calculated by using LINEST function in Microsoft Excel on curves generated from biological triplicates for both the Ala-Nap and R6G assays.

S288C shows substantial decrease in accumulation of Ala-Nap, 33% of accumulation of ADΔ (100%). Similarly, Ala-Nap accumulation was 38%, 43% and 77% for AD-CDR1, AD-CDR2, and AD-MDR1 respectively. All strains showed significant decreases in accumulation of Ala-Nap compared to the negative control (Fig. S2.1b).

An efflux map (Fig. 2.3) was generated for the control strains using the results from the Ala-Nap assay (x-axis) and the R6G results (y-axis). AD-CDR1, AD-CDR2 and S288C have high R6G values (y-axis) and low Ala-Nap values (x-axis) and thus are graphed in the top right area of the map (Fig. 2.3). AD-MDR1 has low Ala-Nap values and no increase in R6G values compared to ADΔ. Thus it is graphed to the right of ADΔ, with no increase in R6G (y-axis, Fig. 2.3).

Using the same assays as demonstrated in Fig. 2.3 and Fig. S2.1, the efflux activities of 38 vaginal clinical isolates were analyzed (Fig. 2.4). Strain 2-76, a susceptible oral isolate from a matched set was used as a control to which data are normalized. Strain 12-99, the matched resistant oral isolate to 2-76, that over-expresses *CDR1*, *CDR2, MDR1,* and *ERG11*, was used as a positive control. Strain DSY1050, which has all efflux pumps deleted, was used as a negative control for pump activity, and SC5314 was included as the wild-type strain.

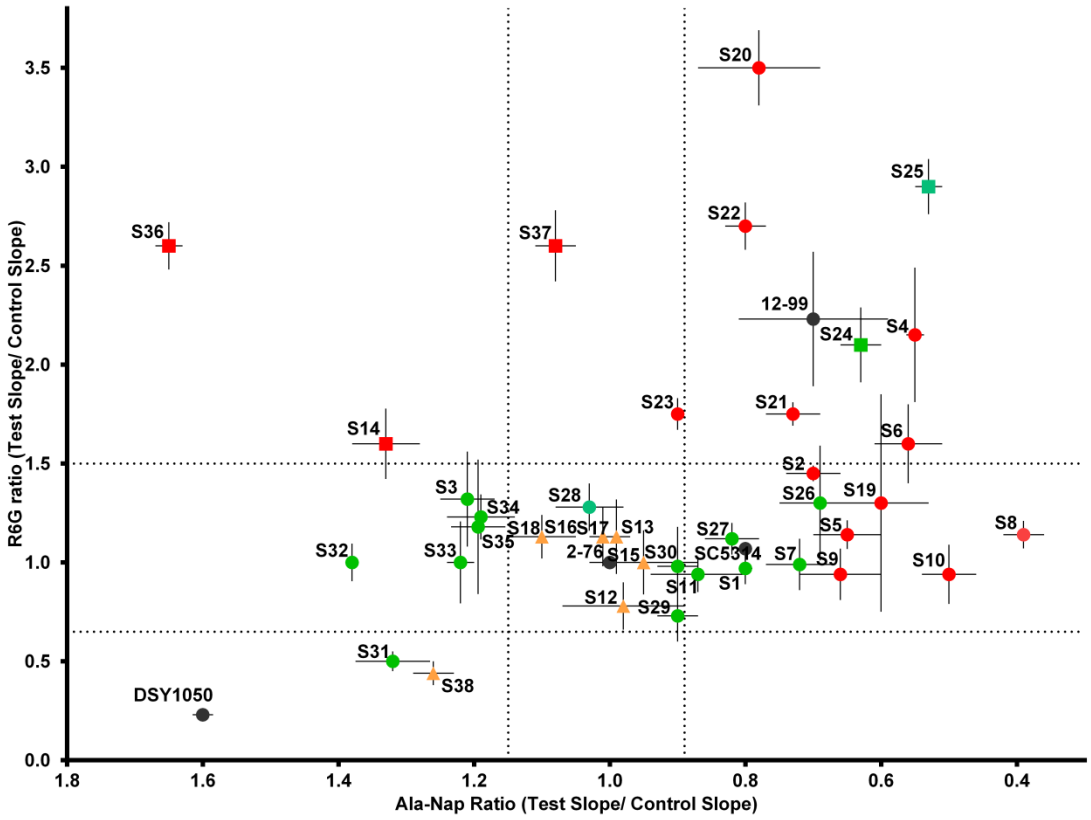


Fig. 2.4. Efflux map of 38 *C. albicans* Vaginal Clinical Isolates.

Controls used were strains 2-76, 12-99, SC5314, and DSY1050 (black circles). Data are normalized to strain 2-76. Values were calculated as described in Fig. 2.3. Green symbols represent FLC susceptible strains, red symbols represent FLC resistant strains, and yellow triangles represent FLC resistant strains over-expressing *ERG11*. Red squares S14, S36, and S37 are unusual in showing R6G efflux but not Ala-Nap efflux. Green Squares (S24, S25) are unusual as they are susceptible isolates showing significant efflux with both Ala-Nap and R6G, most likely due to *CDR4* over-expression. The region between the vertical dotted lines is not significantly different from the control for Ala-Nap and the region between the horizontal dotted lines is not significant from the control for R6G. Significance was calculated using one-way anova with Dunnett's multiple comparison tests. Error bars and slopes were calculated by using LINEST function in Microsoft Excel on curves generated from biological triplicates from both Ala-Nap and Rhodamine 6G assay.

Most of the resistant strains are located to the right and above compared to the strain 2-76 in the efflux map (Fig. 2.4, red circles and squares). Most of the susceptible isolates (green circles) do not show an increase in R6G value but vary in their Ala-Nap values. Susceptible strains S24 and S25 did show high R6G and low Ala-Nap values (green squares). This is likely due to over-expression of gene *CDR4* (Table 2.3), which encodes another ABC-transporter. Previously, it has been shown that *CDR4* does not contribute to drug resistance (41). Strains S36 and S37 (marked with red squares Fig. 2.4), are located in the top left of the map, and have high R6G values. However, the Ala-Nap assay of S36 showed high accumulation (low efflux), while S37 showed no significant change in Ala-Nap levels. This may be due to pumps with altered specificities for Ala-Nap and R6G. In Fig. 2.4, resistant strains S12, S13, S14, S15, S16, and S38 do not show significant efflux pump activity. Table 2.3 and Fig. 2.1 demonstrate that they have over-expression of *ERG11,* which likely contributes to their resistance. In two isolates (S9 and S10), over-expression of *MDR1* was observed to correlate with high Ala-Nap activity and low R6G activity (Fig. 2.4). The resistance mechanism in these two isolates may be due to over-expression of *MDR1*.

In the matched vaginal sets (Fig. 2.5), the resistant isolate in four out of the five pairs (S2/S1, S4/S3, S6/S5 and S8/S7) were mapped above and to the right of their matched susceptible isolate, suggesting that the efflux pumps in these strains contribute to drug resistance. In matched set S8/S7, the resistant isolate is to the right but not significantly above S7 suggesting that efflux pumps that do not transport R6G are involved in drug resistance. In the matched set S11/S12, efflux pumps do not appear to contribute to drug resistance, since S12 is located below its partner S11 suggesting that the efflux activity of

S12 is less efficient than its matched partner S11. When compared to S5, S6 shows no increased FLC resistance (Table 2.3, Fig. 2.1), and increased efflux activity (Fig. 2.5), but does not show increased gene expression, suggesting that S6 expresses efflux pumps unrelated to azole resistance.

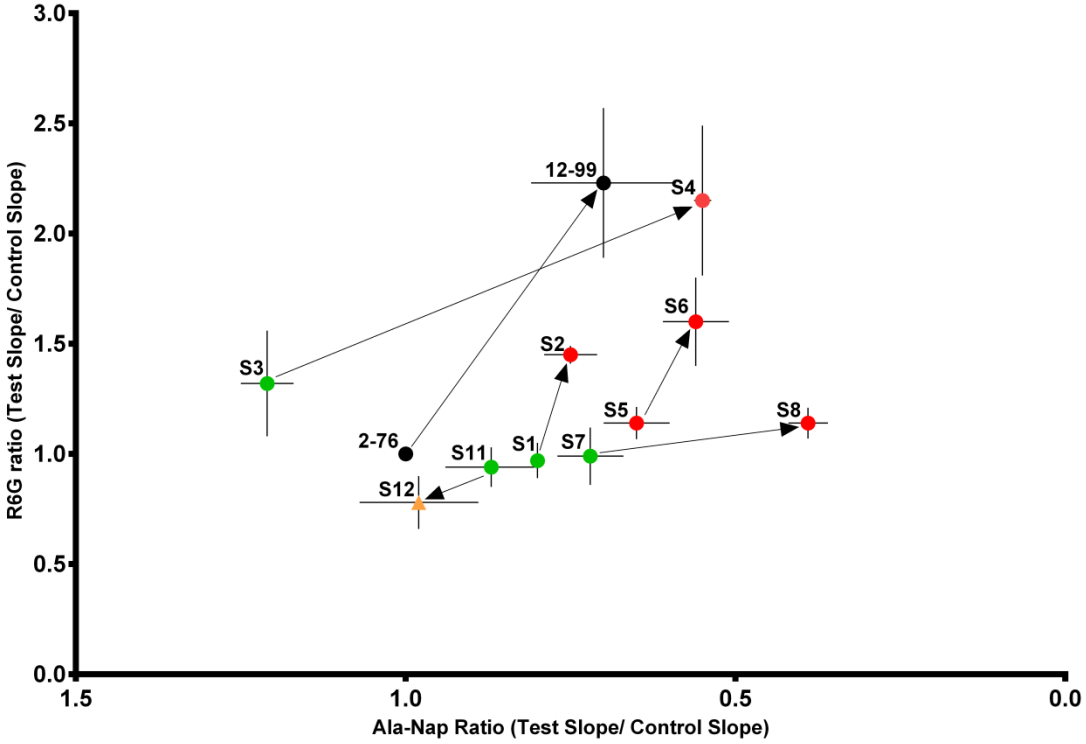


Fig. 2.5. Efflux map of *C. albicans* Vaginal Matched Clinical Isolates.

Strains 2-76 and 12-99 were used as efflux controls and the data are normalized to strain 2-76. Calculations were performed as in Fig. 2.3. Green circles represent FLC susceptible strains, red circles represent FLC resistant strains and the yellow triangles represent FLC resistant strains over-expressing *ERG11*. Error bars and slopes were calculated as described in Materials and Methods. S5 and S6 are both resistant.

2.5 Discussions

The goal of the experiments described in this research was to determine the molecular mechanisms that contribute to drug resistance in vaginal clinical isolates. Thirty-eight *C. albicans* vaginal clinical isolates collected from the Wayne State University Vaginitis Clinic were confirmed to be *C. albicans* and had similar growth rates (data not shown) suggesting that their growth rates were not affecting MIC.

Twenty-five of the 38 isolates were resistant to FLC and/or CLT, while three isolates were resistant to ITC. The smaller number of ITC resistant isolates may be due to the use of ITC clinical break points that are not well defined for vaginal isolates. Clinical breakpoints may differ in the vagina as opposed to oral cavity as described for FLC (2). Alternately, resistance to ITC may be less common in vaginal isolates, which may have implications for treatment. Azole cross-resistance in the isolates (Table 2.3) is consistent with the over-expression of *CDR1*, *CDR2*, *MDR1*, and *ERG11* (Fig. 2.1) and increased efflux activity (Fig. 2.4). All azoles are substrates for the over-expressed efflux pumps. Over-expression of *ERG11* provides additional target enzyme that can contribute to resistance.

Increased levels of mRNA (Fig. 2.1) of the genes encoding the membrane transporters do not always signify increased protein. Therefore, assays that monitor actual efflux activity are a better measure of efflux as a resistance mechanism. In most of the resistant strains, over-expression of efflux pump genes (Fig. 2.1) correlates with increased efflux pump activity (Fig. 2.4). However, qRT-PCR and efflux activity do not correlate in eight strains (S5, S8, S12, S14, S15, S19, S36, and S37). All eight over-express *CDR1* or *MDR1*, yet they show no significant efflux of Ala-Nap and/or R6G

efflux. Over-expression of *ERG11* may contribute to resistance in five of these strains (Fig. 2.1: S12, S14, S15, S36 and S37). The increased expression of pump genes without increased efflux activity may be due to altered substrate specificity, protein instability, or translational defects. The disconnect between gene expression and pump activity emphasized the need for assays that reflect pump activity.

While the efflux activity assays did not detect resistant isolates with *ERG11* over-expression, the efflux assays have many advantages. They are straightforward, quick, and cost effective. Their data analysis is less complex and it measures efflux directly which is the dominant resistance mechanism in these vaginal isolates, as well as the dominant mechanism in oral and systemic isolates (14).

The two efflux assays (Fig. 2.3, Fig. 2.4, and Fig. 2.5) show strong agreement between high efflux rates and azole drug resistance (red circles, Fig. 2.4). Susceptible strains have less efflux and hence in the efflux map were clustered around 2-76 or wild-type SC5314. Thus, these efflux assays should be important tools in the armamentarium for the analysis of drug resistance mechanisms.

This study uses a collection of matched and unmatched vaginal clinical isolates. Matched isolates have been very useful in the analysis of oral and systemic isolates. Six pairs of matched susceptible and resistant vaginal isolates were obtained in the collection. Using MLST, isolates S9 and S10 (Table 2.1) are not the same strain, but have similar resistant MICs. This suggests that the patient was previously exposed to azoles, and that the patient had coinfection with two strains or was super infected with the second strain.

Resistant isolate S5 and S6 are the same strain and have high MIC, suggesting that the patient had previously being exposed to azoles. In the other four pairs of matched

isolates, the isolates are the same strain and the MIC is increased in the later isolate compared to the initial isolate. In three of these four matched pairs (S2/S1, S4/S3, S8/S7), the later isolate show increased expression of *CDR1*, *CDR2* or *MDR1* by qRT-PCR (Fig. 2.2) and increased efflux activity (Fig. 2.5). This implies that long-term treatment with azoles may result in drug resistance by increasing the expressions and activities of efflux pumps.

Azole resistance in *C. albicans* is most commonly due to upregulation of genes that enhances efflux pump activity or upregulation of *ERG11* ((14) and this study). This suggests that resistance might be reversed if drug exposure is withheld, reduced or eliminated. Hypothetically, a second drug class that reduces efficiency of efflux pump activity, if used in combination with azoles, can prevent resistance in vaginal as well as oral and systemic candidiasis.

In conclusion, a combined efflux assay was developed that measures efflux activity rather than gene expression. This new assay should be useful in the future to study drug resistance in pathogenic fungi. This assay and qRT-PCR was used to show that over-expression of membrane transporters and *ERG11* contribute to the molecular mechanisms of drug resistance in vaginal isolates, similar to the mechanisms previously seen in oral and systemic isolates.

2.6 Acknowledgement

We thank our colleagues in the White laboratory for critical discussions and comments on the manuscript. We thank Dr. Richard Cannon and Dr. Dominique Sanglard for giving us the AD-mutant strains and DSY1050 respectively.

2.7 Funding Responsibility

This research was supported by unrestricted Research Funds from the School of Biological Sciences, University of Missouri at Kansas City.

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CHAPTER 3

PHENOTYPIC CHANGES RESULTING FROM THE OVER-EXPRESSION OF ERGOSTEROL BIOSYNTHETIC GENES IN *SACCHAROMYCES CEREVISIAE*

3.1 Abstract

Ergosterol is the major sterol in the cell membranes of different fungi, and its biosynthesis is tightly regulated. Various antifungals target different enzymes in the ergosterol biosynthetic pathway. Each of the 25 genes (*ERG* genes) in the ergosterol pathway of *Saccharomyces cerevisiae* was over-expressed under a galactose inducible promoter. The strains over-expressing nine of the genes (*HMG1*, *ERG1*, *ERG2*, *ERG6*, *ERG9*, *ERG25*, *ERG27*, *ERG28*, and *NCP1*) showed significant decrease in growth rates. Ergosterol biosynthesis can affect different cellular processes including the cell wall, iron and calcium homeostasis, respiration, protein translation and osmotic stress. The slow-growing strains showed severe growth defects compared to wild-type when grown in low levels of iron, low levels of calcium, and high osmotic stress. Low iron affected nine over-expressing strains, low calcium affected 22 strains, and high osmotic stress affected 20 strains. Two over-expressing strains (*ERG2* and *ERG6*) were respiratory incompetent, showing no growth in glycerol. Five strains (*HMG1*, *NCP1*, *ERG28*, *HMG2*, and *ERG12*) showed no growth in Congo red, a cell wall targeting agent. Finally, three over-expressing strains (*ERG2*, *ERG6*, and *ERG24*) were hypersusceptible to cyclohexamide, an inhibitor of protein translation. Over-expression of 11 of 25 *ERG* genes resulted in altered phenotypes under three or more stresses. Since the strains over-expressing *ERG2* and *ERG6* were affected by all six stress agents, they are clearly central to the pathway, and have the most potential as drug targets.

3.2 Importance

Different pathogenic fungi cause oral, pulmonary, systemic, and vaginal infections in immuno-compromised individuals. These infections are treated by antifungals that target the ergosterol biosynthetic pathway. Ergosterol is unique in fungi, and has similar functions to mammalian cholesterol. It regulates the structure, and the functions of the fungal plasma and the mitochondrial membranes. Thus, it is important to study ergosterol biosynthesis. The effects of the over-expression of each of the 25 *ERG* genes in *S. cerevisiae* are analyzed. *S. cerevisiae* is haploid, has high transformation efficiency, and many molecular tools are available. The phenotypes of these strains were analyzed in the presence of each of six stress agents that target several important cellular processes (cell wall biosynthesis, protein translation, respiration, osmotic stress, iron and calcium homeostasis). Eleven over-expressed strains were affected by three or more stress agents. Two strains over-expressing *ERG2* and *ERG6* showed altered phenotypes for all stress agents, and clearly have important functions in the cell. This analysis helps to identify important enzymes in the pathway that may be used as future drug targets.

3.3 Introduction

Infections caused by pathogenic fungi are on the rise in recent times due to increase in frequencies of autoimmune diseases, organ transplants and use of chemotherapy. Most of the pathogenic fungi affect individuals with compromised immune system. Fungi including *Aspergillus* sp. (pulmonary and systemic), *Candida* sp. (oral, vaginal and systemic), and *Cryptococcus* sp. (pulmonary and cerebral) can cause infections (1, 2). The modes of action of available antifungal drugs are conserved in most pathogenic fungi. Azole drugs such as fluconazole are the most common antifungal drug used to treat fungal infections (3). The azoles target 14α-demethylase (Erg11p), which is an important enzyme in the biosynthesis of ergosterol.

Ergosterol is the major sterol present in plasma and mitochondrial membrane of fungi and in some protists. Ergoterol is not present in mammalian cells, which instead use cholesterol. Both ergosterol and cholesterol have similar functions including membrane fluidity, permeability, structure, and function. Proper sterol levels are important for the movement of proteins and other cell components within the membrane. Cell membranes consist of microdomains called lipid rafts, which are formed by association of sterols and sphingolipids. Many biologically important proteins associated with efflux pumps, stress response, sodium and potassium pumps, mating, and nutrient transports are located in these lipid rafts (4). All these cellular processes are important for proper functioning of the cells.

Thus, the biosynthesis of ergosterol is important for cellular structure and function. Ergosterol biosynthesis occurs in the endoplasmic reticulum and involves a cascade of 25 biosynthetic enzymes (Fig. 3.1). The first substrate for the pathway is Acetyl-CoA, an

important product of the tricarboxylic acid cycle (TCA cycle, Fig. 3.1). The ergosterol biosynthetic pathway begins with mevalonate pathway, which is committed in synthesizing farnesyl pyrophosphate (FPP) from Acetyl-CoA. FPP is an important intermediate in the biosynthesis of ubiqinone, dolichol, heme, sterols, and prenylated proteins (5). Hmg1p/Hmg2p, which catalyzes the third step (the rate limiting step) in the mevalonate pathway, is synthetically lethal in *Saccharomyces cerevisiae* (6). The later part of the ergosterol pathway is responsible for synthesizing ergosterol from FPP. Erg1p and Erg11p are the two rate limiting steps in this part of the pathway (6). Erg11p functions in an association with Ncp1p, a NADP-cytochrome p450 reductase (7). Apart from Erg11p, Ncp1p may interact with other cytochromes, for example, Erg5p, Dit2p, cytochrome b5, and heme oxygenase among others. Both *NCP1* and *ERG11* are co-regulated and may regulate the entire ergosterol pathway.

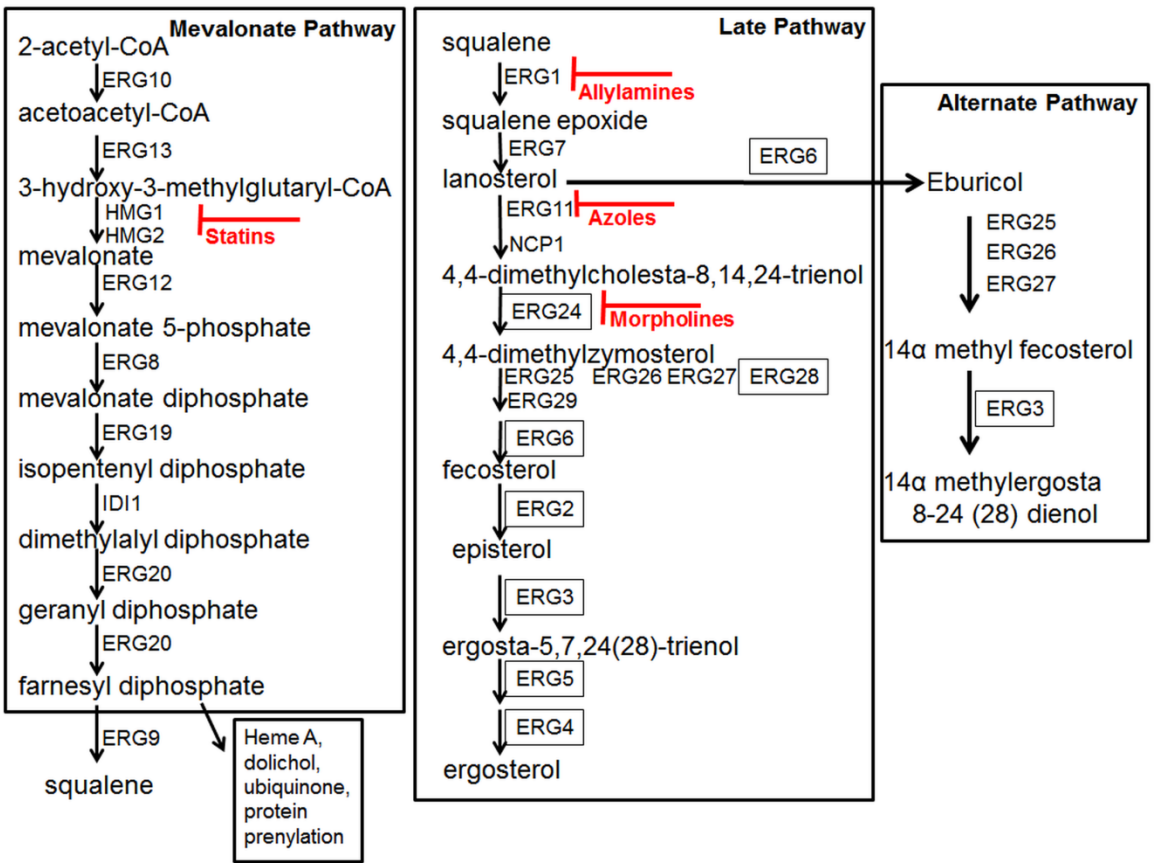


Fig. 3.1 Ergosterol Biosynthetic Pathway.

The box on the left diagrams signifies the mevalonate pathway, which can channel into different biosynthetic pathways. The box in the middle is the late ergosterol pathway terminating in ergosterol. The box on the right is an alternate pathway leading to the toxic sterol (14α methylergosta 8-24-(28) dienol). Boxed gene names denote non-essential genes.

The three rate limiting steps of the ergosterol biosynthetic pathway are the targets of different antifungal drugs: statins target Hmg1p/Hmg2p, whereas Erg1p and Erg11p are targets for drugs allylamines and azoles respectively (8). Apart from these, several other drugs target different parts of the pathway. Morpholines target Erg24p, while polyenes bind to ergosterol in the membrane (9). Due to minimal side effects, azoles are the most common drugs in treating fungal infections.

The incidence of infections caused by azole resistant pathogenic fungi is increasing (10). Factors that may be responsible include patients' exposure to high cumulative dosages, nosocomial transmission and use of azoles in agriculture (11). Recent studies identify the molecular mechanisms of azole resistance. These include altered efflux pump activities (ABC-transporters and major facilitator transporters), and altered ergosterol biosynthesis (2, 12-14). Increased expressions and activities of efflux pumps are common in resistant *C. albicans* clinical isolates (3). Azole drug resistance resulting from altered ergosterol biosynthesis is most commonly the result of over-expression or point mutation of *ERG11* (13, 15). Previous research by many authors have identified point mutations in *ERG11* gene in many fungal species (13, 16). Mutations in *ERG2*, *ERG3* and *ERG6* have been characterized in drug resistant clinical isolates of *Candida* sp. (17-19) and disruption of *ERG3* and *ERG6* lead to altered drug susceptibilities in *Saccharomyces cerevisiae* (17, 20). *S. cerevisiae* is a model system for the biology of human cells as well as pathogenic fungi. In *Saccharomyces,* engineered over-expression of *ERG11* results in azole resistance (21). No *ERG11* point mutation resulting in azole resistance has been identified.

The ergosterol pathway includes seven non-essential genes including *ERG3* and *ERG6* (Fig. 3.1). The deletions of the seven non-essential genes show disruption of ergosterol biosynthesis and accumulation of aberrant sterols (22). Some of these mutants are hyper-susceptible to many stress agents. Δ*erg3* and Δ*erg6* are hyper-susceptible to cycloheximide, (protein translation inhibitor), Congo Red (cell wall inhibitor), and osmotic stress (high Sodium Chloride) (23-25). Δ*erg3* and Δ*erg6*, Δ*erg24* show abnormal calcium homeostasis and have reduced efflux pump activities (23, 26, 27). The other four non-essential genes had no significant phenotypes with these stressors.

In a recent study, Δ*erg6*, Δ*erg24*, and Δ*erg28* showed abnormal mitochondrial structure and are respiratory incompetent (28). The other four non-essential gene deletions show aberrant sterols but showed normal mitochondrial structures.

A large scale survey used a repressible promoter to study the mitochondria of all essential ergosterol genes (*ERG* genes) (26). Eight of 16 essential *ERG* genes (*ERG7*, *ERG8*, *ERG10*, *ERG12*, *ERG13*, *ERG19*, and *NCP1*) exhibit defects in mitochondrial structure and function, as well as accumulation of aberrant sterol intermediates (26).

In wild-type (WT) cells, aberrant sterol intermediates accumulate when *ERG* gene expression is altered (*ERG7*, *ERG11*, *ERG12*, ERG25, and *ERG28*). This can be the result of changes in iron and heme concentrations. Iron and heme are cofactors for Erg11p and Erg5p (29, 30). Therefore, alterations in iron and heme can affect both sterol biosynthesis and function.

The phenotypic studies described above, all use the model organism *S. cerevisiae*. The advantages of *S. cerevisiae* are that it is haploid, has high efficiency of transformation and has many available molecular resources. The aim of the study is to

analyze the *S. cerevisiae* ergosterol pathway and its effects on cell wall, respiration, protein synthesis and plasma membrane. To achieve this, all 25 *ERG* genes were over-expressed and their effects were analyzed using stress agents that include Congo Red (CR), cycloheximide (CHX), sodium dodecyl sulfate (SDS, disrupts plasma membrane), sodium chloride (NaCl), glycerol (GLY, non-fermentative carbon source), ethylene glycol-bis(β-aminoethyl) ether (EGTA, calcium chelator) and ferrozine (iron chelator). Thus, this research will contribute to our understanding of the ergosterol biosynthetic pathway and may identify new targets for future drug designing.

3.4 Results

Cloning and Complementation Analyses of the ERG *Genes*

25 *ERG* genes were inserted downstream of *GAL1*- p in a yeast multi-copy plasmid as described in the Materials and Methods (Fig. S3.1). The activities of the nine non-essential Erg enzymes were analyzed by phenotypic complementation when expressed in the corresponding deletion strains (Δ*hmg1*, Δ*hmg2*, Δ*erg2*, Δ*erg3*, Δ*erg4*, Δ*erg5*, Δ*erg6*, Δ*erg24*, and Δ*erg28*). The complementation studies were performed in Glu-media, which allows a basal level of gene expression from the leaky *Gal1-p*. For complementation, MICs to the antifungals FLC, LOV, FEN, and NYS were performed (Table 3.1). Strains Δ*hmg1*, Δ*erg3*, and Δ*erg6* are resistant to FLC. These phenotypes were restored to the WT susceptible levels in presence of the plasmids expressing the respective genes (Table 3.1). Similarly, NYS resistance observed in Δ*erg3*, Δ*erg4*, Δ*erg5*, and Δ*erg6* strains was restored to susceptible level in presence of the plasmids expressing the corresponding genes. LOV hyper-susceptibility was observed in Δ*hmg1* and Δ*erg6*. The plasmids expressing *HMG1* and *ERG6* complemented the LOV phenotype in both Δ*hmg1* and Δ*erg6*. Plasmids expressing *ERG3* and *ERG6* complemented FEN hyper-susceptibility observed in Δ*erg3* and Δ*erg6*. Strains Δ*hmg2*, Δ*erg24*, and Δ*erg28* showed no phenotypic changes. However, the plasmid carrying *HMG2* complemented the FLC resistance phenotype of Δ*hmg1* (Table 3.1). A slow-growth rate observed in the Δ*erg2* strain was restored to WT growth in the presence of the plasmid expressing *ERG2*.
In Gal-media, over-expression of *ERG11* showed resistance to FLC, and the over-expression of *ERG24* in Δerg24 strain showed resistance to FEN (Table 3.1). Thus,

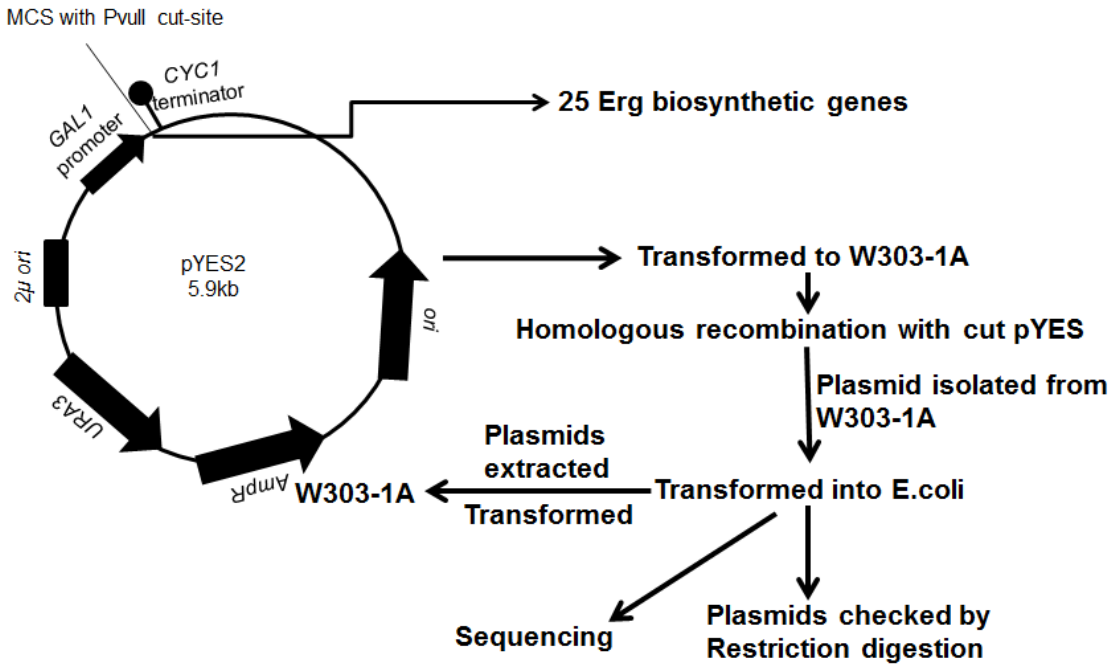
complementation analyses have demonstrated that the plasmids carrying nine genes (*HMG1*, *HMG2*, *ERG2*, *ERG3*, *ERG4*, *ERG5*, *ERG6*, ERG11, and *ERG24*) are functional. The complementation of Δ*erg28* did not show a phenotype that would prove the gene is functional. All 25 plasmids were sequenced, and the sequences were matched with the sequences available in the public databases.

qRT-PCR was used to analyze the mRNA levels for each *ERG* gene under inducing condition (Gal-media). All *ERG* genes were significantly over-expressed when compared to the wild-type (WT) in the presence of galactose (> two-fold, Fig. S3.2).

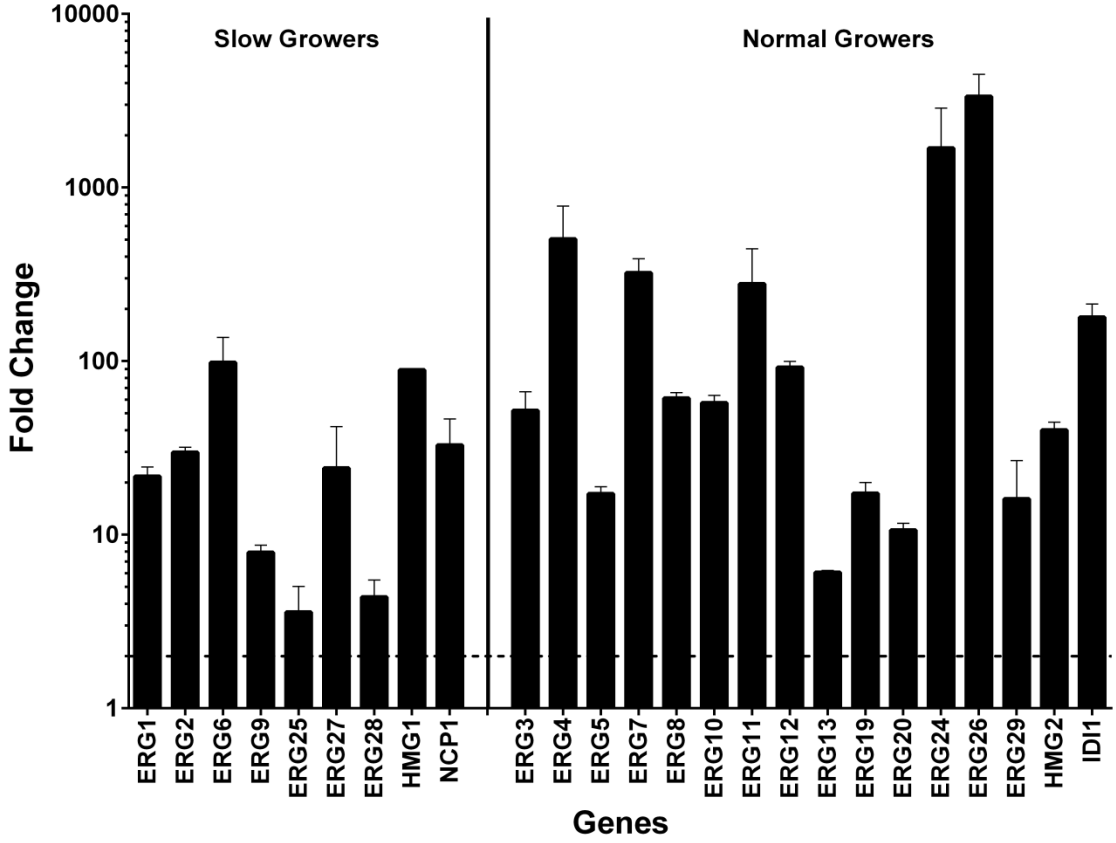
Table 3.1: Complementation of Deletion Strains with Over-Expression Plasmids

Gene	Deletion Phenotype	Complementation Phenotype
HMG1[a]	Susceptible to Lovastatin, Resistance to FLC	WT
HMG2[a]	WT	plasmid compliments Δhmg1
ERG2	Slow-Growth	WT
ERG3	Resistant to FLC, NYS, Susceptible to FEN	WT
ERG4	Resistant to FEN, NYS	WT
ERG5	Resistant to NYS	WT
ERG6	Resistant to FLC, NYS, Susceptible to FEN, LOV	WT
ERG24	WT	WT[b]
ERG11	Non-Viable	WT[c]

a. Synthetic lethal
b. Over-expression leads to FEN resistance
c. Over-Expression leads to FLC resistance. In addition, the endogenous copy for *ERG11* can be deleted in the presence of the *ERG11* over-expressing plasmid.



Supplemental Fig. S3.1. Cloning Strategy.
Strategy used to over-express *ERG* genes. All ergosterol biosynthetic pathway genes were amplified by PCR from W303-1A and inserted downstream of *Gal1*-p and upstream of *CYC1*-t. All cloned plasmids were sequenced and verified with the *Saccharomyces* genome database.



Supplemental Fig. S3.2. Gene Expression Levels of all 25 Over-expressed *ERG* Genes.
The mRNA transcript levels of the over-expressed genes were analyzed in their respective strain using qRT-PCR. The data are normalized to W303-1A (WT). *ACT1* was used as an expression control. The gene expression levels are plotted on a log- scale, and error bars signify standard errors from the triplicate assays. The horizontal line shows significant two-fold over-expression when compared to W303-1A (WT).

Analysis of Growth Phenotypes

The growth rates of all 25 genes over-expressed from plasmids in W303-1A were analyzed after the cells grew in Glu-media or Gal-media for 96 h. In Glu-media, all 25 strains and the WT strain showed similar doubling times (Table S1). In Gal-media, nine out of 25 over-expression strains (*HMG1*, *ERG1*, *ERG2*, *ERG6*, *ERG9*, *ERG25*, *ERG27*, *ERG28*, and *NCP1*) show a significant decrease in growth rates in both W303-1A and BY4741 WT backgrounds (Fig. 3.2). Among the slow-growers, the strains over-expressing *ERG1* (BY4741 background) and *NCP1* (W303-1A background) had the longest doubling times (Fig. 3.2). The doubling time for WT is 4.5 h in Gal-media.

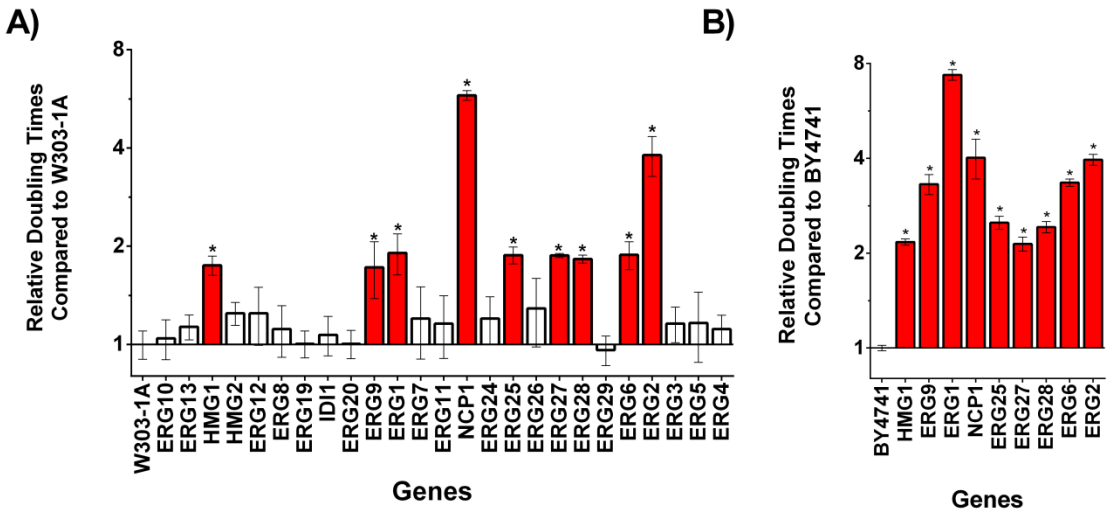


Fig. 3.2. Growth Rates of Strains Over-Expressing *ERG* Genes.

Strains over-expressing *ERG* genes were grown in Gal-media. The growth rates were calculated as described in the Materials and Methods, and normalized to A) W303-1A containing the pYES2 strain or B) BY4741 containing the pYES2 strain. The cells were incubated at 30^{o} C for 96 h with constant shaking and readings were taken every 15 m at 600 nanometers. Error bars represent standard error of the assay done in biological triplicate. One-Way Anova with Holm Sidak's multiple comparison test was performed, (*=$p<0.05$) and the significant values are plotted as red bars.

Requirements of Iron and Calcium

Iron and calcium are important elements required for cell growth. The over-expressing strains were grown in Gal-media in the presence of chelating agent for iron (Ferrozine) and calcium (EGTA). No significant growth was observed in the nine slow-growers in the presence of ferrozine (Fig. 3.3). The growth was restored when $FeSO_4$ was added to Gal-media containing ferrozine (Fig. 3.3). There were no significant differences in ferrozine effects between the remaining 16 over-expressing strains and the WT (Fig. 3.3). In Gal-media, the doubling time of WT is 10 h in the presence of Ferrozine.

In contrast, calcium had an effect on 22 out of 25 over-expressing strains. In the presence of EGTA, no significant growth was observed in the nine slow-growing strains (Fig. 3.4) and 12 of 16 normal growing cells (*IDI1*, *HMG2*, *ERG3*, *ERG4*, *ERG5*, *ERG7*, *ERG11*, *ERG12*, *ERG13*, *ERG20*, *ERG24*, and *ERG29*, Fig. 3.4). The strain over-expressing *ERG8* showed significantly slower, but detectable growth in the presence of EGTA compared to the WT (Fig. 3.4). There were no significant differences in EGTA effects in the remaining three over-expressing strains (*ERG10*, *ERG19*, and *ERG26*). All growth rates were restored in the 25 strains after $CaCl_2$ was added to Gal-media containing EGTA (Fig. 3.4). In Gal-media, the doubling time of WT is 15 h in the presence of EGTA.

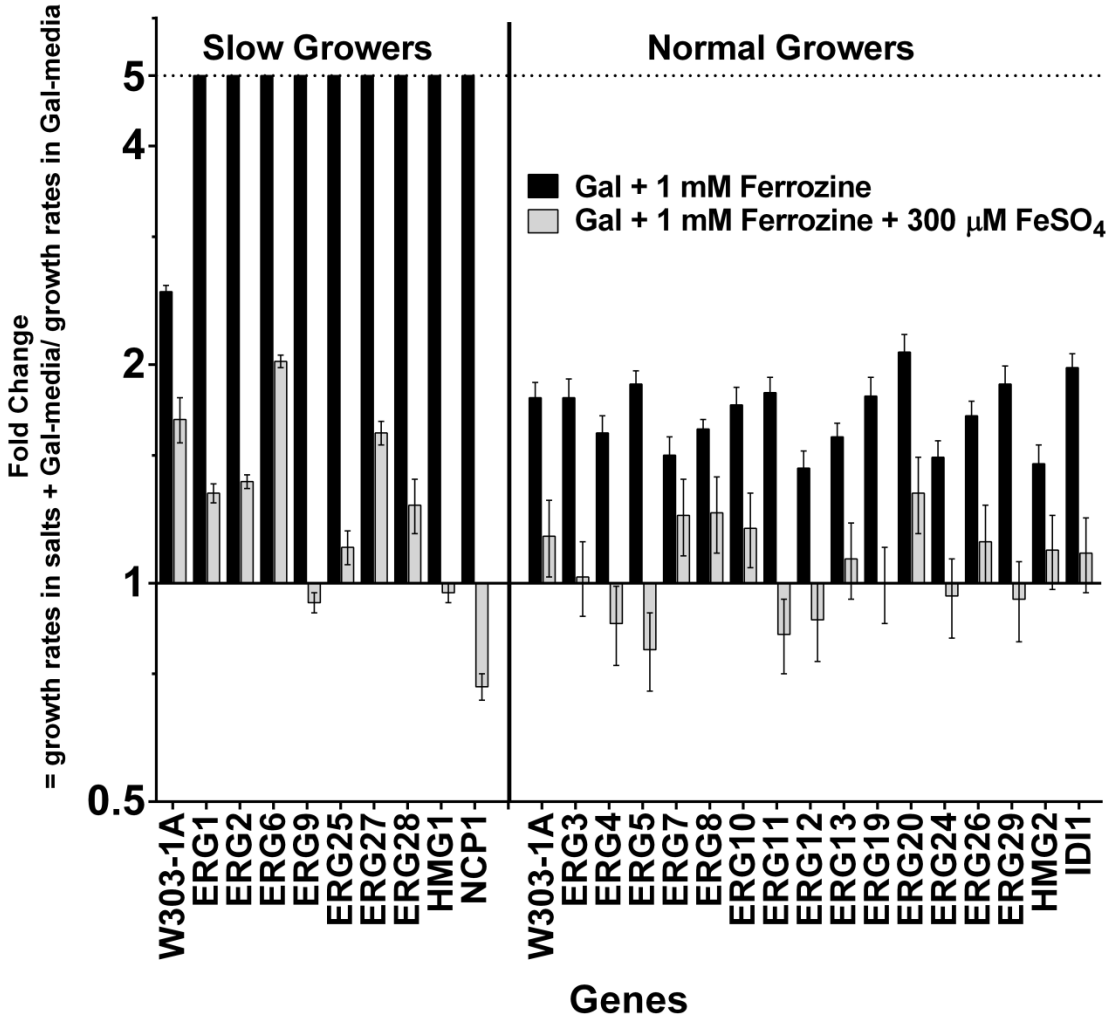


Fig. 3.3. Iron Requirement of Strains Over-Expressing *ERG* Genes.

Growth of the slow-growers (left) and normal growers (right) were tested in Gal-media alone, in 1 mM ferrozine (black-bar), and in 1 mM ferrozine plus 300 μM $FeSO_4$ (grey-bar). The cells were incubated at 30° C for 96 h with constant shaking, and readings were taken every 15 m at 600 nanometers. The growth rates were measured as described in the Materials and Methods. Fold change = the growth rate of the individual strain in salts plus Gal-media / growth rate of individual strain in Gal-media. Strains with fold change graphed at 5 (dotted line) showed no significant growth (see Materials and Methods). The experiments were done in biological triplicate and error bars represent standard errors. One-Way Anova with Holm Sidak's multiple comparison test was performed. All slow-growing cells (left) show significantly compromised growth in ferrozine alone compared to WT in ferrozine alone ($p<0.05$).

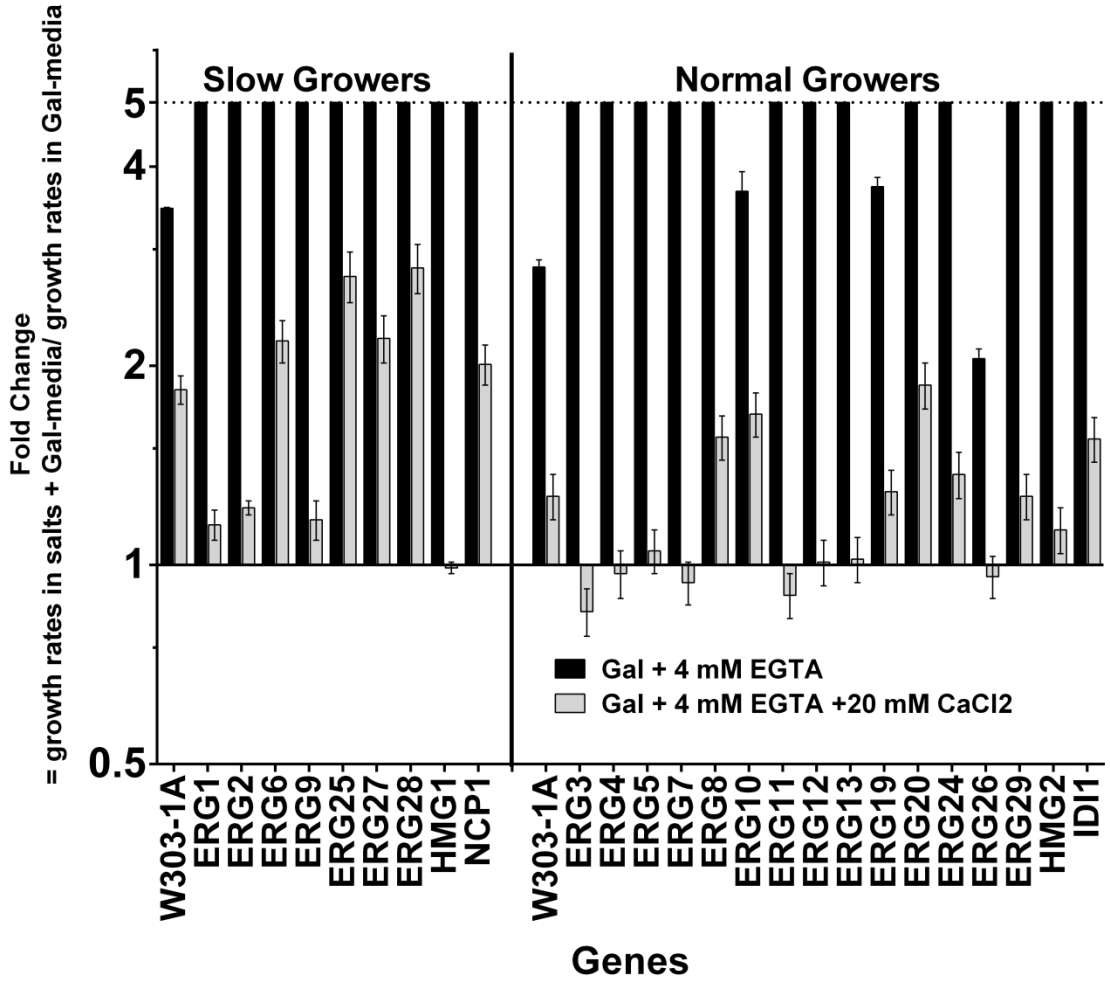


Fig. 3.4. Calcium Requirement of Strains Over-Expressing *ERG* Genes.

Growth of the slow-growers (left) and normal growers (right) were tested in Gal-media alone, in 4 mM EGTA (black-bar), and in 4 mM EGTA plus 20 mM $CaCl_2$ (grey-bar). The cells were incubated at 30^o C for 96 h with constant shaking and readings were taken every 15 m at 600 nanometers. The growth rates were calculated as described in the Materials and Methods. Fold change = the growth rate of the individual strain in salts plus Gal-media / growth rate of individual strain in Gal-media. Strains with fold change graphed at 5 (dotted line) showed no significant growth (see Materials and Methods). The experiments were done in biological triplicate and error bars represent standard errors. One-Way Anova with Holm Sidak's multiple comparison test was performed. All the red-bars excluding *ERG10*, *ERG19*, and *ERG26* show significantly decreased growth rate in presence of EGTA when compared to WT ($p<0.05$).

Tolerance to Hyperosmotic Stress

ERG gene deletion results in hyper-susceptibility to osmotic stress (23). Therefore, the tolerance of cells over-expressing *ERG* genes to osmotic stress was analyzed in Gal-media with high levels of NaCl. In eight out of the nine slow-growing strains (*HMG1*, *ERG1*, *ERG6*, *ERG9*, *ERG25*, *ERG27*, *ERG28*, and *NCP1*), the presence of NaCl further reduced the growth rates of the strains (Fig. 3.5). Interestingly, the slow-growing strain over-expressing *ERG2* is less responsive than WT to NaCl. Slower growth rates in presence of NaCl are also observed for 11 of the 16 normal growing strains (*HMG2*, *ERG4*, *ERG5*, *ERG7*, *ERG10*, *ERG11*, *ERG13*, *ERG19*, *ERG20*, *ERG24*, and *ERG26*). Normal growth in the presence of NaCl is observed in the five remaining over-expressing strains (*IDI1*, *ERG3*, *ERG8*, *ERG12*, and *ERG29*; Fig. 3.5). In Gal-media, the doubling time of WT is 7.5 h in presence of high NaCl.

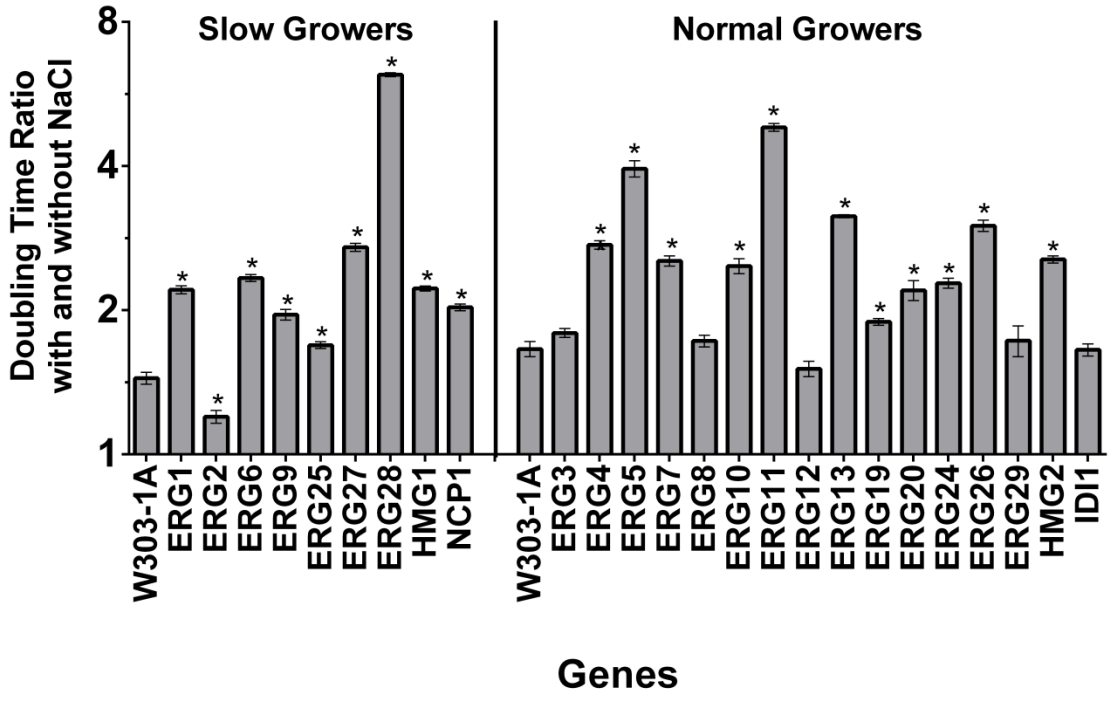


Fig. 3.5. Cellular Response to Osmotic Stress.

Growth of slow-growers (left) and normal growers (right) were tested in the absence and presence of 1.2 M NaCl in Gal-media. The cells were incubated at 30^{o} C for 96 h with constant shaking and readings were taken every 15 m at 600 nanometers. The experiments were done in biological triplicate and error bars represent standard errors. Fold change = the growth rate of the individual strain in Gal-media plus NaCl / growth rate of individual strain in Gal-media. One-Way Anova with Holm Sidak's multiple comparison test was performed. * Represents $p<0.001$ when compared to W303-1A (WT) in presence of salt.

Utilization of Non-fermentable Carbon Source

The deletion strains Δ*erg2* and Δ*erg6* are respiratory incompetent, and are unable to grow in glycerol. After inducing the *ERG* genes for 48 h in Gal-media, all the strains were spotted on agar plates containing Gly-media (Gal-Gly). Among the nine slow-growing strains, the cells over-expressing *ERG2* and *ERG6* show no significant growth (Fig. 3.6A). The remaining seven slow-growing strains and all of the normal growing strains show WT growth (Fig. 3.6B). All strains show WT growth on the Glu-Glu and Glu-Gly control agar plates (Fig. 3.6). The nine slow-growing strains show reduced growth on Gal-Glu plates compared to WT. The slow-growth phenotype in Gal-Glu, and the lack of growth in Gal-Gly for *ERG2* and *ERG6* indicate that the over-expression induced by galactose confers a phenotype that lasts up to 96 h (Fig. 3.2 and Fig. 3.6) and is passed to the daughter cells.

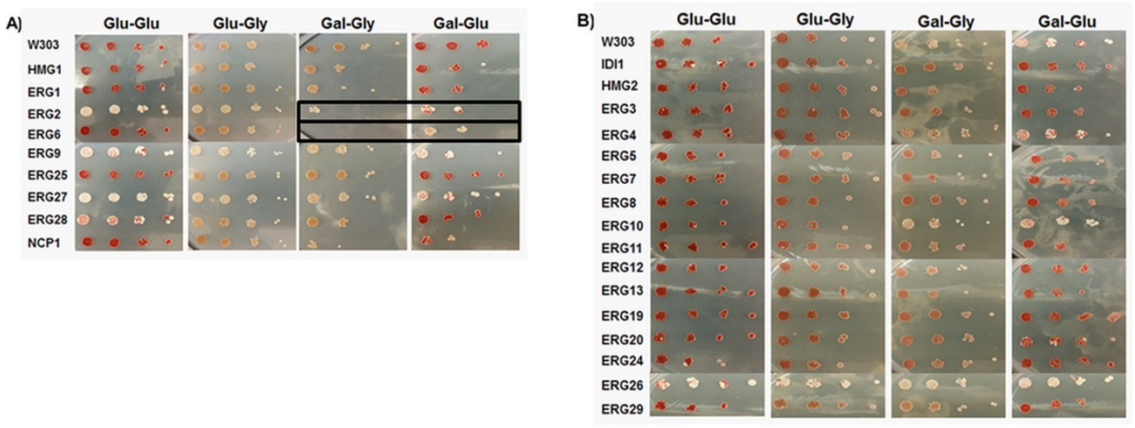


Fig. 3.6. Utilization of Non-Fermentable Carbon Source.

Slow-growers (A) and normal growers (B) were grown in Gal-media or Glu-media, and then were plated on Glu-media or Gly-media agar plates. The plates were incubated at 30^{o} C for 96 h. Glu-Glu and Glu-Gly denote cells grown in glucose media for 24 h and plated on Glu-media and Gly-media agar plates respectively. Gal-Gly and Gal-Glu denote cells grown in Gal-media for 48 h and plated on Gly-media and Glu-media agar plates respectively. The black boxes highlight the phenotypic changes.

Response to the Cell Wall Stress Agents

A defective cell wall can affect the fitness of the cells (31). CR was used to analyze the cell wall stability of all the strains. In Gal-media, five of the nine slow-growing strains (*HMG1*, *ERG1*, *ERG2*, *ERG6*, and *NCP1*), and one normal growing strain (*HMG2*) showed severe growth defects in the presence of CR (Fig. 3.7). All strains, including the WT, show similar susceptibilities to CR in Glu-media.

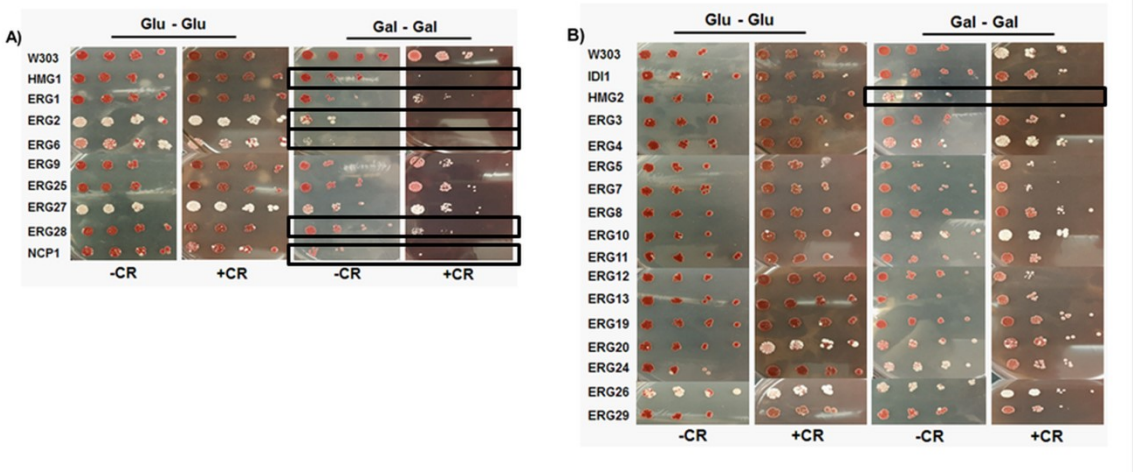


Fig. 3.7. Response to Cell Wall Stress Agents.

Slow-growers (A) and normal growers (B) were grown in Glu-media and Gal-media, and were plated in Glu-media and Gal-media agar plates in the absence (-CR) or presence of 64 µg/ml of Congo Red (+CR). The plates were incubated at 30^{o} C for 96 h. Glu-Glu signifies cells grown in Glu-media for 24 h and plated in Glu-media agar plates. Gal-Gal signifies cells grown in Gal-media for 48 h and plated in Gal-media agar plates. The black boxes highlight the phenotypic changes.

Susceptibilities to CHX and SDS

Abnormal protein synthesis and defective plasma membranes may affect the cell growth in the nine slow-growing strains. CHX targets ribosomal protein translation, while SDS can destabilize plasma membrane (32). CHX hyper-susceptibility is observed in three slow-growing strains (*ERG2*, *ERG6*, and *NCP1*) and one normal growing strain (*ERG24*). MICs to CHX for the strains over-expressing *ERG2*, *ERG6*, *NCP1*, and *ERG24* were 0.125 μg/ml, whereas the MICs to CHX for WT was 0.5 μg/ml. None of the 25 strains were affected by SDS.

3.5 Discussion

In this study, increased expression of the *ERG* genes is analyzed for its effects on cell physiology. Severe growth defects were observed in nine of the 25 over-expressing strains (Fig. 3.2), including three non-essential genes (*ERG2*, *ERG6*, and *ERG28*) and six essential genes (*HMG1*, *ERG1*, *ERG9*, *ERG25*, *ERG27*, and *NCP1*). These growth defects and other phenotypes are summarized in Fig. 3.8 for all the 25 genes. Among the nine slow-growing strains, the growth rates of the strains over-expressing the non-essential genes *ERG2* and *ERG6* are affected by six stress agents, more than any other genes. A recent study demonstrated that total sterol levels decrease in the strain over-expressing *ERG2*, whereas sterol levels increase in the strain over-expressing *ERG6* (33). These altered sterol levels may result in the growth defects observed in *ERG2* and *ERG6* over-expressing strains. *ERG6* is also involved in the production of the toxic sterol, 14α-methyl-3,6-*diol*, from lanosterol (34). Toxic sterol levels are known to increase when FLC inhibits Erg11p (alternate pathway, Fig. 3.1) (34). It is possible that over-expression of *ERG6* may also lead to the accumulation of the toxic sterol, resulting in the observed growth defects.

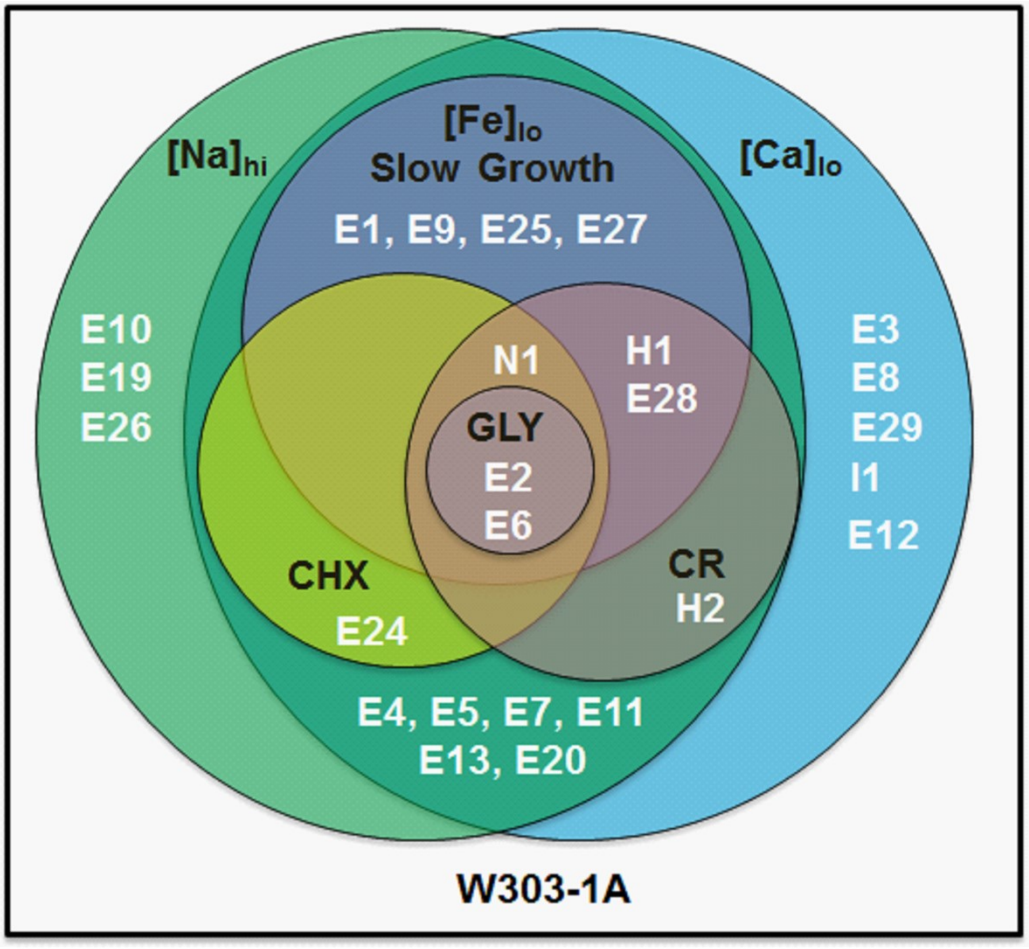


Fig. 3.8. Summary of Phenotypes.

A summary of the phenotypes in strains over-expressing *ERG* genes were compared to W303-1A (WT). Stress agents used: CR (Congo Red, Cell Wall Inhibitor), CHX (Cycloheximide, Protein Translation Inhibitor), $[Na]_{hi}$ (High Sodium Chloride, Osmotic Stress), $[Ca]_{lo}$ (EGTA, Calcium Chelator), $[Fe]_{lo}$ (Ferrozine, Iron Chelator), and GLY (Glycerol, non-fermentative Carbon source). The slow-growth phenotype of the over-expressing strain is also diagramed. All strains with slow-growth phenotype have an altered low iron phenotype. The strains are placed in circles if they demonstrate an altered phenotype from WT for that condition. E signifies an *ERG* gene, the letter I signifies the *IDI1* gene, the letter H signifies the *HMG* genes, and the letter N signifies the *NCP1* gene.

Erg2p is not involved in toxic sterol production. However, the strain over-expressing *ERG2* is the only strain with increased tolerance to osmotic stress (Fig. 3.5), associated with reduced sterol levels (33). Over-expression of *ERG2* may alter the sterol composition of the plasma membrane, making the membrane more resistant to osmotic stress. A previous study demonstrated that strains with non-functional mutations in *ERG2* and *ERG6* were slow-growing and respiratory incompetent (22). The effects of over-expression of *ERG2* and *ERG6* on six different stress agents emphasize the importance of these genes in the ergosterol pathway and may be appropriate targets for future drug development.

The next most significant strain is the strain over-expressing *NCP1*, whose growth is compromised by five of the seven stress agents (EGTA, ferrozine, high salt, CHX, and CR; Fig. 3.8). The phenotypes associated with *NCP1* over-expression may result from potential regulation of the ergosterol pathway, potential regulation of other p450 genes such as *ERG5* or *DIT2*, or pleotropic effects of *NCP1* throughout the cell. While the *ERG11* co-factor *NCP1* over-expression has five phenotypes, the strain over-expressing Erg11p, a rate-limiting step in ergosterol biosynthesis (Fig. 3.1), is only affected by two stress agents (EGTA, and high salt) and shows WT growth.

The strain over-expressing Hmg1p, the first rate-limiting step in the ergosterol pathway (6) (Fig. 3.1), is affected by four stress agents (EGTA, ferrozine, high salt, and CR; Fig. 3.8) and shows severe growth defects when induced in Gal-media. The effects of over-expression of *HMG1* may increase the production of the toxic sterol, or may alter the distribution of FPP into its many downstream pathways, any of which could lead to the growth defects observed (Fig. 3.1).

In *S. cerevisiae*, Hmg1p and Hmg2p are homologs. Unlike the *HMG1* over-expressing strain, the strain over-expressing Hmg2p is affected by only three stress agents (EGTA, high salt, and CR; Fig. 3.8). Though homologs, Hmg1p has longer half-life than Hmg2p (35), These results suggest that over-expression of *HMG1* or *HMG2* is sufficient to produce hyper-susceptibility to CR, osmotic stress, and calcium starvation. However, Hmg2p over-expression is not enough to result in a slow-growth phenotype or hyper-susceptibility to ferrozine.

Erg25p, Erg26p, Erg27p, and Erg28p act as an enzyme complex downstream of Erg11p, which is responsible for ergosterol biosynthesis, as well as the biosynthesis of the toxic sterol (34). In the complex, the strain over-expressing *ERG28* is affected by four stress agents (EGTA, ferrozine, high salt, and CR; Fig. 3.8) and shows severe growth defects when induced in Gal-media. Similarly, strains over-expressing Erg25p and Erg27p are affected by three stress agents (EGTA, Ferrozine, and high salt; Fig. 3.8) and show severe growth defects under inducing conditions in Gal-media. The strain over-expressing Erg26p is affected only by one stress agent (high salt; Fig. 3.8) and shows WT growth in Gal-media (Fig. 3.8). This suggests that over-expression of Erg25p, Erg27p, and Erg28p has a more significant effect on the biosynthesis of ergosterol and/or the toxic sterol than Erg26p. Alternatively, over-expression of either *ERG25*, *ERG27*, or *ERG28*, may lead to the over-expression of the other enzymes in the complex resulting in the higher level of the complete complex and possibly the toxic sterol. Erg25p, Erg27p, and Erg28p may form the core structure of the enzyme complex and altering the expression of these enzymes may affect the complex resulting in the observed phenotypes.

The remaining two slow-growing strains that over-express *ERG9* and *ERG1* are affected by three stress agents (EGTA, ferrozine, and high salt; Fig. 3.8). The late ergosterol pathway begins with Erg9p, an enzyme one step before the rate-limiting enzyme Erg1p. Over-expression of Erg9p and Erg1p may direct FPP towards ergosterol biosynthesis and away from its other cellular roles. Alternately, *ERG9* and *ERG1* over-expression may increase the flow of substrates towards the side pathway synthesizing the toxic sterol from lanosterol resulting in the observed growth defects.

Of the 16 normal growing strains, the growth rate of the strain over-expressing *ERG24* is affected by three stress agents (EGTA, high salt, and CHX; Fig. 3.8). Erg24p is located one-step below Erg11p, and one step above the complex, in the ergosterol pathway (Fig. 3.1). A previous study with Δerg24 strain showed the necessity of calcium for its viability (30). This strain accumulates an aberrant sterol, ignosterol that leads to severe growth defects (30). Over-expression of Erg24p may also accumulate aberrant sterol intermediates, resulting in the observed growth defects in presence of three stress agents.

The majority of stress agents have fewer effects on the normal growing strains compared to the nine slow-growing strains. Only one or two stress agents have significant effects on the growth of the 15 remaining over-expressing strains (Fig. 3.8). Over-expression of these genes has less effects on ergosterol pathway regulation.
Among the stress agents, EGTA and high salt affected the majority of the over-expressing strains. In Gal-media plus EGTA, no significant growth is observed in 22 of 25 strains (Fig. 3.8). Many calcium transporters are located on the cell membrane. The presence of altered sterol intermediates in the plasma membrane may affect the functions

of these transporters by altering the fluidity of the membrane. The majority of the over-expressing strains may have defective import or export of calcium, which may be lethal to cells. Similarly, 20 out of the 25 strains show susceptibility to osmotic stress possibly due to an abnormal sterol composition in the membrane (Fig. 3.8).

The slow-growth rate phenotypes were analyzed in two different laboratory strains of *S. cerevisiae* (BY4741 and W303-1A) with distinctive genotypes. BY4741 has a transposon insertion in the *HAP1* gene and is not capable of growing anaerobically (36). Several phenotypic differences were observed between the two strains (37). For example, BY4741 and W303-1A differ in cell size, plasma-membrane potential, and hygromycin susceptibility (37). Each of the nine slow-growing strains has a decreased growth rate compared to the WT when expressed in both backgrounds (Fig. 3.2A and B).

This study contributes significantly to our understanding of the ergosterol biosynthetic pathway. Several cellular processes including respiration, osmotic stress, cell wall and membrane stability, and cation metabolism are affected in the strains over-expressing the *ERG* genes. Over-expression of nine specific *ERG* genes leads to growth arrest and altered susceptibility to at least three stress agents. Three of these nine (*HMG1*, *ERG1*, and *ERG11*) are the target enzymes for existing antifungal drugs. The remaining six genes (*ERG9, ERG25*, *ERG27, ERG28*, *ERG2*, and *ERG6*) may represent important future drug targets.

3.6 Materials and Methods

Yeast strains, Growth Conditions, and Reagents

The *S. cerevisiae* strain W303-1A (*MAT*a, *leu2-3,112 trp1-1 can1-100 ura3-1 ade2-1 his3-11,15*) was used for most of the experiments. Complementation experiments were done using deletion mutants Δ*hmg1*, Δ*hmg2*, Δ*erg2*, Δ*erg3*, Δ*erg4*, Δ*erg5*, Δ*erg6*, Δ*erg24*, and Δ*erg28* of the *S. cerevisiae* strain BY4741 (*MAT*a *leu2v0 his3v1 ura3v0 met15v0*) (6). Plasmids carrying the *ERG* genes were transformed into W303-1A and selected at 30° C in Glu-media [CSM-ura (1.7 g of yeast nitrogen base without ammonium sulfate without amino acids, 5 g/l of ammonium sulfate, 0.8 g/l of CSM-ura powder) plus 2% glucose)]. Gene expression was induced using Gal-media (CSM-ura plus 2% galactose). Gly-media (CSM-ura plus 3% glycerol) was used as a media containing a non-fermentative carbon source in one experiment. Restriction enzymes used for constructing plasmids were obtained from Promega. Stress agents CR, CHX, ferrozine, EGTA, ferrous sulfate ($FeSO_4$), fluconazole (FLC), fenpropimorph (FEN), lovastatin (LOV), nystatin (NYS) and calcium chloride ($CaCl_2$) were obtained from Sigma Aldrich. Unless indicated, all materials and plastic-wares are from Fisher Scientific.

Plasmids Constructions

Genomic DNA from W303-1A strain was isolated as described before (38). Each *ERG* gene was PCR amplified from the genomic DNA using the oligonucleotides listed in the supplementary Table S1. PCR conditions used were 30 s at 98° C followed by 30 cycles of 10 s at 98° C, 30 s at 60° C, 5 m at 72° C and a final extension of 10 m at 72° C. The amplified products were verified on 0.8% agarose gel. The forward oligonucleotides

have a 30 bp homology to the galactose regulated *GAL1* promoter (*GAL1*-p), while the reverse oligonucleotides have a 30 bp homology to the *CYC1* terminator (*CYC1*-t). Both *GAL1*-p and *CYC1*-t are present in the pYES2 plasmid, which is used for cloning of the *ERG* genes. pYES2 plasmid was a generous gift from Idnurm Lab. The plasmid was digested with PvuII, and each gene was inserted between *GAL1*-p and CYC1-t via homologous recombination in yeast (Fig. S3.1). The yeast cells were transformed using the lithium-acetate method (39) and were plated on Glu-media. The plates were incubated for two days at 30° C. Three random colonies from each transformant were streaked in fresh Glu-media plates and the plasmids were isolated using Thermo-scientific Plasmid DNA extraction kit following manufacturer's protocol. The extracted plasmids were transformed into *E.coli* (TOP TEN competent cells, Sigma Aldrich) per manufacturer's instructions (40). These transformants were plated on Luria-Bertani agar containing 100 µg/ml of ampicillin. The plasmids were then isolated from *E. coli* and sequenced. The sequencing analysis confirmed that the genes' sequence and integration are correct. These plasmids were re-transformed into fresh W303-1A cells and plated on Glu-media. The plates were incubated for 48 h at 30° C and three random colonies of each transformant were selected. These colonies were streaked in fresh Glu-media and used for this study. All the transformants are stored as 30% glycerol stocks at -80° C.

Calculation of DoublingTime

Doubling times were analyzed for the individual strains expressing each of the 25 *ERG* genes in Glu-media and Gal-media. First, the growth curves were generated from the cells growing in both media. In the Glu-media and the Gal-media, a single colony of cells from each transformant was grown at 30° C with constant shaking of 180 rpm for 24

h and 48 h respectively. After incubation, 0.1 OD of cells were plated in 96-well plate (Costar 3699, Fisher Scientific) containing either Glu-media or Gal-media and incubated at 30° C with constant shaking for 96 h in Biotek Take 3 plate reader (Biotek. Inc, USA). ODs were measured every 15 m at 600 nanometers in the plate reader. Overnights were grown in Gal-media for 48 h, instead of 24 h, because the doubling time of *S. cerevisiae* WT (W303-1A) in Glu-media is 3.0 h and in Gal-media is 4.5 h. In addition, the cells over-expressing nine out of the 25 *ERG* genes grew more slowly than WT in Gal-media (Fig. 3.2).

Similar growth curves were performed in Glu-media or Gal-media containing 1 mM ferrozine, 1 mM ferrozine + 300 μM $FeSO_4$, 4 mM EGTA, 4 mM EGTA + 20 mM $CaCl_2$, and 1.2 M NaCl.

Doubling times were calculated using Graphpad prism 6.0. First, the midpoint of the exponential phase (the inflection point) of the growth was analyzed using the double derivative function. Second, the range to generate the doubling time was decided as 5 h before and 5 h after the inflection point for each growth curve. All experiments were done in biological triplicates. Errors in the doubling times are the standard errors of the biological replicates. Strains that did not undergo two divisions in 96 h (OD < 0.4 were considered to have no significant growth, and their doubling time was set at five-fold above the normalizing strain in iron, and calcium utilization experiments.

Measurements of Transcript Levels

Quantitative-reverse transcriptase PCR (qRT-PCR) was used in analyzing the expression of the ergosterol genes in the presence of galactose. A single colony of each of the three transformants was inoculated in Gal-media and incubated at 30° C with 180 rpm

of shaking. Total RNA was extracted from exponentially growing cells in Gal-media by using Qiagen RNeasy kit following manufacturer's guidelines. The concentration and purity of the extracted RNAs were analysed using a Take3 Biotek plate reader (Biotek Instruments. Inc USA). An absorbance ratio (A260/A280) ≥ 2.0 was considered to be pure RNA. Further, the RNA samples were analyzed on a 1.2% agarose gel and the appearance of two distinct ribosomal RNA bands signified RNA integrity.
1 µg of total RNA was treated with DNAseI (Thermo-scientific) following manufacturer's protocol. cDNA was prepared from DNAsed RNA using an Invitrogen cDNA kit with Superscript III Reverse Transcriptase following the manufacturer's instructions.

qRT-PCR was performed in Applied Biosystem 7500 Real-Time PCR System using the oligonucleotides listed in supplementary Table S1. These oligonucleotides were designed using Primer3 software, and all amplicon sizes are in the range of 150-250bp. qRT-PCR was done using Maxima SYBR Green qPCR master mixes (Thermo-scientific) following the manufacturer's protocol. qRT-analysis was performed in triplicate for each biological transformant. The housekeeping genes actin (*ACT1*) and transcription elongation factor (*TEF3*) were used as internal controls. The data were normalized to the wild type W303-1A gene expression. The fold change in the expression was calculated using $2^{-\Delta\Delta ct}$. First, the Ct values were compared to the Ct values of *ACT1*. Second, the normalized values were compared to the WT W303-1A to calculate fold change. Error bars were calculated as previously described (41). A fold change greater than 2.0 was considered significant.

Susceptibility Testing

The strains over-expressing the *ERG* genes were tested for susceptibilities to FLC, FEN, LOV, NYS, CHX, and SDS by using CLSI approved microbroth microdilution protocol that determines minimum inhibitory concentrations (MICs). The MICs are defined as the drug concentration that inhibits 80% cell growth (42). A single colony from each strain was grown at 30° C with constant shaking for 24 h in the Glu-media and for 48 h in the Gal-media. After the growth of the cells in the respective media, 0.1 OD of cells were used as inoculum for performing the MIC analysis. Cells were grown in 96 well plates containing a gradient of drug in two-fold serial dilution in the Glu-media or the Gal-media. Both glucose and galactose plates were incubated at 30° C with shaking for 48 h and 96 h respectively. A positive growth control lacking drug, and a negative growth control lacking cells were included. Cell growth in the wells containing drug was measured by the BioTek plate reader and standardized to the positive growth control. MICs were performed in biological triplicate and the average values are reported.

Spot Assay

A single colony from each strain was inoculated for 24 h in Glu-media and for 48 h in Gal-media at 30° C with shaking. These cells were used as inocula for both GLY and CR experiments.

Cells growing on Glu-media were spotted in a series of six 10-fold dilutions starting at 0.1 OD on either Glu-media (Glu-Glu) or on Gly-media (Glu-Gly) agar plates. Similarly, cells growing on Gal-media were spotted in a series of six 10-fold dilutions starting at 0.1 OD on either Glu-media (Gal-Glu) or in Gly-media (Gal-Gly) agar plates.

Glu-Glu, Glu-Gly and Gal-Glu are important control plates used in this experiment. All plates were incubated for 96 h in 30° C and then imaged with a colored digital camera.

Cells growing in Glu-media or Gal-media were spotted in a series of six 10-fold dilutions starting at 0.1 OD on agar plates containing Glu-media or Gal-media, with or without CR. A stock solution of 10 mg/ml of CR was prepared in sterile water and added to a final concentration of 64 μg/ml. All plates were incubated for 96 h in 30° C and then imaged with a colored digital camera.

Statistical Analysis

All data were analyzed and graphed with Graph-pad prism 6.0. All statistics performed are described in the figure legends.

3.7 Acknowledgement

We thank Alexander Idnurm (University of Melbourne, Australia) for kindly donating pYES2 plasmid, and our colleagues in White Lab for providing helpful and critical input on this manuscript.

3.8 Funding Information

This research was supported by unrestricted research funds from the School of Biological Sciences, University of Missouri- Kansas City to Theodore C. White.

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CHAPTER 4

ALTERED ANTIFUNGAL DRUG SUSCEPTIBILITIES IN *SACCHAROMYCES CEREVISIAE* STRAINS OVEREXPRESSING ERGOSTEROL BIOSYNTHESIS GENES

4.1 Abstract

Ergosterol is an important sterol component of cellular membranes in pathogenic fungi. It is synthesized by 25 biosynthetic enzymes, many of which are antifungal drug targets. The transcription factor Upc2p regulates most of the ergosterol pathway genes. This study analyzes the effects of ergosterol biosynthesis inhibitors in *Saccharomyces cerevisiae* strains overexpressing ergosterol pathway genes. Overexpression of many of the 25 ergosterol pathway genes causes altered susceptibilities to ergosterol inhibitors. In addition, nine genes in the pathway are non-essential, and can be deleted. Altered susceptibilities are observed in the deletion strains, and in the deletion strains in which the genes were overexpressed. Ergosterol is targeted by the polyenes nystatin and amphotericin B. Yet these polyenes behave differently for one overexpression and four deletion strains, suggesting subtle differences in their mode of action. Among all the inhibitors used in this study, the morpholine fenpropimorph effects the most number of strains (four over-expression strains, and three deletion strains), and is therefore the most important inhibitor of the ergosterol pathway. Furthermore, deletion of *UPC2* results in morpholine hyper-susceptibility in both *S. cerevisiae* and *Candida albicans*. In addition, deletion of membrane transporters in *S. cerevisiae* results in morpholine hyper-susceptibility. Thus this study contributes to our knowledge of ergosterol biosynthesis, and may in future help in identifying potential drug targets.

4.2 Introduction

Ergosterol is a major sterol component of the fungal plasma and mitochondrial membranes. It is analogous to cholesterol in mammalian cell membranes. It maintains fluidity, structure, and function of the membrane, forming micro-domains (lipid rafts) in which membrane transporters, and receptors, which are important for different cellular processes are concentrated (1). In the fungus *Saccharomyces cerevisiae*, altered ergosterol biosynthesis correlates with decreases in tolerance to osmotic stress, cell wall biosynthesis, and iron and calcium homeostasis (Chapter 3). Ergosterol biosynthesis occurs in the endoplasmic reticulum (ER) in a cascade of 25 enzymatic reactions. These enzymes are regulated by the zinc-cysteine finger transcription factor paralogs Upc2p/Ecm22p in *S. cerevisiae*, and Upc2p in the pathogenic fungus *Candida albicans* (2). In *Aspergillus fumigatus* , the ergosterol pathway (erg pathway) is regulated by SrbA, a homolog of human SREBP (sterol regulatory element binding protein) (3). Hmg1p, Erg1p, and Erg11p catalyze the three rate-limiting steps in the erg pathway (4). Each of these rate-limiting enzymes is targeted by different drugs, including the statins, which target Hmg1p, the antifungal allylamines, which target Erg1p, and the antifungal azoles, which target Erg11p (5).

Fluconazole (FLC) is the most common azole used in treating *C. albicans* oral, systemic, and vaginal infections, (6, 7), and in treating *Cryptococcus neoformans* pulmonary and cerebral infections (8). However, FLC is not used in treating systemic and pulmonary *Aspergillus* infections, as *Aspergillus* is intrinsically resistant to FLC (9). Instead, other azoles including itraconazole, voriconazole, and posaconazole are used in treating *Aspergillosis* (9).

The antifungal polyenes Amphotericin B (AMB) and Nystatin (NYS) target ergosterol in the fungal membrane, and are used in treating fungal infections. Both AMB and NYS have side-effects that include hepatotoxicity and renal failure (Chapter 1). The antifungal morpholines, including Fenpropimorph (FEN), Tridemorph (TRI), and Amorolfine (AMO) target Erg24p in the erg pathway (10). These drugs are commonly used in agriculture, although AMO can be used in treating fungal skin infections (11). The antifungal allylamines, including terbinafine (TRB) target Erg1p, and are effective against dermatophyte infections (Chapter 1). Lovastatin (LOV) is a statin that targets Hmg1p in both mammals and fungi Statins like LOV are commonly used in humans to lower cholesterol levels (Chapter 1).

Infections caused by azole resistant fungi are increasing for several reasons including, the fungistatic nature of azoles, the use of high drug doses, nosocomial transmission, and the agricultural use of azoles (Chapter 1). In pathogenic fungi, the molecular mechanisms of azole resistance are well characterized that include, overexpression of membrane transporters and altered ergosterol biosynthesis (Chapter 1). In azole resistant fungi, membrane transporters including ABC-transporters (ABC-T) and major facilitator transporter (MFS-T) show increased expression and efflux activities. For example, azole resistant *C. albicans* clinical isolates show increased expression and activities of Cdr1p, Cdr2p (ABC-T), and Mdr1p (MFS-T) (Chapter 2). Additionally, altered ergosterol biosynthesis can correlate with azole resistance. For example, in azole resistant *C. albicans* clinical isolates, overexpression and point mutations in *ERG11* are commonly observed (12, 13). *In vitro* in *S. cerevisiae*, overexpression of *ERG11* under a galactose inducible promoter caused increased FLC resistance (14).

S. cerevisiae is used as a model organism for studying yeast genetics. It is haploid, has high transformation efficiency, and has several molecular resources. A large scale survey analysis was carried out to determine the effects of several antifungal drugs on the deletion library of *S. cerevisiae*. Specific deletion strains are resistant to the medically important antifungals. For example, Δ*erg3* and Δ*erg6* are resistant to FLC and the polyenes AMB and NYS (1).

In a previous study all 25 erg pathway genes (*ERG* genes) were overexpressed in a wild-type background (Chapter 3). Additionally, the seven non-essential *ERG* genes were overexpressed in their respective deletion background. The previous study analyzed these constructs for altered phenotypes relating to cell wall biosynthesis, osmotic stress tolerance, protein biosynthesis, respiration, iron and calcium homeostasis. The present study extends these observations by examining these constructs for their phenotypes relating to the known erg pathway inhibitors LOV, FEN, FLC, TRB, AMB, and NYS. The study also focuses on the effects of morpholines on FLC resistant *Candida albicans* strains. The mechanisms of FLC resistance in these strains are because of increased expression and activities of efflux pumps. Thus this study will characterize erg pathway regulation and may identify novel mechanisms of antifungal resistance.

4.3 Materials and Methods

Strains and Growth Conditions

ERG genes were over-expressed from a galactose inducible promoter in plasmids as described previously (Chapter 3). These plasmids were maintained in *S. cerevisiae* strain W303-1A (*MAT*a, *leu2-3,112 trp1-1 can1-100 ura3-1 ade2-1 his3-11,15*). *ERG* genes mutants Δ*hmg1*, Δ*hmg2*, Δ*erg2*, Δ*erg3*, Δ*erg4*, Δ*erg5*, Δ*erg6*, Δ*erg24* and Δ*erg28* were obtained from the deletion library of *S. cerevisiae* in the background strain BY4741 (*MAT*a *leu2v0 his3v1 ura3v0 met15v0*) (4). All strains were grown in CSM-ura (1.7 g of yeast nitrogen base 5 g/l of ammonium sulfate and 0.8 g/l of CSM-ura powder, Fisher Scientific) + 2% glucose (Glu-media). Expression of *ERG* genes were induced in CSM-ura + 2% galactose (Gal-media). The cells in both Glu-media and Gal-media were incubated at 30^{o} C with constant shaking at 180 rpm. Both glucose and galactose were obtained from Fisher Scientific. All strains are stored in Glu-media + 30% glycerol at -80^{o} C for future reference.

Susceptibility Testing

Susceptibilities to drugs LOV, FLC, TRB, FEN, AMB, AMO, TRI, and NYS were performed using the CLSI approved microbroth microdilution protocol that determines minimum inhibitory concentration (MIC) (15), the concentration of drug that inhibits 80% of cell growth. MICs were performed in both Glu-media and Gal-media. First, a single colony of each strain was grown in Glu-media (for 24 h) and Gal-media (for 48 h) at 30^{o} C with constant shaking at 180 rpm. Strains growing in Gal-media have slower growth rates than strains grown in Glu-media, requiring longer incubation times. After the respective incubation times, 0.1 OD from each culture were used as inoculum for

MIC determination. MICs were performed in 96 well plates (Corning Costar 3699) containing two-fold serial dilutions of respective drugs in both Glu-media and Gal-media. Glu-media plates and Gal-media plates were incubated respectively for 48 h and 96 h at 30^{o} C with constant shaking at 180 rpm. A positive growth control lacking drug, and a negative growth control lacking cells were used. All MICs were done in biological triplicates and the average values of the triplicates are listed in Tables 1,2, 3, and 4. All drugs were obtained from Sigma Aldrich, St louis, MO.

Candida albicans FLC resistant matched oral clinical isolates, and *S. cerevisiae* ADΔ strains (Chapter 2) were grown in CSM complete media (1.7g of yeast nitrogen base 5g/l of ammonium sulfate and 0.8g/l of CSM powder, Fisher Scientific) + 2% glucose.

4.4 Results and Discussions

Overexpression of the *ERG* genes was analyzed in the wild-type strain W303-1A (Table 4.1), and nine strains with deletions of non-essential *ERG* genes in a wild-type strain BY4741 (Table 4.2). The MICs were analyzed in all strains in both Glu-media (data not shown) and Gal-media. An MIC difference of at least four-fold from wild-type (WT) was considered significant. Initially, the overexpression strains were analyzed in Gal-media, for altered susceptibilities to LOV, TRB, FLC, FEN, AMB and NYS when compared to the wild-type (Table 4.1).

Table 4.1: MICs of the Overexpression Strains (µg/ml) in Gal-media

Strains	LOV	TRB	FLU	FEN	NYS	AMB
WT	32	32	16	8	2	2
ERG10	16	32	16	16	2	2
ERG13	16	16	32	8	2	2
HMG1	16	32	4	1	2	1
HMG2	16	64	8	4	2	2
ERG12	16	16	16	8	2	2
ERG8	32	32	16	8	2	2
ERG19	64	32	16	4	2	2
IDI1	32	32	16	8	2	2
ERG20	32	32	16	16	2	2
ERG9	8	8	8	8	2	2
ERG1	32	8	8	2	2	1
ERG7	32	32	8	8	2	1
ERG11	32	32	64	8	2	2
NCP1	16	16	16	0.5	2	2
ERG24	32	32	16	>64	2	1
ERG25	16	32	8	8	2	1
ERG26	32	8	16	8	2	0.5
ERG27	16	16	8	8	2	1
ERG28	16	32	8	4	2	1

ERG29	32	32	16	8	2	2
ERG6	32	16	16	8	0.5	0.01
ERG2	16	32	8	4	2	2
ERG3	32	16	8	4	2	1
ERG5	32	32	8	4	2	2
ERG4	16	16	16	4	2	2

Red box denotes significant resistance
Blue box denotes significant hyper-susceptibility

Table 4.2 : MICs of the Deletion and the Overexpression Strains (µg/ml) in Gal-media

Strains	LOV	TRB	FLC	FEN	NYS	AMB
WT	128	32	8	16	2	4
Δhmg1	32	32	32	32	4	4
Δhmg1+gHMG1	128	32	8	8	2	4
Δhmg2	128	32	16	16	4	4
Δhmg2+gHMG2	128	32	2	8	4	4
Δerg2	128	16	2	16	2	4
Δerg2+gERG2	128	8	8	8	2	4
Δerg3	128	32	64	2	8	8
Δerg3+gERG3	128	16	8	16	4	4
Δerg4	128	16	4	>64	8	4
Δerg4+gERG4	128	32	8	16	2	4
Δerg5	128	32	4	8	16	4
Δerg5+gERG5	128	32	8	8	1	4
Δerg6	32	16	32	0.06	>16	8
Δerg6+gERG6	128	16	4	16	0.5	1
Δerg24	128	64	8	16	2	4
Δerg24+gERG24	128	32	8	>64	2	4
Δerg28	128	32	8	8	2	4
Δerg28+gERG28	128	32	8	8	2	4

Red box denotes significant resistance
Blue box denotes significant hyper-susceptibility

Hyper-susceptibility to LOV, a cholesterol lowering drug, is only observed in the strain overexpressing *ERG9* (Table 4.1). Overexpression of *ERG9* may transcriptionally regulate the LOV targets Hmg1p/Hmg2p or may increase the flow of sterols into the late sterol pathway, and away from biosynthesis of heme, dolichol, and ubiquinone, as well as protein prenylation (Fig. 1.1). Interestingly, LOV MICs are not altered by overexpression of *HMG1* or *HMG2*. Hmg1p and Hmg2p are isoenzymes, and the overexpressing one may be counter-balanced by downregulation of the other enzyme.

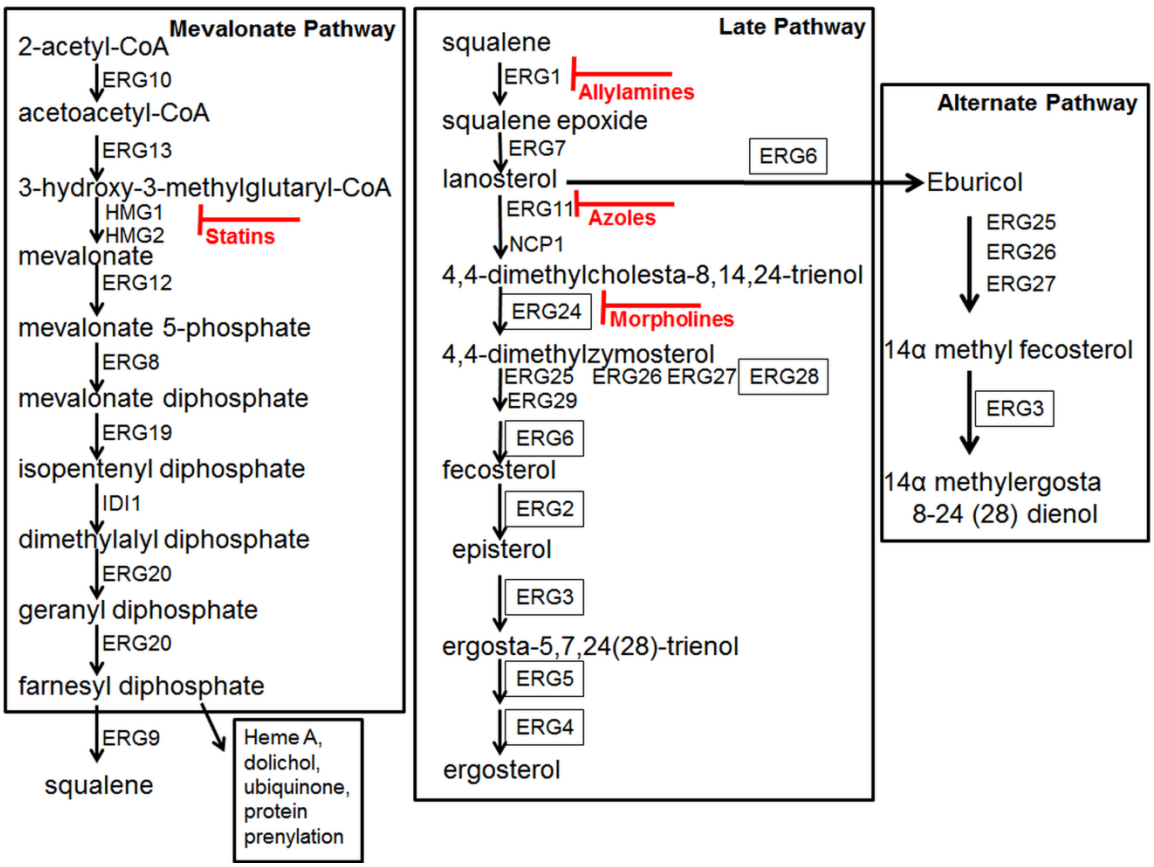

Fig. 4.1.Ergosterol Biosynthetic Pathway.
The box on the left diagrams signifies the mevalonate pathway, which can channel into different biosynthetic pathways. The box in the middle is the late ergosterol pathway terminating in ergosterol. The box on the right is an alternate pathway leading to the toxic sterol (14α methylergosta 8-24-(28) dienol). Boxed gene names denote non-essential genes.

The antifungal TRB inhibits Erg1p, yet the strain overexpressing *ERG1* is hyper-susceptible to TRB. The overexpression of *ERG1* causes a slow growth rate possibly because of the production of the toxic diol (Chapter 3), which may also cause TRB hyper-susceptibility, which is also observed in strains overexpressing *ERG9* and *ERG27* that are also involved in the production of the diol (Fig 1, Table 4.1).

Hyper-susceptibility to the azole FLC is observed in the strain overexpressing *HMG1* in (Table 4.1). This over-expression of *HMG1* may shift the flow of sterol intermediates towards the alternate pathway, which synthesizes the toxic diol (Fig. 4.1). As expected, the strain overexpressing *ERG11* is resistant to FLC (Table 4.1).

Resistance to the agricultural antifungal FEN is observed in the strain overexpressing the target enzyme Erg24p (Table 4.1) Additionally, strains overexpressing *HMG1*, *ERG1*, and *NCP1*, are hyper-susceptible to FEN (Table 4.1). These hyper-susceptible strains are slow growing (Chapter 3), which may affect their MICs to FEN. Overexpression of *HMG1* and *ERG1* may also cause a flow of sterol intermediates into the alternate pathway (Fig. 4.1).

The medically important polyenes AMB and NYS target ergosterol in the membrane. The strain overexpressing *ERG6*, is hyper-susceptible to both polyenes (Table 4.1). This strain is slow growing and respiratory incompetent. The strain also shows aberrant osmotic stress tolerance, cell wall biosynthesis, protein translation, and iron and calcium homeostasis (Chapter 3). Additionally, Erg6p is one step in the biosynthesis of the toxic diol (Fig. 4.1). All of these factors may contribute to the polyene hyper-susceptibility in this *ERG6* overexpressing strain. The strain overexpressing *ERG26*, upstream of *ERG6*,

was hyper-susceptible to AMB but not hyper-susceptible to NYS (Table 4.1). While AMB and NYS both target ergosterol in the membrane, their mode of action may be slightly different.

Overexpression of nine non-essential genes was also analyzed in their respective deletions (Table 4.2) in a different wild-type background (BY4741). The strains were analyzed for their drug susceptibility patterns. As expected, the Δ*hmg1* strain is hyper-susceptible to LOV, and over-expression of the gene complemented the phenotype (Table 4.2). However, the Δ*hmg2* strain has wild-type LOV MIC. A previous study demonstrated that Hmg1p is the predominant isoenzyme, responsible for 84% of the enzyme activity (16) Thus, the effects of LOV on Δ*hmg2* may be masked by *HMG1*. LOV hyper-susceptibility is also observed in the Δ*erg6* strain, which grows slowly, affecting LOV MICs. Again overexpression of *ERG6* restores normal MIC (Table 4.2).

No deletion strains are affected by TRB (Table 4.2). However, overexpression of *ERG2* in the Δ*erg2* strain is hyper-susceptible to TRB (Table 4.2), while overexpression of *ERG2* in the wild-type strain (W303-1A) did not change the MICs (Table 4.1). BY4741 is derived from the laboratory strain S288C that has a Ty1 insertion in *HAP1* gene (17). Hap1p is a transcription factor that regulates the expression of several *ERG* genes including *HMG1*, *HMG2*, and *ERG2* under aerobic and anaerobic conditions (18, 19). Therefore, it is possible that the *HAP1* disruption contributes to the TRB hyper-susceptibility of the Δ*erg2* strain overexpressing *ERG2.*

FLC resistance is observed in Δ*erg3* and Δ*erg6* (Table 4.2). Δ*erg3* and Δ*erg6* strains cannot synthesize the toxic diol (Fig. 4.1), and hence are FLC resistant. Overexpression of *ERG3* and *ERG6* in their respective deletion strains, restore normal FLC MICs.

Additionally, the Δ*hmg1* strain is resistant to FLC. The Δ*hmg1* strain may reduce the flow of sterols through the ergosterol pathway, synthesizing less toxic diol. Overexpressing *HMG1* in the Δ*hmg1* strain restores FLC MICs to wild-type levels (Table 4.2). The strain overexpressing *HMG2* in Δ*hmg2* is hyper-susceptible to FLC (Table 4.2), while the strain over-expressing *HMG2* in W303-1A had normal MICs to FLC (Table 4.1). Similar to the TRB hyper-susceptibility of *ERG2*, disruption of *HAP1* in BY4741 may cause the observed the FLC hyper-susceptibility. The Δ*erg2* strain is hyper-susceptible to FLC. This may result from the slow growth rate observed in Δ*erg2* strain.

FEN targets Erg24p, and overexpression of *ERG24* in Δ*erg24* strain is resistant to FEN (Table 4.2). This is consistent with the observed phenotype of the strain overexpressing *ERG24* in W303-1A strain (Table 4.1). FEN hyper-susceptibility was observed in the deletion strains Δ*erg3* and Δ*erg6* (Table 4.2). Δ*erg3* and Δ*erg6* strains show slow growth, which may affect FEN MICs. Overexpression of *ERG3* and *ERG6* in their respective deleted strains, restore FEN MICs to normal level (Table 4.2).
While AMB and NYS both polyene drugs, the deletion strains including Δ*erg3*, e*rg4*, Δ*erg5*, and Δ*erg6* are resistant to NYS, yet have wild-type MICs to AMB (Table 4.2). These deletion strains lack ergosterol. The differences in susceptibilities of NYS and AMB may reflect at subtle difference in the mechanisms of actions of these two polyenes.

Among all the ergosterol biosynthesis inhibitors, FEN effected four of 25 overexpressing strains and three of nine *ERG* deletion mutants. FEN may thus be an important ergosterol pathway inhibitor. FEN belongs to morpholine class of drugs that include TRI and AMO. The MICs to the different morpholines were analyzed in the wild-

type *S. cerevisiae* strain BY4741 (Table 4.3), where AMO was more active than FEN, which was more active than TRI. From Tables 1 and 2, FEN susceptibilities are affected by many of the genes in the *ERG* pathway. Therefore, the transcription factor *UPC2* that regulates the *ERG* pathway may have significant effects on morpholine MICs. As expected the deletion of paralogs *UPC2* and *ECM22* led to FEN, TRI, and AMO hyper-susceptibility. Complementation with the endogenous *UPC2* locus restored normal morpholine susceptibilities (Table 4.3). Similarly, hyper-susceptibility to FEN, TRI, and AMO was observed in the *C. albicans* Δ*upc2* strain (Δ*upc2*/Δ*upc2*) (Table 4.3), and normal MICs are observed in the complimented strain (Table 4.3).

Table 4.3: MICs of Δupc2 *S. cerevisiae* and *C. albicans* Strains (μg/ml) in Glu-media

Strains	FEN	TRI	AMO
Sc_WT	8	32	2
Sc_Δupc2/Δecm22	1	8	0.5
Sc_Δupc2/Δecm22+ p*UPC2*	8	32	2
Ca_WT	16	64	4
Ca_Δupc2/Δupc2	4	16	1
Ca_Δupc2/*UPC2*	16	64	4

Red box denotes significant resistance
Blue box denotes significant hyper-susceptibility

Morpholine hyper-susceptibility is also observed in *S. cerevisiae* strains with deletion of eight endogenous efflux pumps (ADΔ, Table 4.4). In ADΔ, expression of *CaCDR1* and *CaCDR2* from *PDR5* promoter restored morpholine MICs to normal levels. Expression of *CaMDR1* in ADΔ did not affect the hyper-susceptibility of ADΔ to morpholines (Table 4.4). This suggests that *CaCDR1* and *CaCDR2*, but not *CaMDR1* may efflux morpholines, similar to azoles.

Table 4.4: MICs of *S. cerevisiae* Strains with or without Efflux Pumps (µg/ml) in Glu-media

Strains	FEN	TRI	AMO
Sc_WT	8	32	2
ADΔ	2	8	0.5
AD-CDR1	8	32	2
AD-CDR2	8	32	2
AD-MDR1	2	8	0.5

Red box denotes significant resistance
Blue box denotes significant hyper-susceptibility

Since *CaCDR1* and *CaCDR2* are associated with efflux mediated azole resistance in *C. albicans*, azole resistant clinical isolates may be expected to have altered susceptibilities to morpholines. To test this, four oral clinical isolates from a series of 17 isolates from a single immune-compromised patient obtained during a two-year period were analyzed. These isolates developed resistance to FLC due to prolonged and high drug dosage (20). The isolates used were Strain 1 (initial FLC susceptible isolate), Strain 4 (overexpressing *MDR1*), Strain 13 (overexpressing *ERG11*) and Strain 17 (overexpressing *CDR1*, *CDR2*, *MDR1*, and *ERG11*) (20). Morpholine susceptibilities were not altered in this series (Table 4.5), suggesting that overexpression of *CaCDR1* and *CaCDR2* does not result in morpholine resistance in clinical these clinical isolates. The differences in morpholine susceptibilities between *S. cerevisiae* constructs and *C. albicans* clinical isolates is not obvious.

Table 4.5: MICs of *C. albicans* Clinical Isolates (µg/ml) in Glu-media

Strains	FEN	TRI	AMO	FLC
Ca_WT	16	64	4	0.5
Strain 1	16	64	4	0.5
Strain 4	16	64	4	2
Strain 13	16	64	4	8
Strain 17	16	64	4	64

Red box denotes significant resistance
Blue box denotes significant hyper-susceptibility

In conclusion, this study analyzed the effects of *ERG* gene overexpression and deletion in *S. cerevisiae* on five erg pathway inhibitors. Several interesting phenotypes were observed. First, *HMG1* may play a role in azole resistance apart from overexpression of *ERG11*. Second, TRB resistance does not correlate with increased expression of *ERG1*. Third, AMB and NYS may differ in their mechanism of action, yet they target ergosterol in the membrane. Fourth, morpholines are important erg pathway inhibitors. Fifth, *UPC2* plays an important role in hyper-susceptibility to morpholines. And sixth, *CaCDR1* and *CaCDR2* may play a role in morpholine resistance. Thus this study contributes to our understanding of the erg pathway, and may help to identify new targets for future drug design.

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CHAPTER 5

CONCLUSION AND FUTURE DIRECTION

5.1 Conclusion

In pathogenic fungi, the two most important mechanisms of azole resistance are a) overexpression of efflux pumps, and b) altered ergosterol biosynthesis, both of which are analyzed in this thesis.

In *C. albicans*, overexpression of two types of efflux pump, the ABC-transporters Cdr1p and Cdr2p, and the Major Facilitator transporter Mdr1p, correlate with azole resistance (Chapters 1 and 2). At present, qRT-PCR is the most common tool available in analyzing the expression of the efflux pump genes *CDR1*, *CDR2*, and *MDR1* in clinical isolates of *C. albicans*. qRT-PCR quantifies the mRNA transcript levels without analyzing the protein activities, and is resource intensive. In addition, increased transcript levels do not always correlate with protein synthesis. To quantify the efflux activities in azole resistant isolates, a novel assay was designed that uses a combination of two fluorescent dyes, R6G and Ala-Nap (Chapter 2). While R6G is a substrate for Cdr1p and Cdr2p, Ala-Nap is a cleaved by proteases, releasing β-napthylamide, a substrate for all membrane transporters. The assay combining R6G and Ala-Nap directly measures the activities of membrane transporters. Increased efflux activities correlate with increased expression of the efflux pump genes in a collection of matched and non-matched azole resistant vaginal *C. albicans* clinical isolates (Chapter 2). This assay is less resource intensive than qRT-PCR, and can be used as a diagnostic tool in studying azole resistance.

In these vaginal clinical isolates, azole resistance also correlates with increased expression of *ERG11*, which encodes the azole target enzyme Erg11p (Chapter 2). Erg11p is an important enzyme that catalyzes one of the rate limiting steps in ergosterol biosynthesis (Chapters 1 and 3). Ergosterol is an important component of cellular membranes in fungi and plants, and its biosynthesis is catalyzed by 25 biosynthetic enzymes (Chapters 1, 3, and 4). Several enzymes in the pathway are targeted by medically important antifungal drugs as discussed (Chapters 1, 3 and 4). This dissertation also analyzes the effects of altered ergosterol biosynthesis on different cellular processes, including cell wall biosynthesis, respiration, protein translation, osmotic stress tolerance, and iron and calcium homeostasis in *S. cerevisiae* (Chapter 3). Each of the 25 *ERG* genes was overexpressed under a galactose inducible promoter. Most of the strains overexpressing *ERG* genes have growth defects in the presence of high osmotic stress, or low environmental calcium (Chapter 3). Two strains overexpressing *ERG2* and *ERG6* have abnormal phenotypes in all of the conditions tested. Erg2p and Erg6p may be important regulators in the ergosterol biosynthetic pathway, and might be used as drug targets in the future.

The effects of different ergosterol inhibitors on the strains overexpressing the *ERG* genes were also analyzed (Chapter 4). Altered susceptibilities to different ergosterol inhibitors are observed in some of the strains overexpressing the *ERG* genes. Altered susceptibilities to ergosterol inhibitors are also observed in nine strains that were deleted for non-essential *ERG* genes. The strains overexpressing *ERG11* and *ERG24* are resistant to azoles and morpholines, confirming that Erg11p and Erg24p are substrates of azoles and morpholines respectively. However, TRB resistance is not observed in the strain

overexpressing *ERG1*, the target of TRB. The polyenes NYS and AMB produce altered susceptibilities in different overexpression strains, suggesting a slightly different mode of action.

As described above, increased efflux correlates with azole resistance. Our findings suggest that the expression of the efflux pumps Cdr1p and Cdr2p can also cause increased morpholine resistance. Though cross resistance between azoles and morpholines is not common, morpholine susceptibilities may be effected by increased efflux pump activities.

5.2 Future Directions

In chapter 2, 38 *C. albicans* clinical isolates were analyzed for efflux-mediated molecular mechanisms of azole resistance. Of the 38 isolates, six pairs are matched isolates from the same patient, and in at least four pairs, FLC MICs are increased in the later isolate compared to the initial isolate. A recent study has hypothesized that altered azole import may contribute to azole resistance. Azole import should be analyzed in the matched isolates obtained in chapter 2, comparing import between the resistant and susceptible partners.

Chapter 2 studied clinical isolates obtained from vaginal cavities, which have lower environmental pH. Previous studies have shown that at a lower pH, *C. albicans* are more resistant to antifungals than at a higher pH (1, 2). The molecular mechanisms by which drug susceptibilities are altered by pH, are not known. Gene expression should be analyzed by RNAseq to identify the pH related molecular mechanisms.

In chapter 3, overexpression of *ERG* genes caused slow growth in nine strains. The slow growth rate may be the result of the accumulation of a toxic sterol or blockage of the

ergosterol pathway at specific steps, resulting in sterol intermediate accumulation. External sterols can be added in the growth medium to determine if they restore normal growth in these strains either under aerobic or anaerobic conditions. Finally, a complete sterol analysis could be performed in these slow growing strains.

In chapter 4, the *S. cerevisiae* strains overexpressing the *ERG* genes were analyzed for their susceptibilities to different erg pathway inhibitors. For decades, *S. cerevisiae* has been used as a model organism to study fungal genetics. However, the susceptibilities to erg pathway inhibitors can differ between *S. cerevisiae* and *C. albicans*. For example, the FLC MIC of wild-type *S. cerevisiae* is 16 µg/ml, while the FLC MIC of wild-type *C. albicans* is 0.5 µg/ml. In the future, *C. albicans* strains overexpressing the *ERG* genes should be constructed and analyzed for their susceptibilities to erg pathway inhibitors.

In conclusion, this thesis studies the common molecular mechanisms of drug resistance in pathogenic fungi, and may help to design novel therapies and strategies to counteract the development of drug resistance. This study also describes a novel assay, which can be used to analyze efflux pump mediated drug resistance in *C. albicans*.

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VITA

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