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Response to flucytosine in *Candida glabrata* at the membrane proteome level: role of the DHA family and the transcription factor CgPdr1

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Dissertation guided by Prof. Dr. Miguel Cacho Teixeira (IST-UL)
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Abstract

Candida glabrata is pathogenic fungi that emerged in the last decades as the second most common cause of candidiasis worldwide. The infections caused by this yeast are characterized for a high mortality and morbidity, and the action of drug efflux pumps is impairing treatment effectiveness. Multidrug resistance has emerged in most organisms and poses a severe clinical problem for the treatment because the extensive use of antifungal drugs had led to a huge increase in the number of intrinsically resistant infections with fungal pathogens.

Since resistance often relies on the action of membrane transporters, including drug efflux pumps from ATP-Binding Cassette (ABC) family or from the Drug:H^+ Antiporter (DHA) family, an iTRAQ-based (isobaric Tags for Relative and Absolute Quantification) membrane proteomics analysis was performed to identify all the membrane-associated proteins whose abundance changes in *C. glabrata* cells exposed to the Fluoropyrimidine drug 5-Flucytosine (5-FC). A total of 32 proteins were found to display significant expression changes in the membrane fraction of cells exposed to 5-FC, when compared to cells in the unstressed control conditions, 50 % of which under the influence of the transcription factor CgPdr1, found to be a determinant of *C. glabrata* resistance to 5-FC. These proteins cluster into functional groups associated to cell wall assembly, lipid metabolism, amino acid/nucleotide metabolism, ribosome components and translation machinery, mitochondrial function, glucose metabolism and multidrug resistance transport.

Among the proteins whose concentration was found increased in 5-FC stressed cells, CgFlr1 was elected for further studies. The role of CgFlr1, and of its close homolog CgFlr2, in 5-FC resistance was assessed. Results obtained demonstrate that both proteins confer 5-FC resistance. Despite their high degree of homology, CgFlr1 seems to specifically confer resistance to the fungicide mancozeb, while CgFlr2 appears to confer resistance to azole antifungal drugs and amphotericin B. CgFlr1 was found to be localized in the plasma membrane in *C. glabrata* and when heterologously expressed in *Saccharomyces cerevisiae*, complementing the 5-FC susceptibility phenotype exhibited by the *S. cerevisiae Δflr1* mutant. Additionally, the deletion of *CgFLR1* and *CgFLR2* was found to lead to increased intracellular accumulation of 5-FC, suggesting that these transporters play a direct role in the resistance to 5-FC by extruding this antifungal drug to the extracellular medium.

KeyWords

Candida glabrata, 5-Flucytosine drug resistance, membrane proteome, drug:H+ antiporters.

Resumo

Candida glabrata é um fungo patogénico que emergiu nas últimas décadas como a segunda causa mais comum de candidíase em todo o mundo. O género *Candida* compreende quase duzentas espécies, a maioria das quais são omnipresentes em numerosos habitats naturais e artificiais. As espécies patogénicas de *Candida* são encontradas como comensais no trato oral, gastrointestinal e genital de hospedeiros saudáveis e têm a propensão de se tornar patogénicas quando o hospedeiro se torna imunodeprimido. As infeções causadas por esta levedura são caracterizadas por uma elevada mortalidade e morbilidade. Entre outros fatores de virulência, as espécies do género *Candida* exibem, em comparação com espécies não patogénicas, uma expansão de famílias de transportadores de enzimas extracelulares e transmembranares, bem como um enriquecimento em transportadores de superfície celular com o potencial de conferirem resistência aos fármacos por funcionarem como bombas de efluxo de drogas, prejudicando a eficácia das terapias antifúngicas. *C. glabrata* é filogeneticamente mais próxima da levedura modelo - *Saccharomyces cerevisiae* do que das outras espécies do género *Candida.* No entanto*,* é frequentemente a segunda ou terceira causa mais comum de infeções fúngicas, sendo que em populações como diabéticos e idosos chega a ser o fungo patogénico dominante. Em comparação com outras espécies, *C. glabrata* fornece um modelo promissor para o estudo da base genética da resistência a múltiplos antifúngicos.

A resistência a múltiplos fármacos é definida como a aquisição simultânea de resistência a um grande espectro de substâncias químicas citotóxicas estrutural e funcionalmente não relacionadas, às quais o organismo nunca tinha sido exposto. Este fenómeno está a tornar-se um problema clínico crescente, uma vez que compromete a eficácia da terapêutica antimicrobiana. Para diminuir com sucesso o número de agentes patogénicos resistentes a múltiplos fármacos, é fundamental adquirir um conhecimento extenso dos mecanismos moleculares subjacentes à resistência aos fármacos antimicrobianos. Esta resistência a fármacos pode ser adquirida através de vários mecanismos: (I) alteração dos níveis de expressão e atividade de proteínas da membrana plasmática ou canais e transportadores de membrana; (II) degradação enzimática ou inativação dos fármacos; (III) ativação de sistemas de replicação e reparação de DNA; (IV) impedimento da entrada do fármaco na célula e/ou extrusão ativa do mesmo catalisada por transportadores transmembranares; (V) sequestro do fármaco em vesículas intracelulares; (VI) alteração ou modificação do alvo do fármaco. A ação de transportadores de membrana, incluindo bombas de efluxo da superfamília ATP-Binding Cassette (ABC) e transportadores da família Drug:H+ Antiporter (DHA), é fundamental para a

resistência a múltiplos fármacos. Os transportadores ABC obtêm energia por hidrólise de ATP, enquanto que os transportadores DHA utilizam o gradiente de protões através da membrana plasmática para exportar compostos para fora da célula. O genoma de *C. glabrata* codifica 18 transportadores ABC, seis meios transportadores e 12 transportadores completos, dos quais seis pertencem à subfamília PDR (*Pleiotropic Drug Resistance*) e apenas alguns foram estudados para resistência a fármacos ou atividades de transporte; codifica ainda 15 transportadores DHA1 que codificam para proteínas DHA com 12 segmentos transmembranares. Desde a década de 1950 várias classes de drogas antifúngicas foram desenvolvidas, no entanto, apenas quatro deles são atualmente usados na prática clínica para tratar infeções por *Candida*: análogos de pirimidina, polienos, azóis e equinocandinas. A extensão da resistência aos fármacos antifúngicos varia para as diferentes classes de fármacos. Em espécies de *Candida* há resistência bastante limitada aos polienos e equinocandinas, enquanto que a resistência ao análogo de pirimidina 5-flucitosina e a azóis é mais comum.

Neste trabalho foi estudado o efeito da exposição de células de *C. glabrata* ao fármaco antifúngico 5-flucitosina, ao nível do proteoma de membrana. Hoje em dia, novas abordagens quantitativas que utilizam a Espectrometria de Massa (MS) e a química estável de rotulagem de isótopos oferecem uma alternativa às técnicas tradicionais que empregam a eletroforese bidimensional comparativa para estudos de proteómica de expressão. A MS é uma ferramenta particularmente relevante no caso de proteínas de membrana, que não são detetáveis em géis bidimensionais, porque são em grande parte insolúveis em tampões de focagem isoelétrica. Neste trabalho, realizou-se análise proteómica de membrana baseada em iTRAQ-MS (isobaric Tags for Relative and Absolute Quantification) para identificar e quantificar as proteínas associadas à membrana em células de *C. glabrata* cuja concentração se altera após exposição à 5-flucitosina (5-FC). Um total de 32 proteínas apresentaram diferenças de expressão na membrana depois de expostas a 5-FC, quando comparadas com células em condições controlo. Estas proteínas estão envolvidas nos seguintes processos biológicos: remodelação da parede celular, metabolismo lipídico, metabolismo de aminoácidos/nucleotídeos, componentes ribossomais e maquinaria de tradução, função mitocondrial, metabolismo da glicose e transportadores de membrana.

Por comparação com as alterações que se verificam nas mesmas condições ambientais no mutante *Δpdr1*, com o gene *PDR1* eliminado, observou-se que 50 % das proteínas cuja expressão se altera após exposição a 5-FC estão sob influência do fator de transcrição CgPdr1, que se verificou ser determinante na resistência à 5-FC por *C. glabrata*. Este resultado surpreendente é muito interessante, uma vez que o fator de transcrição CgPdr1 é o principal

determinante de resistência a azóis em *C. glabrata*. A observação de que também poderá estar envolvido na aquisição de resistência a 5-FC sugere a existência de um mecanismo comum à resistência a duas famílias de antifúngicos, o que é preocupante na perspetiva da aquisição de resistência a agentes antifúngicos, sem necessidade de exposição prévia aos mesmos.

Entre as proteínas encontradas com expressão aumentada encontra-se o transportador da família DHA - CgFlr1, que foi eleito para estudos posteriores. Foi estudado o papel do CgFlr1, e do seu homólogo CgFlr2, na resistência à 5-flucitosina. Os resultados obtidos demonstram que ambas as proteínas conferem resistência a 5-flucitosina. Adicionalmente, e apesar do seu elevado grau de homologia, CgFlr1 parece conferir resistência especificamente ao fungicida mancozeb, enquanto CgFlr2 parece conferir resistência a azóis e a anfotericina B. Verificou-se que a proteína CgFlr1 se localiza na membrana plasmática, quando expresso em *C. glabrata*, mas também quando expresso em *S. cerevisiae*, complementando o fenótipo de suscetibilidade a 5-FC exibido pelo mutante *Δflr1*. Verificou-se que a eliminação dos genes *CgFLR1* e *CgFLR2* leva a uma acumulação intracelular de 5-FC maior do que a registada em células da estirpe selvagem, o que sugere que estes transportadores desempenham um papel direto na resistência à 5-FC por expulsão desta droga antifúngica para o meio extracelular.

No global, os resultados descritos neste estudo salientam a importância dos transportadores de múltiplos fármacos da família DHA no fenótipo de resistência a antifúngicos, em particular no que diz respeito a 5-FC. A caracterização dos transportadores CgFlr1 e CgFlr2 como estando envolvidos na resistência a 5-FC reforça a noção de que é necessário estudar os restantes membros desta família em *C. glabrata*, dado o seu provável impacto clínico. Adicionalmente, este estudo realça a importância do recurso a abordagens à escala do genoma, em particular ao nível do proteoma, na identificação e compreensão dos mecanismos de resposta e resistência a antifúngicos. Os processos biológicos e os seus efetores identificados no presente estudo representam alvos promissores para o desenvolvimento de novos sensitizadores da resistência à flucitosina, que poderão permitir a utilização terapêutica de 5-FC em mais baixas concentrações e sem riscos tão elevados de falência da terapêutica, limitando o desenvolvimento de resistência a 5-FC em *C. glabrata* que habitualmente acontece com elevada rapidez.

Palavras-Chave

Candida glabrata, resistência ao fármaco flucitosina, proteoma de membrana, antiportadores droga:H^+

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Thesis Outline

This dissertation is divided into four chapters.

In Chapter 1, an introduction of the current knowledge of the genus *Candida*, its phylogeny, epidemiology and treats, focusing mainly on *Candida albicans* and *Candida glabrata*, the two most prevalent species responsible for human candidemias. The multidrug resistance phenomenon and the role of the multidrug transporters of the ABC and MFS superfamilies is discussed, having as background, the far more well-studied yeast *Saccharomyces cerevisiae*. The mode of action of the five families of antifungal agents is also discussed, highlighting the resistance mechanisms known for each antifungal family. Finally, the iTRAQ-based proteomics is also presented.

Chapter 2 is focused on the experimental methods and approaches used to achieve the goals of the present study.

The Chapter 3 presents the results obtained in all assays. Begins with the role of the transcription factor CgPdr1 in the resistance to 5-Flucytosine and other antifungal drugs. After that, characterization of the *C. glabrata* membrane proteome and the response to stress induced by 5-Flucytosine at the membrane proteome level. Assessment of the role of CgPdr1 in *C. glabrata* response to 5-Flucytosine are also performed. Finally, the evaluation of the role of four *C. glabrata* DHA transporters CgFlr1, CgFlr2, CgTpo1_1 and CgTpo1_2 in the resistance to 5-Flucytosine and other antifungal drugs is tested.

In Chapter 4 the results obtained throughout this work are discussed in light of previous knowledge and the new facts learned about MDR efflux pumps in antifungal drug resistance in *C. glabrata.* Finally, the conclusions of the present work are presented, as well as some suggestions for further work on this subject.

Most of the results displayed in this thesis were published in Frontiers in Microbiology [Pais, P.*, Pires, C.*, Costa, C., Okamoto, M., Chibana, H., Teixeira, M.C., *Membrane proteomics analysis of the Candida glabrata response to 5-Flucytosine: Unveiling the role and regulation of the drug efflux transporters CgFlr1 and CgFlr2*, Frontiers in Microbiology.2016. 7:2045. (*these authors have contributed equally to this work)].

Abbreviations

5-FC – flucytosine or 5-Flucytosine

5-FU – 5-fluorouracil

5-fluoro-UMP – 5-fluorouridine monophospate

4-NQO – 4-Nitroquinoline 1-Oxide

2-DE – Two-dimensional Eletrophoresis

AAA – Amino-Acid Analysis

ABC – ATP Binding Cassette

CID – Collision-induced Dissociation

CYP – Cytochrome P-450

DF – Frequency in Dataset

DHA – Drug:H^+ Antiporter

DMSO – Dimethyl Sulfoxide

FDA – Food and Drug Administration

GFP – Green Fluorescent Protein

GOF – Gain-of-function

GRAS – "General Regarded As Safe"

iTRAQ – isobaric Tags for Relative and Absolute Quantification

MCPA – 2-methyl-4-chlorophenoxyacetic acid

MDR – Multidrug Resistance

MFS – Major Facilitator Superfamily

MMB – Minimal Medium for BY4741

MMG – Minimal Growth Medium

MMTS – Methyl Methane Thiosulfonate

MRP – Multidrug Resistance-associated Protein

MS – Mass Spectrometry

NBD – Nucleotide Binding Domain

OD – Optical Density

OMP – Orotidine-5’-phosphate

PDR – Pleiotropic Drug Resistance

PEMT – Phosphatidylethanolamine Methyltransferase

RF – Frequency Reference Genome

rRNA – Ribossomal RNA

SGD – *Saccharomyces* Genome Database

TCEP – Tris(2-carboxyethyl) Phosphine

TEAB – Tetraethylammonium Bromide

TMD – Transmembrane Domain

UDP – Uridine Diphosphate

UMP – Uridine Monophosphate

UPRT – Uracil Phosphoribosyl Transferase

WGD – Whole Genome Duplication

YPD – Yeast Peptone Dextrose

1 Introduction

1.1 The genus *Candida*

1.1.1 Phylogeny

The genus *Candida* comprises almost 200 *Candida* species [1] and a wide range of phylogenetically unrelated anamorphic fungi. Yeasts of the polyphyletic, artificial genus *Candida* include plant endophytes, insect symbionts and opportunistic human pathogens, most of which are ubiquitous in numerous natural and artificial habitats [2]. The genomes of *Candida* species show enormous variations in size and phenotypic outcome, however, the predicted numbers of protein-coding genes are very similar in all the species [1].

As result of the multigene analysis applied to some *Candida* species by Butler *et al*. [3], a phylogenetic tree was constructed and showed in Figure 1.1.

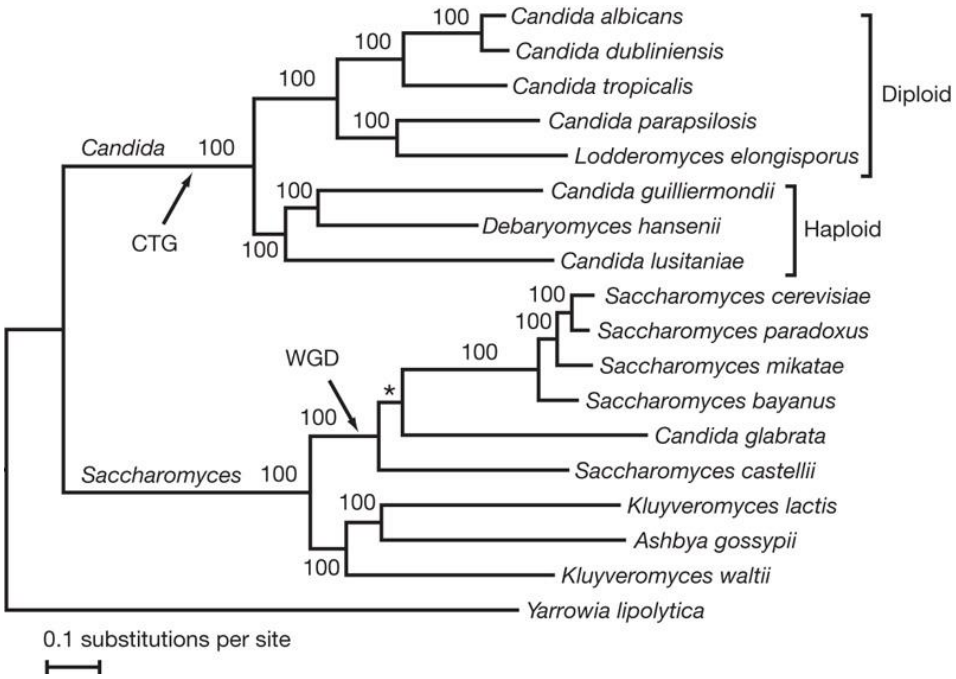


Figure 1.1 - Phylogenetic tree of sequenced *Candida* and *Saccharomyces* clade species constructed and described by Butler *et al.* [3]. CTG: the CUG codon in this clade encodes the amino acid serine instead of leucine**, WGD:** whole genome duplication**, *** marks a branch that was constrained based on syntenic conservation.

As show in Figure 1.1, almost all *Candida* species belong to a single *Candida* clade, with the exception being *Candida glabrata*. This clade is characterized by the exclusive translation of CUG codon as serine rather than leucine. *C. glabrata* is phylogenetically closer to *Saccharomyces* species than other *Candida* species [4]. Therefore, the years of post-genomic

research dedicated to *Saccharomyces cerevisiae* are expected to provide reliable clues to guide *C. glabrata* functional studies [5] and make *C. glabrata* a good model organism for the study of fungal pathogenesis and for the identification of antifungal drug targets and drug resistance mechanisms [5]. An additional characteristic of *C. glabrata* that distinguishes it from other pathogenic *Candida* species is its haploid genome [6].

1.1.2 Pathogenic *Candida* species

The genus *Candida* includes unicellular fungi that have been used in industrial fermentation processes, mainly food and beverage industries, and are listed in "Generally Regarded As Safe" (GRAS) organism by the Food and Drug Administration (FDA) of the USA [2]. However, they have the propensity to become pathogens [7] and are the most common cause of opportunistic fungal infection worldwide [3] with high morbidity and mortality [8].

Although the genus *Candida* contains over 150 heterogeneous species only a small part is implicated in human candidiasis, since there are few it can grow at 37 ^{o}C and become successful pathogens or commensals on humans [6]. Pathogenic *Candida* species are found as commensals in the gastrointestinal and genital tract of healthy hosts and have the propensity to become pathogens when the host is immunocompromised [2].

Colonization rates increase with severity of illness and duration of hospitalization [9] and the currently higher predisposition of human hosts to contract fungal systemic infections are further related to increased aged population, higher number of surgical interventions and the widespread and increased use of immunosuppressive therapy and broadspectrum antibiotic therapy, leaving the human hosts exposed to fungal infections [10, 11].

Besides the host's predisposition to infections, *Candida* pathogenicity is also facilitated by several virulence factors that worsen this problem. These pathogens are able to form biofilms and adhere to host cells and medical devices [12]. Another virulence factor is the production of extracellular enzymes, namely proteinases and phospholipases and this proteolytic and lipolytic activity has been linked with tissue invasion [12, 13]. Furthermore, *Candida albicans* can adapt and switch among different phenotypes including smooth, rough and irregular wrinkle at high frequency [14]. Finally, *Candida* species show expansions of extracellular enzyme and transmembrane transporters families as well as an enrichment of cell-surface transporters with the potential to confer drug resistance [3]. These gene families are not found in

Saccharomyces species or are present in *S. cerevisiae* but significantly expanded in pathogens, explaining the importance of the extracellular activities in virulence and pathogenicity [3].

Although *Candida* species are the most common cause of fungal infection, only four of these species account for 90 % of *Candida* infections: *C. albicans, C. glabrata, Candida tropicalis* and *Candida parapsilosis* [15]. *C. albicans* is the most predominant cause of fungal infections [16], while *C. glabrata*, the object of this study, is the second.

1.1.3 *Candida glabrata*

Initially, this species was classified within the genus *Torulopsis*, but in 1978 *Torulopsis glabrata* was reclassified to the genus *Candida*, due to its association with human infections [4].

C. glabrata is a non-dimorphic haploid yeast that does not form pseudohyphae at temperatures above 37 ^{o}C. Its blastoconidia are smaller than that of *C. albicans* and this is a useful characteristic to distinguish one colonies from the others [17].

The genome of this haploid yeast comprises 13 chromossomes [18] and shows a significally greater degree of gene loss, compared with *S. cerevisiae,* resulting from a regressive evolution [19, 20]. *C. glabrata* can also be distinguished from *C. albicans* by its small-subunit ribosomal RNA (rRNA) [20].

Although *C. glabrata* is phylogenetically closer to *S. cerevisiae* it is often the second or third most common cause of invasive fungal infections [4, 21]. The colonization by *Candida* spp. is almost universal, but in some populations, such as diabetics and the elderly, *C. glabrata* may even be the dominant pathogen [22]. In comparison with other *Candida* species, *C. glabrata* provides a promising model for studying the genetic basis of multidrug resistance [4].

1.2 Multidrug Resistance Phenomenon

Multidrug resistance (MDR) is defined as the simultaneous acquisition of resistance to a large spectrum of structurally and functionally unrelated cytotoxic chemicals, to which the organism had never been exposed before [23]. This phenomenon occurs in a diversity of organisms, from the simplest to the most complex, and is becoming an increasing clinical problem, especially for immunocompromised patients, and for the control of plant pathogens and weeds [23, 24].

The emerge of widespread MDR is a challenge for therapeutics, food preservation and crop protection [25]. On the other hand, MDR can be advantageous from the industrial point of view, conferring tolerance to chemical stress in microbial strains and cell lines used in biotechnological processes [24]. To successfully decrease the numbers of multidrug resistant pathogens extensive knowledge of the molecular mechanisms underlying microbial drug resistance is fundamental [26].

Drug resistance on cells may be acquired through several mechanisms that succeed in overcoming citotoxicity by: (i) changed levels of expression and activity of plasma membrane or endomembrane channels and transporters; (ii) enzymatic degradation or inactivation of the drugs; (iii) DNA replication and repair systems; (iv) preventing the drug from entering the cell and active extrusion of the drugs through proteins which catalyze transmembrane drug transport; (v) sequestration of drug in intracellular vesicles; (vi) alteration or modification of drug target [26-28].

There is a consensus among authors that MDR emergence is mainly due to active membrane transporters that pump a broad spectrum of chemically distinct, cytotoxic molecules out of cells [23, 29]. Several membrane proteins that mediate active drug extrusion have been identified and thoroughly studied in different organisms; however, many of the molecular mechanisms behind the activity of multidrug efflux pumps in MDR remain unknown [20, 23, 26, 30-32].

The eukaryotic model *S. cerevisiae* has been widely used to study drug resistance phenomenon (in which the multidrug resistant phenotype is also referred to as pleiotropic drug resistance or PDR) [31].

1.2.1 Multidrug Resistance Transporters

There are several membrane transport systems involved in drug resistance, both in prokaryotes and eukaryotes, but efflux-mediated drug tolerance seems to be the major factors responsible for clinical multidrug resistance [33, 34]. The first ever described multidrug efflux pump was the mammalian P-glycoprotein [34]. This is an ATP-driven pump that provides resistance to a broad range of drugs, including some anticancer chemotherapeutic agents [27, 35].

Multidrug efflux pumps are present in the plasma membrane of multiple species and the importance of these transporters is evidenced by the fact that genome sequence analysis revealed that multidrug efflux transporters constitute more than 10 % of the transporters

present in *S. cerevisiae* and *C. albicans* [34-36]. These transporters recognize a wide variety of structurally and pharmacologically unrelated drugs and extrude them from the cytoplasm to the outside of the cell or within cell compartments, against their concentration gradient, thus providing resistance to the chemicals. Over the years, the overexpression of many other MDR membrane transporters has been shown to enhance cell resistance to drugs, thus indicating that MDR by efflux pumps is not an exceptional phenomenon, but a highly-conserved defense mechanism [35-37].

The membrane efflux pumps involved in MDR belong mostly to two superfamilies of transporters: the ATP-Binding Cassette (ABC) and the Major Facilitator Superfamily (MFS) [24, 36]. The ABC transporters are energized by ATP hydrolysis, while MFS transporters use the proton gradient across the plasma membrane to export compounds out of the cell [29]. A schematic representation of the ABC and MFS transporters is shown in Figure 1.2.

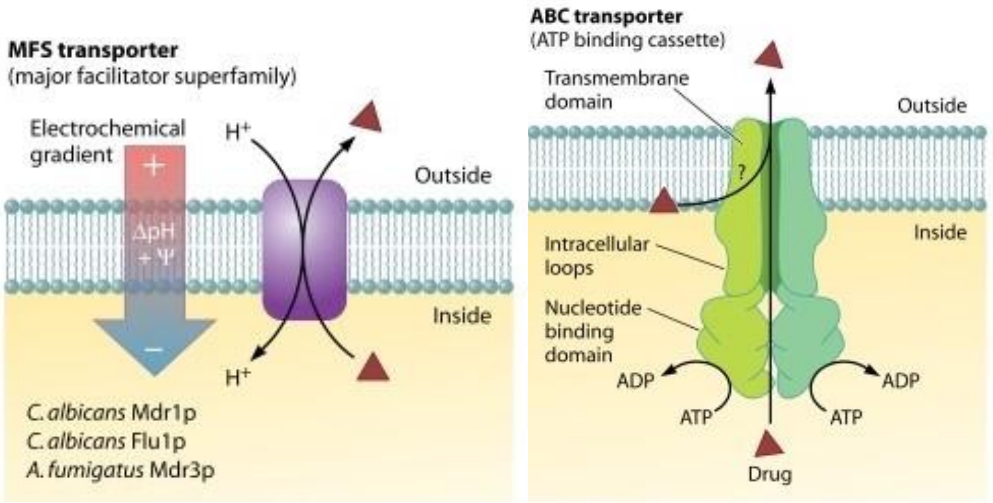


Figure 1.2 - Schematic representation of the ABC and MFS transporter, adapted from Canon *et al.* [29]

1.2.2 The ABC superfamily

ABC superfamily proteins constitute one of the largest protein families, which are ubiquitous to all living cells, from bacteria to higher eukaryotes [38]. The number of known members exceeds already 10,000 and this number increases with each new genome sequence release [39]. The ABC proteins use the free energy of ATP hydrolysis to actively transport a wide range of different molecules (such as hydrophobic drugs, lipids, peptides or proteins) [23, 37, 40].

The majority of the so far described ABC proteins are involved in the ATP-dependent transport of a broad spectrum of substrates across biological membranes, but some of them have been described as ion channels across membranes or as ion receptors or even involved in mRNA translation and ribosome biogenesis [39]. These transporters have specific ligands that go from amino acids, to inorganic ions and polypeptides. Nevertheless, some of them seem to have evolved towards a broader specificity, being also thought to be involved in antibiotic and drug resistance [23]. The structure of ABC transporters consists in four domains: two Nucleotide Binding Domain (NBDs) and two Transmembrane Domains (TMDs) [37]. A schematic representation of the ABC transporter is showed in Figure 1.3.

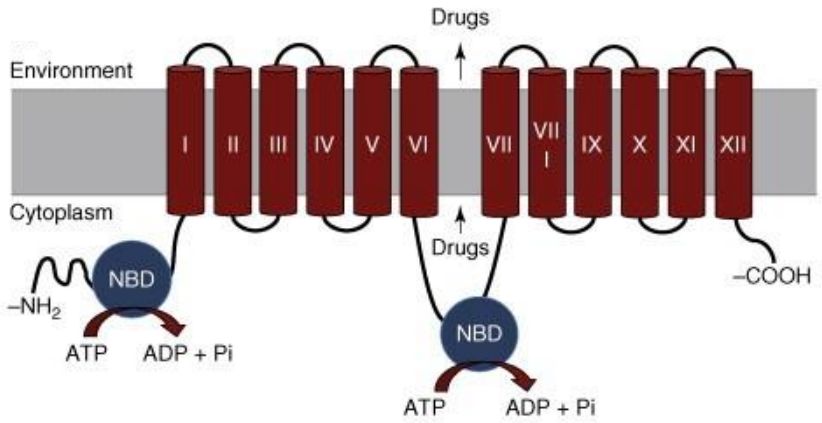


Figure 1.3 - Schematic representation of domain arrangements of ABC transporters from yeasts species, adapted from Sá-Correia *et al.* [24]. NBD – Nucleotide Binding Domain**.**

The TMDs are hydrophobic, consist of usually six transmembrane α-helical segments that are responsible for binding the ligand and can be expressed as multidomain proteins or as separate polypeptide chains. The arrangement of the NBDs and TMDs within the pump polypeptide varies according to the type of ABC protein [29].

Given the role of ABC transporters in multidrug resistance, it is important to understand the molecular mechanisms behind these transporters. This knowledge is crucial to design inhibitors for these multidrug pumps [24].

1.2.2.1 ABC transporters in *S. cerevisiae*

In the *S. cerevisiae* genome, the products of 30 genes are classified as ABC proteins. Of these proteins, 22 are classified as primary active transporters. Among these, 16 proteins are about twice as long as the other six. The larger proteins have 12 transmembrane-spanning domains and probably arose by duplication of the half-size ABC transporters with six TMDs [25]. ABC proteins in *S. cerevisiae* are classified into three main subfamilies: Pleiotropic Drug Resistance (PDR), MDR and Multidrug Resistance-associated Protein (MRP). Of all ABC transporters encoded by *S. cerevisiae* genome, nine belong to the PDR family [41].

The ABC transporters of *S. cerevisiae* as well as their description and localization are summarized in Table 1.1, and their distribution among cellular organelles is presented in Figure 1.4 [42].

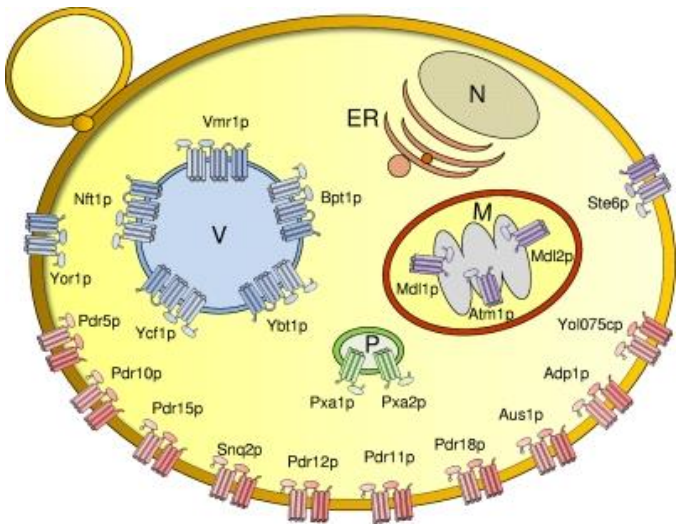


Figure 1.4 - Localization of *S. cerevisiae* ABC transporters, adapted from Paumi *et al.* [42].
ER – endoplasmatic reticulum; **M –** mitochondrion; **N –** nucleous; **P -** peroxisomes; **V -** vacuole.

Table 1.1 - *S. cerevisiae* ABC transporters genes and their product description. Listed in the *Saccharomyces* Genome Database (SGD) (www.yeastgenome.com).

Gene	ORF	Gene product description
ADP1	*YCR011c*	Putative ATP-dependent permease.
ATM1	*YMR301c*	Mitochondrial inter membrane transporter.
AUS1	*YOR011w*	Plasma membrane sterol transporter.
BPT1	*YLL015w*	Involved in the transport of unconjugated bilirubin vacuolar transport and in heavy metal detoxification.
MDL1	*YLR188w*	Mitochondrial inner membrane transporter of peptides generated upon proteolysis of mitochondrial proteins.
MDL2	*YPL270w*	Mitochondrial inner membrane transport required for respiratory growth at high temperature.
NFT1	*YKR103/104*	Putative transporter of the multidrug resistance-associated protein (MRP) subfamily.
PDR5	*YOR153w*	Plasma membrane multidrug transporter involved in steroid transport, cation resistance and cellular detoxification during exponential growth.
PDR10	*YOR328w*	Multidrug transporter involved in the Pleiotropic Drug Resistance network.
PDR11	*YIL013c*	Multidrug transporter involved in the multiple drug resistance that mediates sterol uptake when sterol biosynthesis is compromised, required for anaerobic growth.
PDR12	*YPL058c*	Plasma membrane multidrug transporter involved in the extrusion of weak organic acids.
PDR15	*YDR406w*	Plasma membrane multidrug transporter implicated in general stress response for cellular detoxification.
PDR18	*YNR070w*	Putative transporter implicated in Pleiotropic Drug Resistance.
PXA1	*YPL147w*	Implicated in the transport of long-chain fatty acids into peroxisomes.
PXA2	*YKL188c*	Implicated in the transport of long-chain fatty acids into peroxisomes.
SNQ2	*YDR011w*	Plasma membrane multidrug transporter involved in multidrug resistance and resistance to singlet oxygen species.
STE6	*YKL209c*	Plasma membrane transporter required for the export of α-factor.
VMR1	*YHL035c*	Vacuolar membrane protein involved in multiple drug resistance and metal sensitive.
YBT1	*YLL048c*	Bile acid transporter.
YCF1	*YDR135c*	Vacuolar glutathione S-conjugate transporter.
YOL075c	*YOL075c*	Protein of unknown function.
YOR1	*YGR281w*	Plasma membrane multidrug transporter that mediates export of many different organic anions.

The transcriptional regulation of the majority of the multidrug efflux pump encoding genes is controlled by the Zn_2Cys_6 zinc finger transcription factors Pdr1 and its homologue Pdr3. As in most other Zn_2Cys_6 regulators, the N-terminal cysteine rich zinc finger is necessary for actual DNA binding. Given their role as regulators of drug efflux pump expression level, Pdr1 and Pdr3 exert a strong influence on the MDR phenotypes of yeast cells [43, 44]. Yrr1p and Pdr8, two other Zn_2Cys_6 and the basic-leucine zipper transcription factor Yap1 are involved in the regulation of

the PDR network [45]. Other zinc finger transcriptional regulators and multidrug transporters that are involved in the PDR network can be found in Figure 1.5.

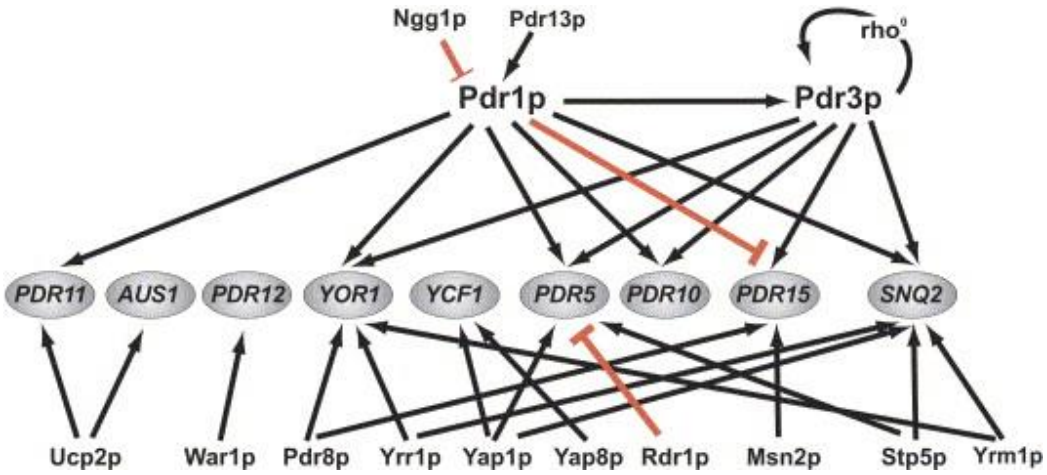


Figure 1.5 - The PDR network in yeast. Genes in the centerline represent MDR-related target genes of the transcriptional regulators depicted above and below. Red lines indicate a negative regulatory impact, while black lines ending with an arrow indicate positive regulation, adapted from Jungwirth *et al.* [45].

1.2.2.2 ABC transporters in *C. glabrata*

The *C. glabrata* genome encode 18 ABC transporters, six half transporters and 12 full transporters, in which six belong to the PDR subfamily [25] and only a few have been studied to drug resistance or transport activities [46]. The ABC transporters Cdr1 and Pdh1 (or Cdr2) have been shown to be involved in acquired azole resistance in *C. glabrata* clinical isolates and seem to be regulated by a common mechanism, probably with the involvement of CgPdr1 [47]; and CgAus1 (an ortholog of *S. cerevisiae* Aus1 and Pdr11 ABC transporters) was recently described as a putative sterol importer that help protect *C. glabrata* from azole toxicity [47, 48].

The CgPdr1, transcription factor of *C. glabrata* represents the single best homologue of the Pdr1/Pdr3 pair of proteins found in *C. glabrata* [47]. *CgPDR1* shares more sequence similarity with *ScPDR1* but exhibits the drug-dependent transcription induction seen for *ScPDR3* [47].

1.2.3 The Major Facilitator Superfamily

The Major Facilitator Superfamily is a very large and ancient superfamily found throughout nature, from bacteria to mammals [23], but compared with the ABC transporters are involved in multiple drug resistance (among other cellular functions) but the understanding of the function and regulation of the MFS-MDR proteins is poorly understand [24, 31]. It is a superfamily energized by the electrochemical gradient across membranes where diverse substrates show affinity to these transporters in a proton motive force dependent mechanism of symport, antiport or uniport [23, 24].

When the *S. cerevisiae* genome sequence was disclosed, 28 genes were predicted to be MFS-MDR transporters. Surprisingly, only three of these transporters had already been described to that date [36, 49, 50]. In yeast, the MFS-MDR transporters function by proton antiport and are classified in two groups according to the number of predicted transmembrane spans (TMS): the drug:H^+ antiporter-1 (DHA1) and the drug:H^+ antiporter-2 (DHA2) showed in Figure 1.6 [24, 25, 29]. The DHA1 family is characterized by 12 spanner (left in Figure 1.6) and the DHA2 family is predicted to have 14 spanner (right in Figure 1.6) [24,25].

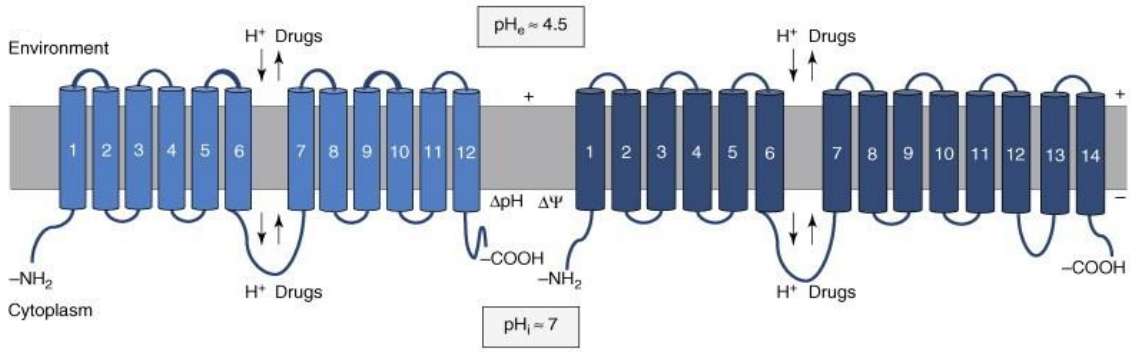


Figure 1.6 - Domain arrangements of MFS transporters from yeasts species. DHA1 family (left) and DHA2 family (right), adapted from Sá-Correia *et al.* [24].

1.2.3.1 MFS-MDR transporters in *S. cerevisiae*

Based on structural considerations DHAs were proved to be MDR determinants in *S. cerevisiae*. It has been observed in some studies that multidrug systems appear to fulfill primary functions unrelated to drug transport, which are consistent with experimental evidences showing that transcription of some MFS-MDR genes is not stimulated by the respective chemical stress [51, 52]. The DHA1 and DHA2 families of *S. cerevisiae* are listed in Table 1.2 and Table 1.3 respectively.

When the genome of *S. cerevisiae* was analyzed, a classification in clusters of the DHA transporters was obtained. Members of DHA1 family were segregated in two clusters (plus one isolated protein – Hol1) and three clusters were discriminated for DHA2 family [49]. Figure 1.7 displays the phylogenetic relationship of DHA1 and DHA2 proteins among several yeast species highlighting the sequence similarities of *S. cerevisiae* transporters and those predicted to play a similar role in *C. glabrata* [25].

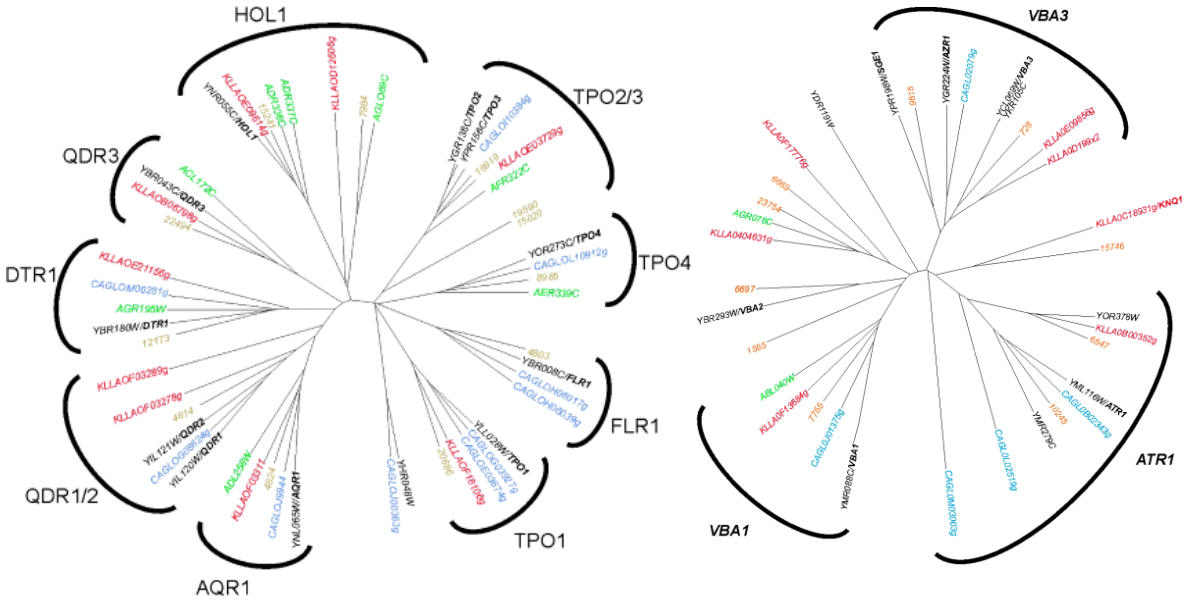


Figure 1.7 - Phylogenetic relationship of the DHA1 family (left) and the DHA2 family (right) in various yeast species. Genes from *S. cerevisiae* are black and the *C. glabrata* genes are blue. Adapted from Gbelska *et al.* [25].

Table 1.2 - Characteristics of DHA1 family members. Adapted from Sá-Correia *et al.* [24]

Gene	ORF	Predicted gene product description	Predicted determinant of resistance to
AQR1	*YNL065w*	Protein located at the plasma membrane and in multiple internal membrane structures, proposed to catalyze amino acid excretion.	Ketoconazole, Crystal violet, Quinidine, Quinine, Short-chain monocarboxylic acids and Barban.
DTR1	*YBR180w*	Pro-spore membrane bisformyl dityrosine transporter, essential for spore-wall synthesis.	Quinine, Quinidine, Propionic acid, Butyric acid and Benzoic acid.
FLR1	*YBR008c*	Plasma membrane protein.	Fluconazole, Cycloheximide, 4-nitroquinoline 1- oxide (4-NQO), benomyl, Methotrexate, Diazoborine, Cerulenin, Diamide, Diethylmaleate, Menadione, Mancozeb and Paracetamol
HOL1	*YNR055c*	Plasma membrane transporter proposed to be involved in histidinol and Na^+ uptake.	None know.
QDR1	*YIL120w*	Plasma membrane transporter involved in quinidine excretion.	Quinidine, Fluconazole, Ketoconazole and Barban.
QDR2	*YIL121w*	Plasma membrane transporter involved in quinidine excretion and in potassium uptake.	Quinidine, Barban, Ketoconazole, Cisplatin and Bleomycin.
QDR3	*YBR043c (AQR2)*	Plasma membrane transporter involved in quinidine and polyamines excretion.	Quinidine, Barban, Cisplatin, Bleomycin, Manganese, Spermine and Spermidine.
TPO1	*YLL028w*	Plasma membrane polyamine transporter; catalyzes uptake of polyamines at alkaline pH and excretion at acidic pH.	Spermine, Putrescine, Spermidine, Quinidine, Cycloheximide, 2-methyl-4-chlorophenoxyacetic acid (MCPA), 2,4 dichlorophenoxyacetic acid (2,4-D), Mycophenolic acid, Nystatine, Artesunate, Caspofungin, Indomethacin and Ibuprofen.
TPO2	*YGR138c*	Plasma membrane polyamine-transport protein specific for spermine.	Spermine, Weak organic acids (acetic, propionic, benzoic and octanoic acids).
TPO3	*YPR156c*	Plasma membrane polyamine-transport protein specific for spermine.	Spermine, Weak organic acids (acetic, propionic, benzoic and octanoic acids).
TPO4	*YOR23c*	Plasma membrane polyamine-transport protein.	Spermine, Quinidine and Cycloheximide.
YHK8	*YHR048w*	Plasma membrane protein.	Itraconazole and Fluconazole.

Table 1.3 - Characteristics of DHA2 family members. Adapted from Sá-Correia *et al.*[24]

Gene	ORF	Predicted gene product description	Predicted determinant of resistance to
ATR1	*YML116w, SNQ1*	Plasma membrane protein.	4-nitroquinoline-1-oxide(4-NQO), Aminotriazole.
AZR1	*YGR224w*	Plasma membrane protein.	Acetic acid, Propionic acid, Crystal violet, Ketoconazole, Fluconazole and Polymyxin B.
SGE1	*YPR198w, NOR1*	Plasma membrane protein.	Crystal violet, Ethidium bromide, Methylmethane sulfonate, Malachite green, 10-N-nonyl acridine orange.
VBA1	*YCL069w*	Vacuolar membrane permease of basic amino acid in the yeast vacuole.	None know.
VBA2	*YMR088c*	Vacuolar membrane permease of basic amino acid in the yeast vacuole.	None know.
VBA3	*YBR293w*	Vacuolar membrane permease of basic amino acid in the yeast vacuole.	None know.
VBA4	*YDR119w*	Vacuolar membrane protein.	None know.
VBA5	*YKR105c*	Putative vacuolar membrane protein.	None know.
-	*YMR279c*	Putative MFS-MDR transporter.	None know.
-	*YOR378w*	Putative MFS-MDR transporter.	None know.

1.2.3.2 MFS-MDR transporters in *C. glabrata*

When *C. glabrata* was compared with *S. cerevisiae*, despite the phylogenetic similarities, exhibited some differences. In *C. glabrata* there are 15 DHA enconding genes that codify for DHA proteins and are listed in Table 1.4 and Table 1.5.

Table 1.4 - Characteristics of DHA1 family members of *C. glabrata.* Listed on the Génolevures (http://www.genolevures.org).

Gene	ORF	Predicted gene product description	Predicted determinant of resistance to
AQR1	*CAGL0J09944g*	Plasma membrane MFS-MDR protein.	Short-chain monocarboxylic acids and Quinidine.
DTR1	*CAGL0M06281g*	Putative dityrosine transporter, required for spore wall synthesis.	None known.
FLR1	*CAGL0H06017g*	Plasma membrane MFS-MDR protein.	Fluconazole, Diazaborine, Benomyl, Methotrexate, and other drugs.
FLR2	*CAGL0H06039g*	Plasma membrane MFS-MDR protein.	Fluconazole, diazaborine, benomyl,
QDR1/2	*CAGL0G08624g*	Putative MFS-MDR protein.	Quinidine, Barban, Cisplatin and Beomycin.
TPO1_1	*CAGL0G03927g*	Polyamine transporter that recognizes.	Spermine, Putrescine and Spermidine.
TPO1_2	*CAGL0E03674g*	Polyamine transporter that recognizes.	Spermine, Putrescine and Spermidine.
TPO2/3	*CAGL0I10384g*	Polyamine transport protein specific for spermine.	Spermine.
TPO4	*CAGL0L10912g*	Polyamine transport protein recognizes.	Spermine, Putrescine, and Spermidine.
YHK8	*CAGL0J00363g*	Putative MFS-MDR protein.	None known.

Table 1.5 - Characteristics of DHA2 family members of *C. glabrata*. Listed on the Génolevures (http://www.genolevures.org).

Gene	ORF	Predicted gene product description	Predicted determinant of resistance to
ATR1_1	*CAGL0B02343g*	Putative MFS-MDR protein.	Aminotriazole and 4-nitroquinoline-N-oxide.
ATR1_2	*CAGL0L02519g*	Putative protein of unknown function.	None known.
ATR1_3	*CAGL0M03003g*	Putative protein of unknown function.	None known.
AZR1	*CAGL0B02079g*	Plasma membrane MFS-MDR protein.	Azole drugs such as Ketoconazole and Fluconazole.
VBA1	*CAGL0J01375g*	Permease of basic amino acids in the vacuolar membrane.	None know.

There are two predicted *TPO1* orthologues and two predicted *FLR1* orthologs in the *C. glabrata* genome. *ScFLR1* homologs in *C. glabrata* include ORFs *CAGL0H06017g* (*CgFLR1*) and *CAGL0H06039g* (*CgFLR2*), with 59 % and 54 % identity between their deduced amino acid sequences, respectively. On the other hand, *ScTPO1* has two *TPO1* homologs, *CAGL0G03927g (CgTPO1_1)* and *CAGL0E03674g* (*CgTPO1_2*), in *C. glabrata* with 70 % and 73 % identity between their deduced amino acid sequences, respectively [53].

FLR1 is known as a Yap1 target gene and has three Yap1 response elements (YREs) in its promoter region at positions -148 (YRE1), -167 (YRE2), and -364 (YRE3) [54]. YRE3 is a palindrome known to function on either strand of many YAP1 target genes and is found upstream of several homologous genes in *C. glabrata* including *CgFLR1*. However, none of the YRE consensus sequences was found within the 1-kb upstream region of *CgFLR2* [54].

According to Chen *et al*. [55] *CgFLR1* was considered to be the closest *FLR1* homolog in *C. glabrata* and the disruption of *CgFLR1* did not affect sensitivity to fluconazole, cycloheximide, 4-NQO, hydrogen peroxide, cerulenin, cadmium or chloramphenicol. The *CgFLR1* deletion led to only very minor increases in sensitivity to benomyl, diamide and menadione. An important fact CgFlr1 was involved in benomyl resistance in *C. glabrata* and CgYap1 regulates the transcription of *CgFLR1_1* gene in response to benomyl stress [55].

1.2.4 The CgPdr1 transcription factor

The *C. glabrata* Pdr1 transcription factor is the single ortholog of two *S. cerevisiae* zinc cluster transcription factors ScPdr1 and ScPdr3, which regulates the expression of genes involved in multidrug resistance [56]. Similarly to Pdr1 in *S. cerevisiae*, CgPdr1 may bind constitutively to the promoters of a repertoire of genes and activate a subset of them, depending on the mechanism by which it is activated [56].

CgPdr1 has been linked to the clinical acquisition of azole antifungal drugs resistance [57], since single point mutations in the functional domains of *CgPdr1*, called gain-of-function (GOF) mutations, have been found in numerous azole resistant *C. glabrata* clinical isolates, these point mutations resulting in constitutive increased transcription of the drug efflux pump encoding genes *CgCDR1, CgPDH1* and *CgSNQ2* [58]. Interestingly, these GOF mutations in *CgPdr1* were further found to be important for pathogen-host interactions, as they are associated with enhanced virulence [58, 59].

1.3 Antifungal Drugs and Fungicides explored in the course of this study

Several antifungal drug families are currently used. They are classified according to their chemical nature and grouped based on their mechanism of action, spectrum of activity and side effects. Figure 1.8 shows the targets of the available antifungal drugs [60].

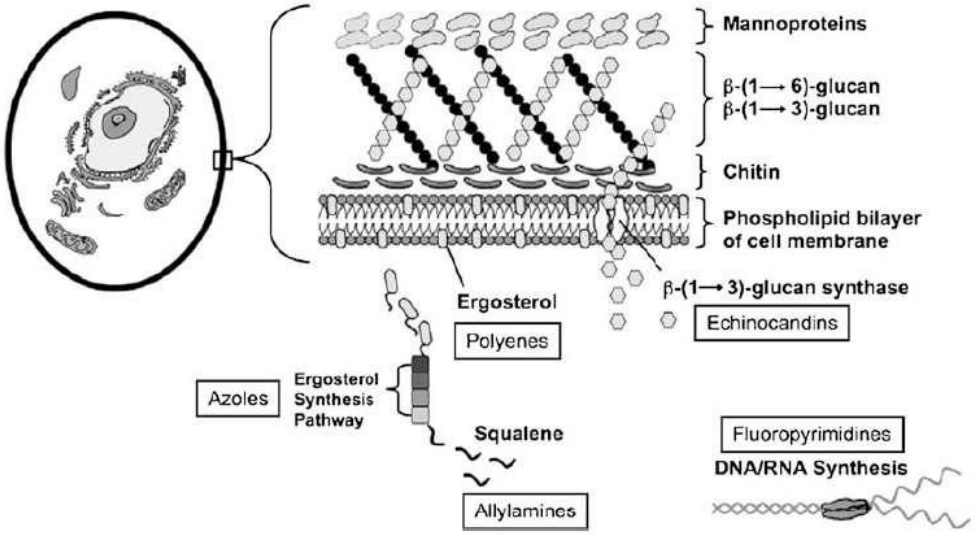


Figure 1.8 - Targets of antifungal agents. Adapted from Thompson *et al.* [60].

Since the 1950s several classes of antifungal drugs were developed, however, only four of them are currently used in clinical practice to treat *Candida* infections: fluorinated pyrimidine analogs, polyenes, azoles and echinocandins [29]. The extent of antifungal drug resistance varies for the different drug classes. In *Candida* species there is fairly limited resistance to the polyenes, and echinocandins, whereas resistance to the fluorinated pyrimidine analog - 5-Flucytosine (5-FC) and azoles is more common [29].

In this work the influence of 5-FC on the activity of the transcription factor CgPdr1 and on the expression of *CgFLR1*, *CgFLR2*, *CgTPO1_1* and *CgTPO1_2* genes were studied; furthermore, the role of the drug:H^+ antiporters CgFlr1, CgFlr2, CgTpo1_1 and CgTpo1_2 in the resistance to three of the four classes of antifungal drugs was assessed (Table 1.6).

Table 1.6 - Drugs used in this study and respective classes.

Antifungal class		Drug
Azoles	Imidazoles	Miconazole
		Thioconazole
		Clotrimazole
		Ketoconazole
	Triazoles	Fluconazole
		Itraconazole
Polyene		Amphotericin B
Fluorinated pyrimidine analog		5-Flucytosine
Dithiocarbamate		Mancozeb

1.3.1 Polyene – Amphotericin B

Amphotericin B has been widely used in clinical practice since the 1950s [61, 62], however its high toxicity, especially nephrotoxicity, probably due to a poor selectivity between fungal ergosterol and mammalian cholesterol, has compromised its use; but, for life threatening fungal disease, the polyene drug amphotericin B is a common choice despite toxic side-effects [63] due such characteristics indispensable for an effective drug such as high antifungal activity, broad antifungal spectrum fungicidal activity and very rare induction of fungal resistance as well as the ability to overcome multidrug resistance in fungi [64].

Polyenes, including amphotericin B are fungicide amphipathic drugs, with both hydrophobic and hydrophilic sides [65]. These antifungal drugs act by binding to ergosterol present in the plasma membrane, creating pores [65-67]. This action produces an altered permeability and leakage of vital cytoplasmic components, ending in the death of the cell [68].

Resistance to amphotericin B is rare among *Candida* species. Resistant isolates are confined mostly to non-*albicans* species such *C. krusei* and *C. glabrata*, what appears to be an exception is *C. lusitaniae* where intrinsic resistance is frequent [69, 70]. The mechanisms behind this resistance have not been studied in detail and since this drug targets ergosterol present in the plasma membrane, it is likely that a lack or decrease in the membrane content of this sterol, replacement of polyene-binding sterols and alteration in membrane sterol organization are involved in resistance [67]. Mutations in the enzymes involved in the ergosterol biosynthesis like Erg3, cause loss-of-function and have been associated with an accumulation of intermediates of the ergosterol biosynthesis pathway instead of ergosterol itself [65, 71], making it difficult for the drug to bind and create the pores that are ultimately responsible for fungal cell death [65, 66].

The *ERG6* gene also seems to be involved in the resistance to polyenes since the mutation in the *C. glabrata ERG6* gene was found to result in its poor polyene susceptibility [72].

1.3.2 Azoles

Azoles are five-membered nitrogen heterocyclic ring compounds containing at least one other non-carbon atom of either nitrogen, sulfur or oxygen and are the most used today when treating fungal infections, especially, topical imidazoles for mucosal or skin infections and oral-parenteral triazoles for invasive and refractory mucosal infections [47]. The azoles family can be classified into two groups: the imidazoles (clotrimazole, miconazole, ketoconazole and thioconazole) and the triazoles (fluconazole, itraconazole, voriconazole and posaconazole) [73] and the big difference between these two groups is the mechanism of inhibition of the cytochrome P-450 (CYP) dependent lanosterol 14-α-demethylase, while in imidazoles the N_3 compound binds to the heme iron CYP, the N_4 of the triazoles bind to the heme group. This confers to triazoles comparatively to imidazoles higher specificity [47].

Within the subfamily of the imidazole antifungal drugs, clotrimazole is widely used in treatment of superficial mycoses [74], vaginal infections and oral thrush [75]. Discovered in 1969, cannot be given parenterally because has poor oral absorption [76]. Ketoconazole is a synthetic antifungal drug normally used for life threatening systemic infections [77] and used mostly to prevent and treat fungal skin infections, especially in immunocompromised patients [78]. Discovered in 1978, this antifungal drug presents good oral absorption and a broad spectrum of activity [76]. Miconazole acts by combination of two mechanisms: ergosterol biosynthesis inhibition, the direct mechanisms of action of azole antifungal drugs, and direct membrane damage of the fungal cells [79]. Discovered in 1969, has poor bioavailability because of its poor dissolution and absorption in the gastrointestinal tract, but is a useful topical drug for the treatment of superficial mycoses; however, it is also given as a systemic antifungal agent when amphotericin B or ketoconazole are either ineffective or contraindicated [76, 80]. Thioconazole is mostly used in treatment of topical mycoses, in particular women's yeast vaginal infections [74, 78].

Within the subfamily of the triazole antifungal drugs, fluconazole is currently the most widely used antifungal azole drug due to excellent bioavailability, tolerability and low-level side effects. It is active against most *Candida* species with the exception of *C. glabrata* and *C. krusei* isolates [60]. Fluconazole was formulated in 1981 and it is available in both oral and intravenous

formulations, which have identical pharmacokinetics [76, 81]. Itraconazole, belonging to the the same class as fluconazole, is used to treat superficial and systemic fungal infections [60]. Discovered in 1986 it is an antifungal drug with broad spectrum of activity, good availability but is only available in oral form [60, 76].

The increase of the prophylactic use of azoles in recent years has led to an escalation of azole drug resistance occurrences [71]. The emergence of *C. glabrata* as one of the most prevalent pathogens responsible for candidemia parallels the introduction in the early 1990s of triazoles and of many imidazoles [47].

The mode of action of azole antifungal drug is based on the ergosterol biosynthesis pathway inhibition. Azoles act by inhibiting the CYP-dependent enzyme lanosterol 14α-demethylase, encoded by *ERG11*, necessary for the conversion of lanosterol to ergosterol [73]. This leads to the depletion of the ergosterol membrane content and to the accumulation of ergosterol precursors, such as toxic 14-α-methylated sterols (lanosterol, 4,14- dimethylzymosterol and 24-methylenedihydrolanosterol) [82]. The absence of ergosterol production leads to significant damage to the cell by increasing the cell membrane permeability, which can cause cell lysis and death [66].

Three mechanisms of secondary azole resistance have been described in *C. albicans*: reduced azole accumulation by active extrusion of the drug, alteration or overexpression of the binding site (lanosterol 14-α-demethylase, encoded by *ERG11*) and loss-of-function of enzymes downstream the ergosterol pathway (defective Δ-5,6-desaturase, encoded by *ERG3*), allowing the accumulation of less toxic sterols in the presence of azoles [33].

C. glabrata has emerged as a common cause of fungal infection and it is reported that this yeast has intrinsically low susceptibility to azole antifungals such as fluconazole [47]. In *C. albicans* the most prevalent mechanisms of resistance is active efflux depending on the genes that defined a role in the azole efflux [83]. *CDR1* and *CDR2* code for ABC transporters seem to reduce accumulation of many azoles and *MDR1*, encoding an MFS-MDR transporter, reduces accumulation of fluconazole [33].

Fluconazole is the most used azole antifungal drug due to low toxicity, availability in both oral and intravenous formulations and excellent activity *versus* most yeast species. 5-Flucytosine represents an attractive alternative or complement to azoles due to its excellent activity against *C. glabrata* [84].

1.3.3 Fluoropyrimidine – 5-Flucytosine

Pyrimidine analogs comprise a unique representative, 5-fluorocytosine (5-FC) [85] or flucytosine which is a synthetic antimycotic drug, was first synthetized in 1957 but its antifungal properties discovered in 1964 [84]. 5-Flucytosine has emerged as an attractive alternative or complement to azoles because it reveals an excellent activity against *C. glabrata* isolates [84], but it cannot be used in monotherapy and should always be combined with another antifungal, usually an azole or polyene drug [85]. It can be administrated both orally and intravenously and it is well tolerated in moderated doses, but when it is used in high doses it can be toxic [84] and the conversion of 5-FC to fluorouracil by gut bacteria contributes to its elevated toxicity [86].

5-Flucytosine is metabolized via the pyrimidine salvage pathway (Figure 1.9 – adapted from Edlind *et al.* [84]) where it acts as a subversive substrate with the subsequent production of toxic nucleotides and disruption of DNA and protein synthesis [84, 87]. Studies on susceptible fungi, as *S. cerevisiae* as a model, shows that 5-FC is taken up into the cell by one or more cytosine permeases (the most relevant is *FCY2* [88]) where it is converted to 5-fluorouracil (5-FU) by cytosine deaminase (encoded by *FCY1*) [89]. Subsequent modifications to 5-fluorouridine monophosphate (5-fluoro-UMP) by uracil phosphoribosyl transferase (UPRT) (encoded by *FUR1*) and to 5-fluoro-dUMP ultimately result in the disruption of protein and DNA synthesis [82, 84, 87].

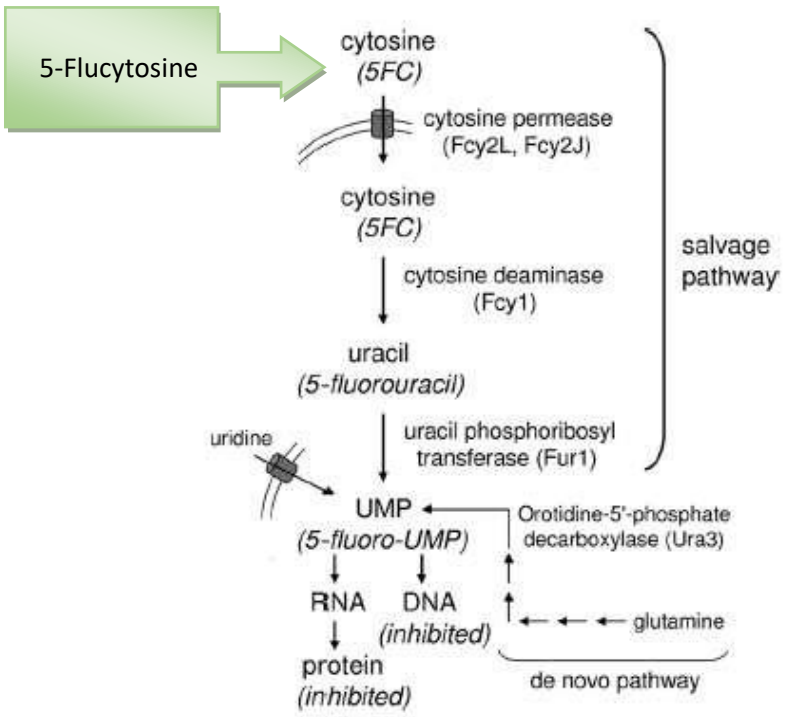


Figure 1.9 - Salvage pathway for 5-Flucytosine uptake and conversion to UMP (or 5-fluoro-UMP) in yeast. Also show (in abbreviated form) the alternative pathways for UMP production via the *de novo* pathway or uridine uptake, adapted from Edlind *et al.* [84]. UMP – uridine monophosphate.

In *Candida* species, resistance can be primary, when it is related with decreased drug uptake of the drug by cytosine permease, encoded by *FCY2* gene, and secondary, when there is a limitation in the conversion of 5-FC to 5-FU, or to 5-FUMP by alterations in enzyme cytosine deaminase or uracil phosphoribosyltransferase activity encoded by *FCY1* and *FUR1* genes, respectively [33, 67].

1.4 iTRAQ-based Proteomics

The identification and measurement of the changes occurring at the proteome level in response to stress is essential in the understanding of the underlying cellular processes.

Today, new quantitative approaches are emerging that utilize MS and stable isotope-labeling chemistry, offering a departure from traditional techniques employing comparative two-dimensional electrophoresis (2-DE) [90]. This tool is particularly relevant in the case of

membrane proteins, which are not detectable in 2-DE gels, because they are mostly insoluble in isoelectric focusing buffers. Ross *et al.* [90] developed a multiplex set of reagents for quantitative analysis that place isobaric mass labels at the N-termini and lysine side chains of peptides in a digest mixture (Figure 1.10). The reagents are differentially isotopically labeled such that all derivatized peptides are isobaric and chromatographically indistinguishable, but yield signature or reporter ions following collision-induced dissociation (CID) that can be used to identify and quantify individual members of the multiplex set. In this first study, a 4-plex system was developed, based on the use of four different isobaric tags, which enable the determination of relative proteins levels in three yeast extracts and provide the ability to measure the absolute quantity of specific target proteins through the use of internal peptide standards.

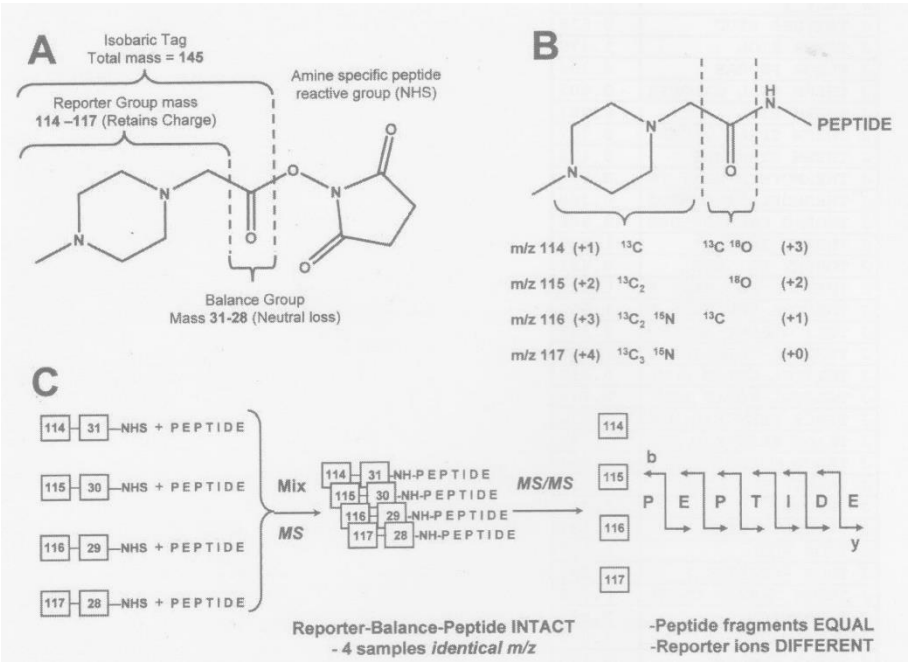


Figure 1.10 - Isobaric Tags for Relative and Absolute Quantification (iTRAQ). *A* shows the components of the multiplexed isobaric tagging chemistry which consist a reporter group, a balance group and a peptide-reactive group; *B* shows a differential isotopic atoms and *C* illustrate the isotopic tagging used to arrive at four isobaric combinations with four different reporter group masses, adapted from Ross *et al.* [90].

The chemistry of the complete molecule of multiplex isobaric tagging (Figure 1.10) consists of a reporter group (based on N-methylpiperazine), a mass balance group (carbonyl) and a peptide-reactive group [N-hydroxy succinimide (NHS) ester], that produce abundant MS/MS signature ions (Figure 1.11 - *m/z* 114.1, 115.1, 116.1 and 117.1) and the relative areas of these peaks correspond with the proportions of the labeled peptides. The use of isobaric peptides

circumvents the ambiguity encountered when trying to identify differentially labeled peptide pairs [90].

The protocol for labeling the tryptic peptides consists of total protein reduction and alkylation, digestion with trypsin, and derivatization of total peptide with the isobaric reagents. Proteins were identified on the basis of having at least one peptide whose individual ion score was $p<0.05$. The ratios of identified peptides from different strains were computed from signature ion peak areas [90].

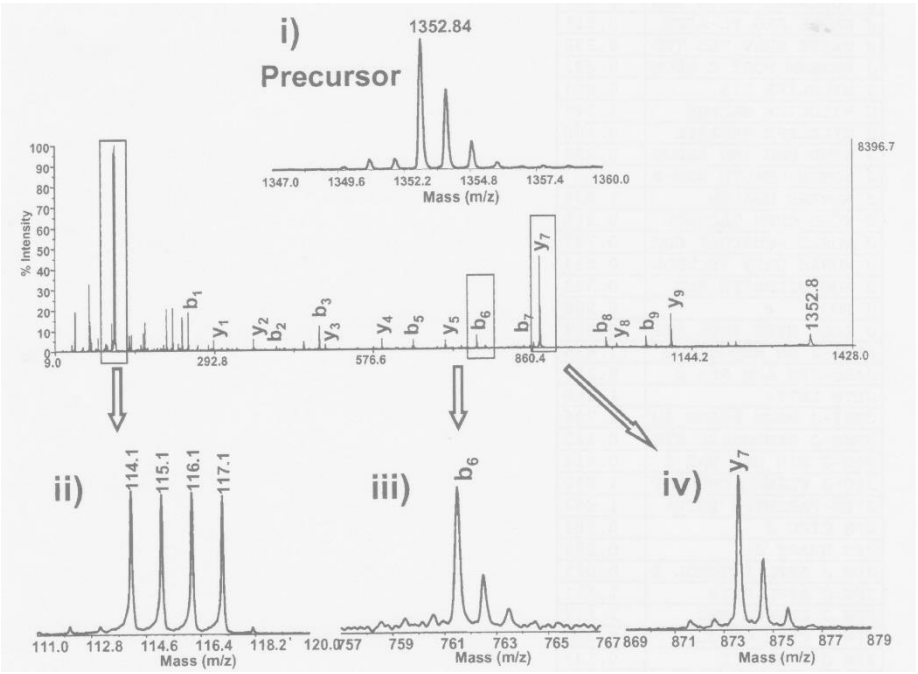


Figure 1.11 - Example MS/MS spectrum of peptide TPHPALTEAK from a protein digest mixture prepared by labeling four separate digests with each of the four isobaric reagents and combining the reaction mixtures in a 1:1:1:1 ratio. Components of the spectrum illustrated are (*i)* isotopic distribution of the precursor [(M+H)+, *m/z* 1352,84)], *(ii)* low mass region showing the signature ions used for quantitation, *(iii)* isotopic distribution of the b_6 fragment, and *(iv)* isotopic distribution of the y_7 fragment ion**, adapted from Ross *et al.* [90].**

2 Materials and Methods

2.1 Cell Culture

2.1.1 Strains

To accomplish the objectives of this study, a group of *C. glabrata* strains were used as described in Table 2.1. They were constructed and generously granted by Professor Hiroji Chibana, from the University of Chiba, Japan, and Professor Thomas Edlind [47], from the Drexel University, USA.

Table 2.1 - *Candida glabrata* strains used in this study.

Strain	Genotype/Description	Source
KUE100	Wild type	Hiroji Chibana, in collaboration
KUE100_*Δcgflr1*	*Δcgflr1*	
KUE100_*Δcgflr2*	*Δcgflr1*	
KUE100_*Δcgtpo1_1*	*Δcgtpo1_1*	
KUE100_*Δcgtpo1_2*	*Δcgtpo1_2*	
66032	Wild type	Thomas Edlind [47]
66032_*Δcgpdr1*	*Δcgpdr1*	

S. cerevisiae parental strain BY4741 (MATa, ura3Δ0, leu2Δ0, his3Δ1, met15Δ0) and the derived single deletion mutant BY4741_Δflr1 were obtained from Euroscarf (http://web.uni-frankfurt.de/fb15/mikro/euroscarf/). All the strains used in this study were stocked at -80 °C in rich growth medium Yeast Peptone Dextrose (YPD) (described in 2.1.3) supplemented with 30 % glycerol (v/v) (Merck). To obtain fresh cell culture, a portion of the frozen cellular material was transferred to plates of solid YPD and incubated at 30 °C until visible cell growth. The obtained cultures were then maintained at 4 °C until further use.

2.1.2 Cloning of the *C. glabrata CgFLR1_1* gene (*ORF CAGL0H06017g*).

The pGREG576 plasmid from the Drag & Drop collection [91] was used to clone and express the *C. glabrata* ORF *CAGL0H06017g* in *S. cerevisiae*, as described before for other heterologous genes [92-94]. pGREG576 was acquired from Euroscarf and contains a galactose inducible promoter (*GAL1*), the yeast selectable marker *URA3* and the *GFP* gene, encoding a Green Fluorescent Protein (GFPS65T), which allows monitoring of the expression and subcellular localization of the cloned fusion protein. *CAGL0H06017g* DNA was generated by PCR, using genomic DNA extracted from the sequenced CBS138 *C. glabrata* strain, and the following specific primers:

5’ – GAATTCGATATCAAGCTTATCGATACCGTCGACA*ATGTATATCGGTGCATTTCAGGAC* - 3’ and

5’ – GCGTGACATAACTAATTACATGACTCGAGGTCGAC*TCATGAATCTGGACTAAATCTTG* - 3’.

The designed primers contain, besides a region with homology to the first 24 and last 23 nucleotides of the *CAGL0H06017g* coding region (italic), nucleotide sequences with homology to the cloning site flanking regions of the pGREG576 vector (underlined). The amplified fragment was co-transformed into the parental *S. cerevisiae* strain BY4741 with the pGREG576 vector, previously cut with the restriction enzyme SalI, to obtain the pGREG576_*CgFLR1* plasmid. The recombinant plasmid pGREG576_*CgFLR1* was obtained through homologous recombination in *S. cerevisiae* and verified by DNA sequencing. The GAL1 promoter present in the pGREG576_*CgFLR1* plasmid was then replaced by the copper-induced MTI C. glabrata promoter, giving rise to the pGREG576_MTI_*CgFLR1* plasmid. The MTI promoter DNA was generated by PCR, using genomic DNA extracted from the sequenced CBS138 *C. glabrata* strain, and the following specific primers: 5’-TTAACCCTCACTAAAGGGAACAAAAGCTGGAGCTCTGTACGACACGCATCATGTGGCAATC -3’ and 5’-GAAAAGTTCTTCTCCTTTACTCATACTAGTGCGGCTGTGTTTGTTTTTGTATGTGTTTGTTG -3’. The recombinant plasmids pGREG576_*CgFLR1*, and pGREG576_MTI_*CgFLR1* were obtained through homologous recombination in *S. cerevisiae* and verified by DNA sequencing.

2.1.3 Growth media

The rich medium used for the cultivation of *C. glabrata* cells was YPD, containing, per litre: 20 g glucose (Merck); 20 g yeast extract (Himedia); 10 g peptone (Difco). *C. glabrata* cells minimal growth medium – MMG – containing, per litre: 20 g glucose (Merck); 2.7 g $(NH_4)_2SO_4$ (Merck); 1.7 g yeast nitrogen base (Difco) without amino acids or $(NH_4)_2SO_4$. No amino acids were added to this medium. *S. cerevisiae* cells were batch-cultured at 30 °C, with orbital agitation (250 rpm) in MMB (Minimal Medium for BY4741) medium, with the following composition (per liter): 1.7 g yeast nitrogen base without amino acids or NH_4^+ (Difco, Franklin Lakes, NJ), 20 g glucose (Merck, Kenilworth, NJ), and 2.65 g $(NH_4)_2SO_4$ (Merck), 20 mg / L methionine, 20 mg / L histidine, 60 mg / L leucine, 20 mg / L uracil (all from Sigma).

All the previous referred growth media were prepared in deionized water and autoclaved for 15 min at 121 ^oC and 1 atm. Solid media, derived from any of the above, were prepared by adding 20 $g.L^{-1}$ agar (Iberagar).

2.1.4 Antifungal drugs

Stock solutions of the antifungal drugs used in this study were prepared immediately before the drug was added to the medium. The antifungal drugs were obtained from Sigma and dissolved in Dimethyl Sulfoxide (DMSO) (Sigma); the concentrations of the stock solutions and range of drug concentrations used are in Table 2.2.

2.2 Susceptibility assays

The susceptibility to antifungal drugs of the several *C. glabrata* and *S. cerevisiae* laboratorial strains was assessed by spot assays.

Cell suspensions of the *C. glabrata* strains were prepared by cultivation in liquid MMG at 30 ^{o}C with orbital agitation (250 rpm), in the absence of drugs until the standardized culture Optical Density at 600 nm ($OD_{600\ nm}$) of 0.8 ± 0.08 was reached. In the case of *S. cerevisiae* cell suspensions, harboring the pGREG576 and pGREG576_*CgFLR1* plasmids, strains were prepared by cultivation in liquid MMB, without uracil for plasmid maintenance. The cultures were then diluted to a standardized $OD_{600\ nm}$ of 0.05 ± 0.005 and two subsequent dilutions of 1:5 and 1:25 were made.

The cell suspensions were applied as 4 µL droplets (spots) into the surface of the agarized MMG or MMB-U medium, supplemented with adequate concentrations of the antifungal drugs tested (Table 2.2).

Table 2.2 - Concentrations of the stock solutions and range of drug concentration used in the spot assays for 66032 and Δ*cgpdr1*[a] and spot assays for Kchr606 Wt, Δ*cgflr1* and Δ*cgflr2*[b].

Drug	Concentration of the stock solution (mg.mL^{-1})		Range of drug concentrations (µg.mL^{-1})	
	a)	b)	a)	b)
Itraconazole	10	10	60-100	120-160
Miconazole	1	1	0.25-0.75	0.5-0.75
Clotrimazole	50	50	5-10	12-17
Tioconazole	1	1	0.1-0.5	0.5.0.9
Ketoconazole	5	5	35-55	60-70
Fluconazole	60	20	125-175	200-275
5-Flucytosine	1	1	0.01-0.02	0.01-0.025
Amphotericin B	1	1	0.4-0.6	0.4-0.6
Mancozeb	Not tested	1	Not tested	3-4

The plates were prepared adding to the MMG medium the adequate volumes of the stock solutions referred (Table 2.2) and the controls were prepared with the addition of

DMSO (Sigma) but without the addition of drugs. After inoculation, the agar plates were incubated at 30 °C for 2 to 5 days, during such period the growth as visually inspected.

2.3 CgFlr1 Subcellular Localization Assessment

The subcellular localization of the CgFlr1 protein was determined based on the observation of BY4741 *S. cerevisiae* or KUE100. *C. glabrata* cells transformed with pGREG576_*CgFLR1*. These cells express the CgFlr1_GFP fusion proteins, whose localization was determined using fluorescence microscopy. Before that, the plasmids placed into the cell by the following method: Inoculate 5 mL of MMB-U with a colony 2–3 mm in diameter from a fresh plate and grow overnight at 30˚C with shaking (16-18 h). Dilute to an $OD_{600\ nm}$ of 0.2 ± 0.02 in 100 mL of fresh MMB-U and continue to grow until the $OD_{600\ nm}$ reaches 0.6 ± 0.06. Place cells in 50 ml tubes and centrifuge (1000 rpm, 5 min). Discard supernatants and thoroughly resuspend the cell pellets in 25 mL of distilled H_2O. Pool and centrifuge again (1000 rpm, 5 min). Decant supernatant and resuspend the cell pellet in 1 ml of freshly prepared, sterile 100 mM LiAc in TE. Transfer the cells to a 1.5 mL tube and centrifuge at top speed for 5 s. Remove the supernatant and add 500 µL of 100 mM LiAc in TE. In a separate 1.5 mL tube add 0.1 µg of plasmid DNA and 100 µg of salmon sperm DNA. Add 100 µL of yeast competent cells to each tube and mix well by vortexing. Add 600 µL of sterile freshly made PEG/LiAc (40 % PEG + 100 mM LiAc in 1x TE) solution to each tube and vortex at high speed for 10 s to mix. Incubate at 30°C for 30 min with shaking at 200 rpm. Add 70 µL of DMSO. Mix well by gentle inversion whitout vortex. Heat shock for 15 min in a 42°C water bath. Chill cells on ice for 1–2 min. Centrifuge cells (5 s, 14000 rpm) and remove the supernatant and resuspend the cells in 0.5 mL of sterile 1xTE buffer. Plate 100 µl and incubate plates up side down at 30°C until colonies appear.

S. cerevisiae cell suspension was prepared by cultivation in MMB-U medium, containing 0.5 % glucose and 0.1 % galactose, at 30 °C, with orbital shaking (250 rpm) until a standard culture $OD_{600\ nm}$ of 0.4 ± 0.04 was reached. At this point, cells were transferred to the same medium containing 0.1 % glucose and 1 % galactose, to induce protein expression.

C. glabrata cell suspensions were prepared in MMB-U medium, until a standard culture $OD_{600\ nm}$ of 0.4 ± 0.04 was reached and transferred to the same medium supplemented with 50 µm $CuSO_4$ (Sigma), to induce protein overexpression.

After 5 h of incubation, the distribution of CgFlr1_GFP fusion protein in *S. cerevisiae* or in *C. glabrata* living cells was detected by fluorescence microscopy in a Zeiss Axioplan microscope (Carl Zeiss MicroImaging, Oberkochen, Germany), using excitation and emission wavelength of 395 and 509 nm, respectively. Fluorescence images were captured using a cooled CCD camera (Cool SNAPFX, Roper Scientific Photometrics, Sarasota, FL).

2.4 Extraction and Multiplexed Membrane Protein Quantitation

2.4.1 Cell cultivation

Wild-type 66032 and *⊿cgpdr1* deletion mutant were cultured in 500 mL liquid MMG at 30 ºC with orbital agitation (250 rpm) in the absence of drugs until the standardized culture $OD_{600\ nm}$ of 0.8 ± 0.08 was reached. Adequate volumes of cellular suspensions were then harvested by filtration in order to obtain an $OD_{600\ nm}$ of 0.4 ± 0.04 for a volume of 1 L of liquid MMG medium. Cells were then transferred to fresh medium in the absence of stress (control conditions) or in the presence of 5-Flucytosine 4 µg/mL, and cultured for 1 h at 30 ºC with orbital agitation (250 rpm). Cells were harvested by centrifugation at 8000 rpm for 5 min at 4 ºC. The cell pellet was stocked at -80 ºC. This process was repeated to obtain independent biological triplicates.

2.4.2 Membrane proteome extraction

The cell pellets were resuspend in 2 mL of A Buffer (for each 2 mL of A Buffer must joined 100 µL of ABC protease inhibitors and 20 µL of D protease inhibitor) and an equal volume of glass beads was added. For 15 min, cycles of 30 s/30 s on ice/vortex were done, followed by incubation on ice for 5 min. Then, 1 mL of A Buffer was added and 10 min of 30 s/30 s on ice/vortex cycles were carried out. The mixture was centrifuged (8000 rpm, 5 min, 4 ºC) and the top phase collected and put on ice. The resulting pellet was resuspended in 2 mL of A Buffer and subjected to cycles of 30 s/30 s on ice/vortex. The mixture was centrifuged (8000 rpm, 5 min, 4 ºC) and this supernatant was put together with previous. A Buffer was added to this mix to a final volume of 8 mL. This final mix was ultracentrifuged on a Beckman XL-90 ultracentrifuge at 24000 rpm, 90 min, 4 ºC. The supernatant was removed and the pellet was washed with 8 mL of Na_2CO_3 (0.1 M) and incubated on ice for 30 min with orbital agitation (60 rpm). After that, the mixture was ultracentrifuged at 26000 rpm, 60 min, 4 ºC and the top

phase removed. The pellet was again washed with 8 mL of 50 mM Tetraethylammonium bromide (TEAB) and ultracentrifuged. This procedure was repeated 2 more times and, finally, the supernatant was resuspended in 325 µL of TEAB (50 mM) with urea (8 M).

2.4.3 Expression proteomic analysis using iTRAQ

Although this part of the work was carried out as a paid service, the whole procedure is described here, in detail. Samples were submitted in 8 M urea, 50 mM of TEAB at varying volumes that were adjusted to all equal 50 µL, adding 8 M urea. Sonicate each 15 s with 0.5 s pulsing and reduce proteins adding 5 µL of 50 mM of [tris(2-carboxyethyl) phosphine)] (TCEP). The mixture was incubated at 60 ^{o}C, 60 min and then was cooled; after that, was added 2.5 µL of 200 mM methyl methane thiosulfonate (MMTS) and incubate 10 min at room temperature. Finally, 140 µL of 500 mM TEAB was added to each sample, followed by 10 µL of 1 mg/mL Lys-C. After incubation at 37 ^{o}C, for 3 h, 10 µL of 1 mg/mL trypsin were added, followed by overnight incubation at 37 ^{o}C. For the peptide clean-up and quantitation, was done a macro-spin desalt the digests with C18 spin columns and dried the effluents in speed vac, then dissolve back up in 65 µL of 500 mM TEAB and remove 2 µL each for amino-acid analysis (AAA) quantitation (also will nanodrop 2 µL).

iTRAQ Labelling based on the AAA quantitation was performed as follows: mutant and wild-type pools were generated, and equal amounts of each sample for **three** 8-plex experiments were labeled. The three iTRAQ experiments were run through the SCX cartridge, after which the mass spectrometry analysis was performed on a TripleTOF 5600 mass spectrometer.

2.5 ^{3}H-5-Flucytosine accumulation assays

To assess whether the role of the transporters under study in 5-Flucytosine resistance is associated to a reduction in their intracellular accumulation, ^{3}H-5-Flucytosine accumulation assays were carried out in the *C. glabrata* wild type strain KUE100 and the derived *Δcgflr1*, *Δcgflr2*, *Δcgtpo1_1* and *Δcgtpo1_2* deletion mutants.

Cells were grown at 30 ^{o}C with orbital agitation (250 rpm) in minimal medium MMG until an $OD_{600\ nm}$ of 0.8 ± 0.08 was reached. Adequate volumes of the cellular suspensions were then harvested by filtration in order to obtain an $OD_{600\ nm}$ of 0.5 ± 0.05 in 2 mL of liquid MMG fresh medium. After being resuspended, 1.6 mL of cellular suspensions were transferred and 1 µM

of ^{3}H-labeled 5-FC (American Radiolabeled Chemicals, USA) and 3 mg/L of cold 5-FC (Sigma) were added to the cell suspension. Cells were then incubated for 30 min at 30 ^{o}C with orbital agitation (170 rpm). Intracellular and extracellular ^{3}H-5-Flucytosine were monitored in specific time intervals (1, 5, 10, 20 and 30 min). To access intracellular accumulation of ^{3}H-5-Flucytosine, 200 µL of cellular suspension were harvested at each time interval by filtration through pre-wetted glass microfibers (Whatman GF/C), washed twice with 4 mL of ice cold TM buffer [0.1 M MES hydrate (Sigma)] and immersed in 7 mL of scintillation liquid (Beckman). At the same time intervals, 70 µL of the cell suspension were centrifuged at 13000 rpm for 2 min and 50 µL of the supernatant obtained were collected and added to 7 mL of scintillation liquid (Beckman), to access the extracellular ^{3}H-5-Flucytosine concentration. The radioactivity was measured in a Beckman LS 5000TD scintillation counter.

2.6 Gene expression analysis

In this work Real Time RT-PCR was used to estimate the expression level of *CgFLR1*, *CgFLR2*, *CgTPO1_1* and *CgTPO1_2* genes. *C. glabrata* wild-type 66032 and derived *Δcgpdr1* deletion mutant cells were used to inoculate fresh MMG medium with and without 5 µg/mL 5-Flucytosine to an initial $OD_{600\ nm}$ of 0.2 ± 0.02. Growth curves of these cultures were followed by measuring culture $OD_{600\ nm}$ and cells were harvested after 1 h of incubation. The pellets obtained from centrifugation (8000 rpm, 4 ^{o}C, 5 min) were immediately frozen and stored -80 ^{o}C until further use.

2.6.1 Total RNA extraction and quantification

mRNA extraction from *C. glabrata* cells, cultivated as above mentioned, was performed using the hot phenol method [95] and acidic pH. To start, pellets were resuspended in 900 µL of AE buffer [10 mM Tris-HCl (Sigma), 0.5 mM EDTA (Aldrich)] and transferred into 2 mL eppendorfs. Then, 90 µL of SDS 10 % (w/v) (Sigma) and 800 µL of phenol (Sigma) were added to the suspension, followed by a short vortex. Incubation at 65 ^{o}C for 4 min was followed by incubation in dry ice until phenol crystals were visible. The mixture was centrifuged (15000 rpm, 4 ^{o}C, 5 min) and the top phase was collected. This was followed by two successive extractions with 450 µL of phenol (Sigma) and a 450 µL of a 24:1 chloroform/isoamilic alchool solution. The top phase was collected and 90 µL of sodium acetate 3 M (Merck, pH 5.3) was added. mRNA was purified by ethanol 100 % precipitation (1 mL) and kept at -20 ^{o}C for about

20 min. The product was then centrifuged (15000 rpm, 4 ^{o}C, 20 min) and the pellet washed with 750 µL of ethanol 70 % (v/v) and dried in a Speed Vacuum Concentrator Plus (Eppendorf). The obtained mRNA was dissolved in 50 µL of sterile water and stored at -20 ^{o}C until further use. All the material and solutions used in this protocol were treated with diethyl pyrocarbonate – DEPC (Sigma) to inhibit RNase activity.

Total RNA concentration of each sample was quantified in a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies) and dilutions of the samples were prepared, with sterile deionized water, so that the concentration of RNA used in the real time quantitative RT-PCR was 500 ng.µL^{-1}.

2.6.2 Real Time RT-PCR

Real Time RT-PCR is a technique that allows the quantification of mRNA levels from specific genes, based on a first step of reverse transcription followed by the measurement of cDNA amplification during PCR in real time. This real-time measurement is possible with the use of unspecific fluorophores, in this case SYBR® Green, which becomes fluorescent upon binding to double-stranded DNA. The data thus generated can be analyzed by computer software to calculate relative gene expression in several samples.

To analyze the expression of the *C. glabrata* genes *CgFLR1*, *CgFLR2*, *CgTPO1_1* and *CgTPO1_2*, on cells cultivated in the presence of inhibitory concentrations of 5-FC comparatively with cells cultivated in the absence of drugs, a real-time RT-PCR was performed with the RNA extracted previously (section 2.5.1). The first step of this procedure is the synthesis of cDNA from total RNA samples. In this step the MultiScribe™ reverse transcriptase is used to perform the reverse transcription of the RNA samples, in a mixture prepared as described in Table 2.3.

Table 2.3 - Real Time RT-PCR - First Step: Reverse transcription reaction mixture (Applied Biosystems).

Component	Volume per reaction (µL)	Final Concentration
TaqMan RT buffer (10X)	1.0	1X
$MgCl_2$ (25 mM)	2.2	5.5 mM
dNTP's mixture (2.5 mM)	2.0	500 µM per dNTP
Random hexamers (50 µM)	0.5	2.5 µM
RNase inhibitor (20 U.L^{-1})	0.2	0.4 U.L^{-1}
MuiltiScribe reverse transcriptase (50 U.µL^{-1})	0.25	1.25 U.µL^{-1}
Ribonucleic Acid (RNA) sample (500 ng.µL^{-1})	2	100 ng.µL^{-1}
RNase-free water	1.85	-
Total	**10**	**-**

The reverse transcription reaction was executed using a thermal cycler block (7500 Real-Time PCR System – Applied Biosystems) and accordingly to the parameters described in Table 2.4.

Table 2.4 - Real Time RT-PCR - First step: Thermal cycling parameters (Applied Biosystems).

	Step		
	Incubation	**Reverse transcription**	**Reverse transcriptase inactivation**
Time	10 min	30 min	5 min
Temperature	25 ºC	48 ºC	95 ºC

After finishing the thermal cycling, the cDNA samples were immediately placed on ice and stored at -20ºC until further use. In order to be used in the second step, the cDNA samples obtained were diluted so that the quantity of cDNA in each reaction was kept around 10 ng.

The second step of real time RT-PCR step was carried out using SYBR® Green reagents and the mixture used was prepared as described in Table 2.5.

Table 2.5 - Real Time RT-PCR - Second step: reserve transcription reaction mixture (Applied Biosystems).

Component	**Volume per reaction (µL)**
SYBR Green PCR master mix (2x)	12.5
Forward primer (4 pmol.µL^{-1})	2.5
Reverse primer (4 pmol.µL^{-1})	2.5
cDNA	2.5
Water	5.0
Total	**25**

The reaction was carried out using a thermal cycler block (7500 Real-Time PCR System – Applied Biosystems) following these same conditions: 10 min at 95 ºC, then 40 cycles repeating the set of two steps: 15 s at 95 ºC and 1 min at 60 ºC.

Five sets of primers were used to analyze each of the cDNA samples obtain in the first step. The five sets of primers correspond to the four genes under study, *CgFLR1*, *CgFLR2*, *CgTPO1_1*, *CgTPO1_2* and *CgACT1*, encoding the housekeeping protein actin (Table 2.6). Primers for the amplification of cDNA were designed using Primer Express Software (Applied Biosystems). The *CgACT1* mRNA expression was used as an internal control accounting for variability in the initial concentration and quality of the total RNA as well as in the conversion efficiency of the reverse transcription reaction.

Table 2.6 - Real Time RT-PCR - Second step: Primers of *CgACT1, CgFLR1, CgFLR2, CgTPO1_1* and *CgTPO1_2* genes.

Gene	Primer	Sequence
CgACT1	Forward	5'-AGAGCCGTCTTCCCTTCCAT-3'
	Reverse	5'-TTGACCCATACCGACCATGA-3'
CgFLR1	Forward	5'-TCTTATTCACGATGCTACAAATTGG-3'
	Reverse	5'-GAATCACAAGGCCAGCAAAGTT-3'
CgFLR2	Forward	5'-GCAGCGGCATTCCCATTAT-3'
	Reverse	5'-CGGGATACTTTTTTGTGCTCAAT-3'
CgTPO1_1	Forward	5'-CGCTGCTTCCCCAGTTATCT-3'
	Reverse	5'-CTAGCACCACGTCTACCGTAA-3
CgTPO1_2	Forward	5'- AGGACCCGCTCTATCGAAAAA-3'
	Reverse	5'- GCTGCGACTGCTGACTCAAC-3'

The measurement of gene expression in this second step is the binding of the SYBR ® Green 1 dye (contained in the SYRB® Green PCR master mix) with the double-stranded DNA. In the thermal cycle, when the DNA is denatured the dye is released and the fluorescence is drastically reduced. Then, during the extension, the primers anneal and the PCR product is generated, leading to the biding of the dye to the double-stranded product, which results in a not increase in the fluorescence detected by the instrument and registered in an amplification plot. The software used to perform this analyzes is the *7500 Systems SDS Software* from Applied Biosystems.

An example of an amplification plot is represented on Figure 2.1.

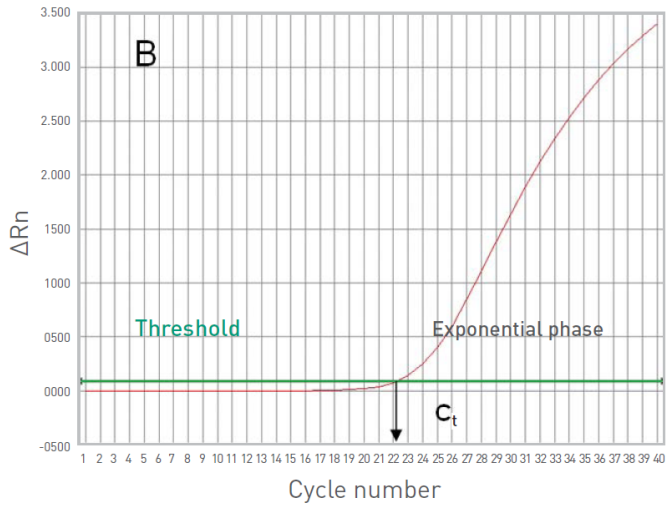


Figure 2.1 - Graphical representation of real-time PCR data. Rn is the fluorescence of the reporter dye divided by the fluorescence of a passive reference dye. ΔRn is Rn minus the baseline and is plotted against PCR cycle number, adapted from the Applied Biosystems "Real-time PCR: understanding Ct" application note" (http://www.thermofisher.com/content/dam/LifeTech/Documents/PDFs/PG1503-PJ9169-CO019879-Re-brand-Real-Time-PCR-Understanding-Ct-Value-Americas-FHR.pdf).

For each sample the software computes an amplification plot (Figure 2.1) from which a C_t value is calculated. C_t (threshold cycle) is the intersection between an amplification curve and a

threshold line (Figure 2.1). It is a measure of the concentration of target in the PCR reaction. The C_t values obtained for the target genes and the reference gene, *CgACT1*, and used to calculate relative expression fold-differences using the following equations:

$$\Delta C_t \text{ sample} = C_t \text{ (target)s} - C_t \text{ (reference)s}$$

$$\Delta C_t \text{ control} = C_t \text{ (target)c} - C_t \text{ (reference)c}$$

$$\Delta\Delta C_t = \Delta C_t \text{ sample} - \Delta C_t \text{ control}$$

$$\text{Fold difference} = 2^{-\Delta\Delta C_t}$$

3 Results

3.1 Role of the transcription factor CgPdr1 in the resistance to 5-Flucytosine and other antifungal drugs

In order to evaluate the involvement of the transcription factor CgPdr1 in the susceptibility to 5-FC and other antifungal drugs, spots assays were carried out. The susceptibility of the *⊿cgpdr1* strain was compared to that of the corresponding parental strain 66032 in the absence or presence of inhibitory drug concentrations (Figures 3.1 and 3.2). In these susceptibility assays the following drugs were used: fluconazole, ketoconazole, miconazole, clotrimazole, tioconazole, itraconazole, amphotericin B and 5-FC.

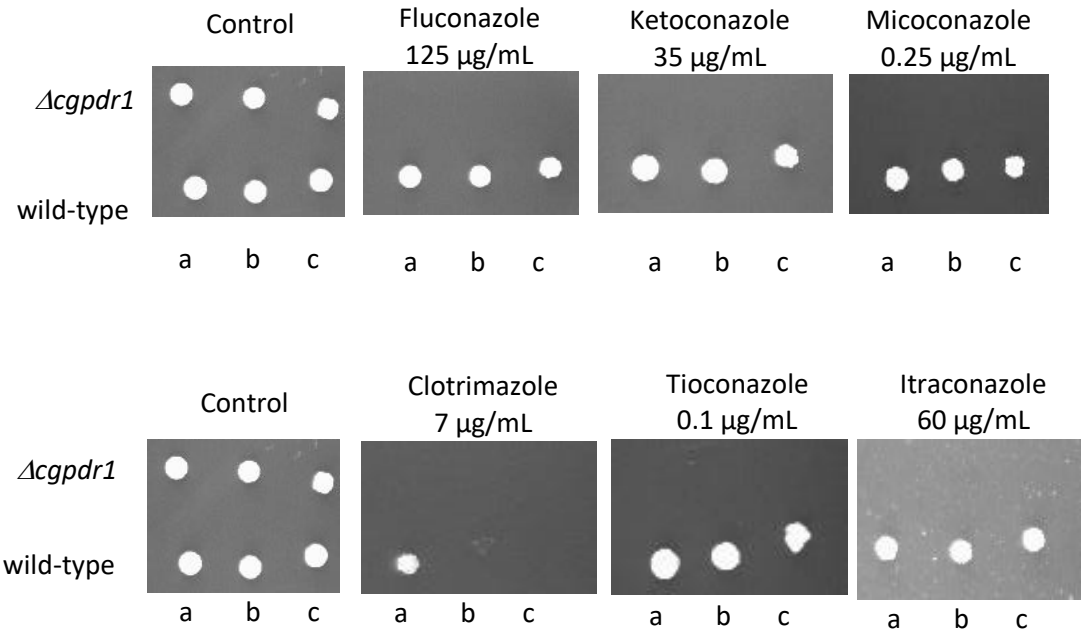


Figure 3.1 - Spot assays comparing the growth susceptibility to the azole antifungal drugs fluconazole, ketoconazole, miconazole, clotrimazole, tioconazole and itraconazole, at the indicated concentrations, of the 66032 and the mutant *⊿cgpdr1*, in MMB agar plates. Results of assays performed in the absence of drugs are presented as controls. Cell suspensions used to prepare the spots were grown in the absence of antifungal drugs, until an $OD_{600\ nm}$ of 0.4 ± 0.04 was attained (exponential phase). Cell suspension used to prepare the spots in (b) and (c) were 1:5 and 1:25 dilutions of the cell suspension used in (a) ($OD_{600\ nm}$ of 0.05 ± 0.005), respectively.

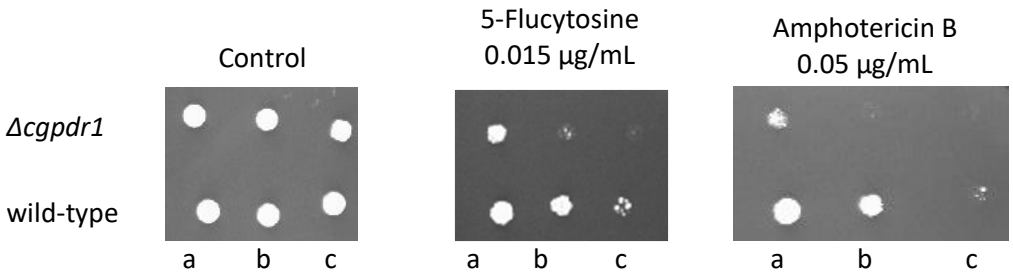


Figure 3.2 - Spot assays comparing the growth susceptibility to antifungal drugs 5-FC and amphotericin B, at the indicated concentrations, of the 66032 and the mutant *Δcgpdr1*, in MMB agar plates. Results of assays performed in the absence of drugs are presented as controls. Cell suspensions used to prepare the spots were grown in the absence of antifungal drugs, until an $OD_{600\ nm}$ of 0.4 ± 0.04 was attained (exponential phase). Cell suspension used to prepare the spots in (b) and (c) were 1:5 and 1:25 dilutions of the cell suspension used in (a) ($OD_{600\ nm}$ of 0.05 ± 0.005) respectively.

The transcription factor CgPdr1 was found to be involved in resistance to the azole drugs fluconazole, ketoconazole, miconazole, clotrimazole, tioconazole and itraconazole, as previously demonstrated [56, 96], but also to amphotericin B and 5-FC, since the growth of the *Δcgpdr1* mutant, in the presence of the selected antifungal drugs, is visibly inhibited comparatively to the growth observed for the wild-type strain (Figure 3.2).

3.2 Characterization of the *C. glabrata* membrane proteome

The membrane proteome of *C. glabrata* cells exposed to 5-Flucytosine-induced stress was compared to that of unstressed cells. The *C. glabrata* membrane enriched fraction was found to include 624 detectable proteins, comprising around 10 % of the predicted *C. glabrata* proteome of the first membrane proteome-wide analysis carried out in *C. glabrata* and is an invaluable repository of information on the functional analysis of these proteins.

Categorization, based on the biological process Gene Ontology, of the proteins identified in membrane-enriched fractions of *C. glabrata* cells, using the GoToolBox software (http://genome.crg.es/GOToolBox/), enabled the identification of the most statistically significant (p-value<0.01) GO terms enriched in the dataset under analysis (Figure 3.3).

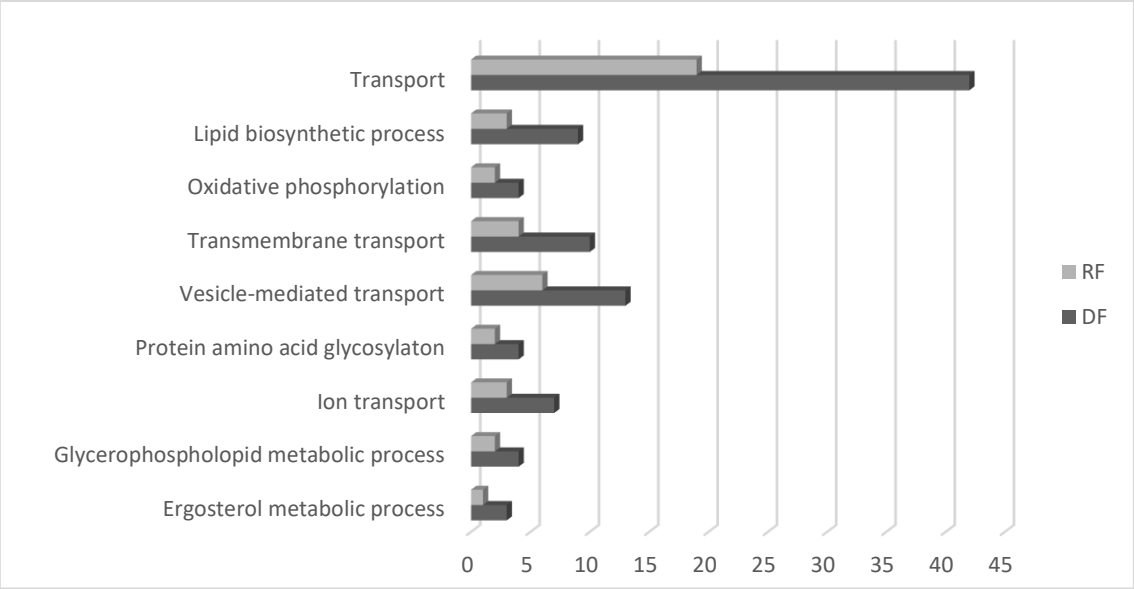


Figure 3.3 - Categorization, based on biological process taxonomy of Gene Ontology, of the proteins identified in membrane enriched fractions of *C. glabrata* cells. These genes were clustered using the GoToolBox software (http://genome.crg.es/GOToolBox/), and the most highly ranked statistically significant (p-value<0.01) GO terms are displayed. The frequency in dataset (DF) within each class is indicated by the black bars, compared to the frequency registered for the *C. glabrata* reference genome (RF), indicated by the grey bars, gene frequency being the percentage of the genes in a list associated to the specific Go term.

As expected, proteins recovered in the membrane-enriched fraction are related with membrane-associated metabolic processes, such as synthesis of ergosterol and phospholipids but also transmembrane transporters.

3.3 *C. glabrata* response to stress induced by 5-Flucytosine, at the membrane proteome level

In *C. glabrata* cells exposed to inhibitory concentrations of 5-FC when compared to the same cell growing in the absence of stress, 32 proteins were found to exhibit more than 1.5-fold increased or decreased concentrations. Among these membrane-associated proteins, 21 were found to display decreased concentrations and 11 increased concentrations (bold) in 5-FC challenged cells (Table 3.1).

Table 3.1 - Relative expression changes registered for membrane associate proteins in *C. glabrata* wild-type and *Δcgpdr1* strains upon exposure to 5-Flucytosine.

C. glabrata protein (ORF) name	*S. cerevisiae* homologs	Description of the function of the *C. glabrata* protein or of its *S. cerevisiae* homolog[a]	Wild-type fold change (upon 5-Flucytosine stress)	Δ*cgpdr1* fold change (upon 5-Flucytosine stress)
Glucose Metabolism				
PDC1	*PDC1*	Major of three pyruvate decarboxylase isozymes	0.54	0.39
CAGL0L01485g	*GSF2*	ER localized integral membrane protein that may promote secretion of certain hexose transporters, including Gal2p	0.60	0.43
PGK1	*PGK1*	3-phosphoglycerate kinase, key enzyme in glycolysis and gluconeogenesis	0.20	0.63
CAGL0L02497g	*FBA1*	Fructose 1,6-bisphosphate aldolase, required for glycolysis and gluconeogenesis	0.58	0.47
CAGL0G06138g	*YCK1*	Palmitoylated plasma membrane-bound casein kinase I isoform; involved in morphogenesis, proper septin assembly, endocytic trafficking, and glucose sensing	0.44	0.90 *
Mitochondrial Function				
CAGL0I03190g	*RIP1*	Ubiquinol-cytochrome-c reductase; a Rieske iron-sulfur protein of the mitochondrial cytochrome bc1 complex	**1.68**	0.28
CAGL0F04213g	*AAC2*	Major ADP/ATP carrier of the mitochondrial inner membrane	0.64	1.00
MDM10	*MDM10*	Subunit of both the ERMES complex that links the ER to mitochondria, that functions in import and assembly of outer membrane beta-barrel proteins	0.51	0.91
CAGL0L06490g	*PHB2*	Subunit of the prohibitin complex (Phb1p-Phb2p), a inner mitochondrial membrane chaperone that stabilizes newly synthesized proteins	0.63	0.85
YIM1	*YIM1*	Protein of unknown function; null mutant displays sensitivity to DNA damaging agents	0.24	0.48

* Fold change outside of the chosen cut-off intervals (0.71 < fold change < 1.4).

[a] Description according to CandidaGenome or YeastGenome databases.

Up regulated proteins are indicated in bold.

Table 3.2 (continuation) - Relative expression changes registered for membrane associate proteins in *C. glabrata* wild-type and *Δcgpdr1* strains upon exposure 5-Flucytosine.

C. glabrata **protein (ORF) name**	***S. cerevisiae*** **homologs**	**Description of the function of the *C. glabrata* protein or of its *S. cerevisiae* homolog[a]**	**Wild-type fold change (upon 5-Flucytosine stress)**	***Δcgpdr1*** **fold change (upon 5-Flucytosine stress)**
Amino Acid / Nucleotide Metabolism				
CAGL0B03047g	*ILV5*	Bifunctional acetohydroxyacid reductoisomerase and mtDNA binding protein; involved in branched-chain amino acid biosynthesis	0.50	0.70
URA3	*URA3*	Orotidine-5'-phosphate (OMP) decarboxylase, involved in the biosynthesis of pyrimidines; converts 5-FOA into 5-fluorouracil, a toxic compound	0.44	2.92
URA1	*URA1*	Ortholog(s) have dihydroorotate oxidase (fumarate) activity, role in 'de novo' pyrimidine nucleobase biosynthetic process	0.26	2.62
Ribosome components and Translation Machinery				
DBP2	*DBP2*	ATP-dependent RNA helicase of the DEAD-box protein family	**3.94**	2.15
CAGL0E03938g	*RPL4B*	Ribosomal 60S subunit protein L13B	**1.56**	1.30
CAGL0K07414g	*RPL20A/RPL20B*	Protein component of the small (40S) ribosomal subunit	**1.70**	0.97
CAGL0J03234g	*RPS24B*	Ribosomal 60S subunit protein L4B	**1.52**	1.05
NOP1	*NOP1*	Nucleolar protein, component of the small subunit processome complex, which is required for processing of pre-18S rRNA	**1.64**	0.86
RPS16	*RPS16A/RPS16B*	Protein component of the small (40S) subunit	**1.97**	1.27
CAGL0G01078g	*RPL33B*	Protein component of the small (40S) ribosomal subunit	**1.78**	1.37
CAGL0E02013g	*RP28A*	Protein component of the small (40S) ribosomal subunit	0.59	0.48
CAGL0L06886g	*RPL13B*	Ribosomal 60S subunit protein L13B	0.62	0.96
CAGL0A03278g	*RPL19B*	Ribosomal 60S subunit protein L19B	0.34	0.55

[a] Description according to CandidaGenome or YeastGenome databases

Up regulated proteins are indicated in bold.

Table 3.3 (continuation) - Relative expression changes registered for membrane associate proteins in *C. glabrata* wild-type and *Δcgpdr1* strains upon exposure 5-Flucytosine.

C. glabrata **protein (ORF) name**	***S. cerevisiae*** **homologs**	**Description of the function of the *C. glabrata* protein or of its *S. cerevisiae* homolog[a]**	**Wild-type fold change (upon 5-Flucytosine stress)**	***Δcgpdr1* fold change (upon 5-Flucytosine stress)**
Lipid Metabolism				
CAGL0L03828g	*CYB5*	Cytochrome b5, involved in the sterol and lipid biosynthesis pathways; acts as an electron donor to support sterol C5-6 desaturation	**2.14**	0.98
CHO2	*CHO2*	Phosphatidylethanolamine methyltransferase (PEMT), catalyzes the first step in the conversion of phosphatidylethanolamine to phosphatidylcholine	**1.56**	1.56
CAGL0K03509g	*HFD1*	Hexadecenal dehydrogenase; involved in the conversion of sphingosine 1-phosphate breakdown product hexadecenal to hexadecenoic acid	0.29	0.24
Cell wall assembly				
CAGL0M08206g	*YJL171c*	GPI-anchored cell wall protein of unknown function	0.59	0.50
Multidrug Resistance Transporters				
CAGL0H06017g CgFLR1	*FLR1*	Plasma membrane transporter of the major facilitator superfamily; member of the 12-spanner drug:H(+) antiporter DHA1 family	**2.08**	1.89 **
CAGL0I04862g CgSNQ2	*SNQ2*	Plasma membrane ATP-binding cassette (ABC) transporter, multidrug transporter involved in multidrug resistance and resistance to singlet oxygen species	0.61	0.75
CAGL0M01760g CgCDR1	*PDR5*	Plasma membrane ATP-binding cassette (ABC) multidrug transporter actively regulated by Pdr1p; also involved in steroid transport	0.30	0.1
CAGL0G00242g	*YOR1*	Predicted plasma membrane ATP-binding cassette (ABC) multidrug transporter	0.51	0.44
CgQDR2	*QDR2*	Plasma membrane transporter of the major facilitator superfamily; member of the 12-spanner drug:H(+) antiporter DHA1 family	0.57	0.31

** Fold change quantification considered as not reliable (p-value > 0.05).

[a] Description according to CandidaGenome or YeastGenome databases

Up regulated proteins are indicated in bold.

Based on their function, predicted based on homology to their *S. cerevisiae* counterparts, proteins whose expression was seen to change in *C. glabrata* cells exposed to 5-FC were clustered into seven groups: glucose metabolism, mitochondrial function, amino acid/nucleotide metabolism, cell wall assembly, ribosome components and translation machinery, lipid metabolism and multidrug resistance transporter (Figure 3.4)

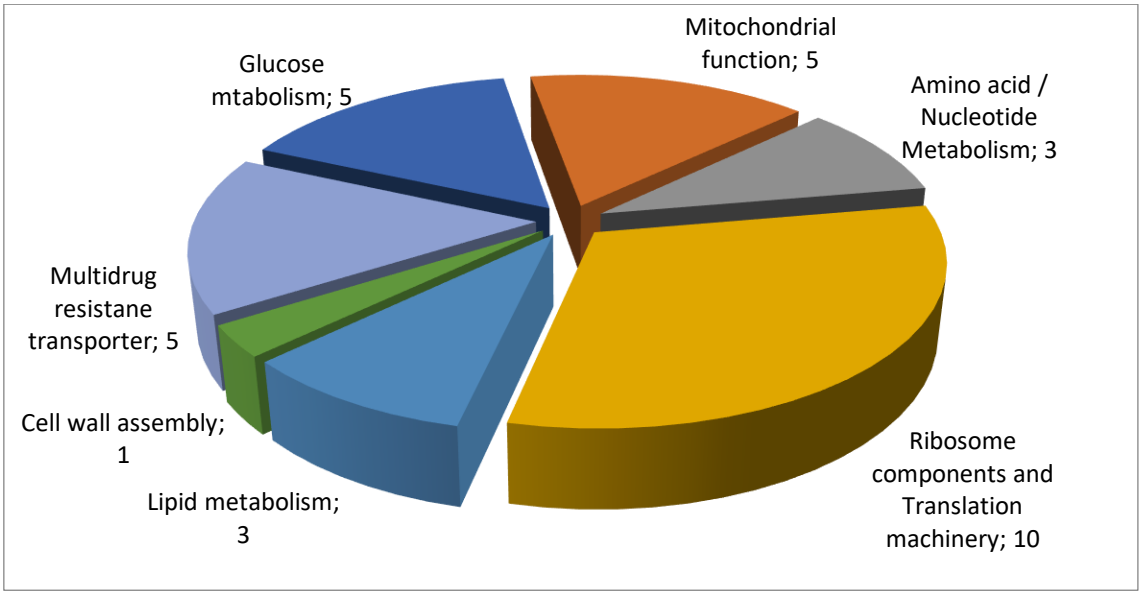


Figure 3.4 - Major functional groups found to have significant expression changes in the membrane-enriched proteome upon exposure to 5-FC in *C. glabrata*.
Proteins with significant expression changes include Ribosome components and translation machinery (10 proteins), Glucose metabolism (five proteins), Multidrug resistance transporter (five proteins), Mitochondrial function (five proteins), Amino acid/Nucleotide Metabolism (three proteins), Lipid metabolism (three proteins) and Cell wall assembly (one protein).

The expression of glucose metabolism and mitochondrial function related proteins is mostly repressed in the presence of 5-FC. The expression of one amino acid biosynthetic protein, Ilv5, and two pyrimidine biosynthetic proteins, Ura1 and Ura3, was also found to decrease in cells exposed to this antifungal agent. The expression of proteins involved in ribosome biogenesis and translation was found to increase in 5-FC stressed cells. Additionally, changes in the expression of a few proteins involved in lipid and cell wall metabolism were registered (Table 3.1), that adaptation to exponential growth in the presence of toxic concentrations of 5-FC lead to extensive cell wall remodeling and strengthening [97].

Finally, a group of five multidrug transporters was found to exhibit altered levels of expression in 5-FC stressed cells. Four of them, previously implicated in azole drug resistance [46, 47, 94, 98],

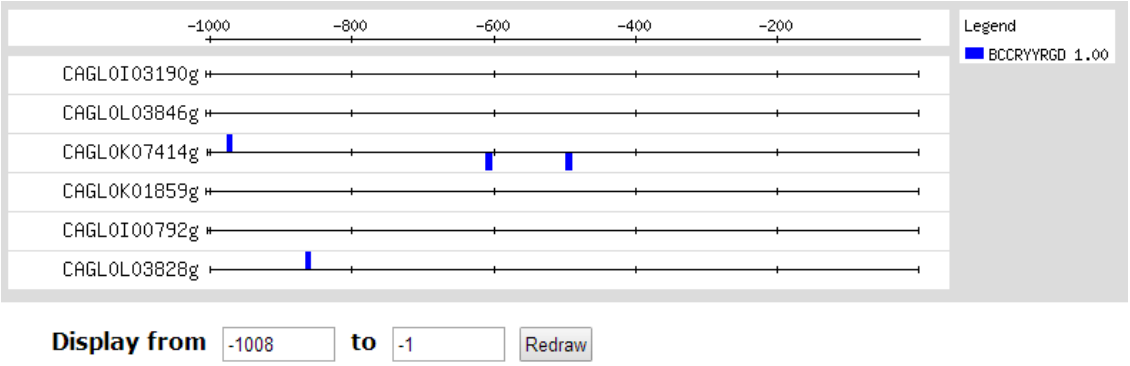
were actually found to display decreased concentrations, while the fifth, CgFlr1, was found to exhibit a more than 2-fold increased concentrations upon *C. glabrata* exposure to 5-FC.

3.4 Role of CgPdr1 in the *C. glabrata* response to 5-Flucytosine

The analysis of the membrane-enriched fraction of the *C. glabrata* proteome obtained from cells exposed to 5-Flucytosine in the absence of the transcription factor CgPdr1 was assessed and compared to that of the *C. glabrata* wild type cells exposed to 5-Flucytosine. Among the 32 proteins whose expression was seen to change in the wild-type strain, eight proteins - CgPgk1, CgYck1, CgAac2, CgMdm10, CgYim1, CgUra1, CgUra3, CgRpl13B and CgRpl19A - were found to be at least 1.5-fold repressed by CgPdr1, possibly in an indirect fashion, while eight proteins were found to be at least 1.5-fold activated by CgPdr1 (Table 3.1). For the remaining 18 proteins, no significant change could be detected in the current experiment.

Among the eight proteins which were found to be positively controlled by CgPdr1, CgCdr1 and CgQdr2 had been previously characterized as direct targets of CgPdr1 action [46, 94, 99]. The remaining six, include Cyb5, involved in ergosterol biosynthesis, Rip1, a component of the mitochondrial respiratory chain, and Dbp2, Nop1, Rpl20B and Rps16A, components of the translational machinery, had not been identified so far as CgPdr1 targets. In order the evaluate the possibility that the expression of these six proteins is directly controlled by CgPdr1, the occurrence of CgPdr1 binding sequences was assessed in the promoter regions of the encoding genes [100]. Interestingly, CgPdr1 binding sites were found in the *CYB5* (CCCGTCAGG in position -858) and *RPL20B* (TCCACTAGT, CCCATTGGT and TCCATTAGT in positions -969, -603 and -490, respectively) promoter regions (Table 3.2), suggesting that at least these two genes are indeed directly up-regulated by CgPdr1 in *C. glabrata* cells responding to 5-Flucytosine.

Table 3.4 - Analysis of the promoter sequences of the proposed six new CgPdr1 targets. The CgPdr1 binding sequences found to occur in the analysed promoters are displayed in blue. The analysis was carried out using the RSAT tools (http://rsat-tagc.univ-mrs.fr/rsat/).



3.5 Evaluation of the role of four *C. glabrata* DHA transporters, CgFlr1, CgFlr2, CgTpo1_1 and CgTpo1_2, in the resistance to 5-Flucytosine and other antifungal drugs

Based on the identification of CgFlr1 as displaying increased concentration in response to 5-FC stress in *C. glabrata*, the role of this protein, and of its closest homologs CgFlr2, CgTpo1_1 and CgTpo1_2, in 5-FC resistance was investigated. To do so, the effect of the deletion of *CgFLR1* or *CgFLR2* in *C. glabrata* susceptibility to 5-FC and other antifungal drugs was assessed. Based on the obtained results, an attempt to evaluate the subcellular localization of the encoded proteins was carried out and the effect of the expression of these four genes in the accumulation of ^{3}H-5-Flucytosine was assessed. Finally, the effect of 5-FC in the expression of the genes under study was evaluated at the transcript level.

3.5.1 Characterization of the effect of CgFlr1 and CgFlr2 expression in antifungal drug resistance

The involvement of CgFlr1 and CgFlr2 in antifungal drug resistance was evaluated, through susceptibility assays, considering a total of nine antifungal drugs of four different families were tested. Specifically, six azole antifungal drugs, the imidazoles: miconazole, clotrimazole, tioconazole and ketoconazole and the triazoles: fluconazole and itraconazole, the polyene antifungal drug amphotericin B, the fluoropyrimidine antifungal drug 5-Flucytosine and the dithiocarbamate fungicide mancozeb were used.

The results obtained reveal that *CgFLR2* confers resistance to all tested azoles, except for miconazole and itraconazole, and to amphotericin B, while *CgFLR1* does not (Figure 3.5). Both *CgFLR1* and *CgFLR2* were found to confer resistance to 5-FC, although the effect of *CgFLR2* is much stronger (Figure 3.6). Finally, *CgFLR1* confers resistance to Mancozeb, whereas *CgFLR2* does not (Figure 3.6).

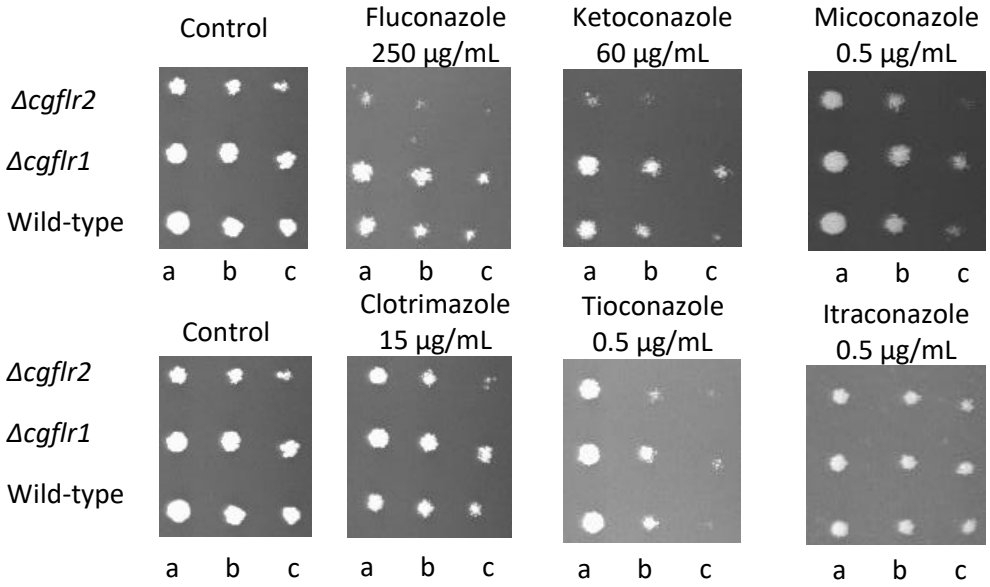


Figure 3.5 - Spot assays comparing the growth susceptibility to the azole antifungal drugs fluconazole, ketoconazole, miconazole, clotrimazole, tioconazole and itraconazole, at the indicated concentrations, of the *Δcgflr1* and *Δcgflr2*, in MMB agar plates. Results of assays performed in the absence of drugs are presented as controls. Cell suspensions used to prepare the spots were grown in the absence of antifungal drugs, until an $OD_{600\ nm}$ of 0.4 ± 0.04 was attained (exponential phase). Cell suspension used to prepare the spots in (b) and (c) were 1:5 and 1:25 dilutions of the cell suspension used in (a) ($OD_{600\ nm}$ of 0.05 ± 0.005), respectively.

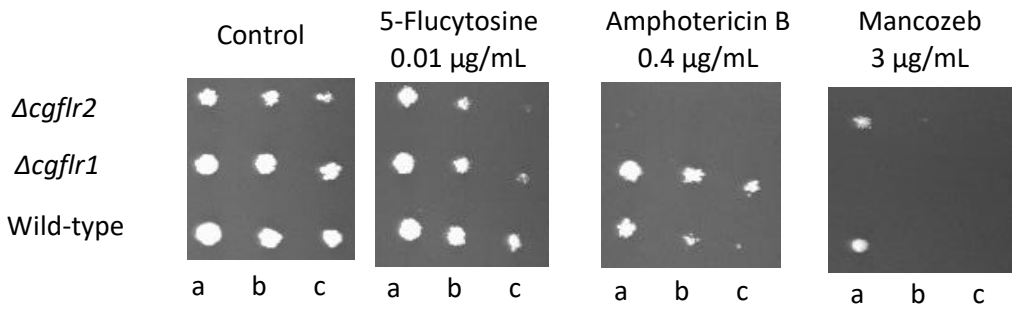


Figure 3.6 - Spot assays comparing the growth susceptibility to antifungal drugs 5-FC, amphotericin B and mancozeb, at the indicated concentrations, of the *Δcgflr1* and *Δcgflr2*, in MMB agar plates. Results of assays performed in the absence of drugs are presented as controls. Cell suspensions used to prepare the spots were grown in the absence of antifungal drugs, until an $OD_{600\ nm}$ of 0.4 ± 0.04 was attained (exponential phase). Cell suspension used to prepare the spots in (b) and (c) were 1:5 and 1:25 dilutions of the cell suspension used in (a) ($OD_{600\ nm}$ of 0.05 ± 0.005), respectively.

3.5.2 CgFlr1_1 is targeted to the plasma membrane in *S. cerevisiae*, being able to complement its homolog Flr1

The importance of the transporters under analysis in antifungal drug resistance was verified by complementation experiments, except for the case of *FLR2*, whose cloning was not finished in time for this thesis. In this study, the role of CgFlr1 was further evaluated, using *S. cerevisiae* as a heterologous host. *S. cerevisiae* BY4741 wild-type or *Δflr1* cells harboring the pGREG576_ *CgFLR1* plasmid were grown to mid-exponential phase in minimal medium, and then incubated in the same medium containing 0.1 % glucose and 1 % galactose, to promote protein over-expression. In these conditions the expression and localization of CgFlr1 was analyzed through fluorescence microscopy. The CgFlr1_GFP fusion was found to be predominantly localized to the cell periphery (Figures 3.7 and 3.8). Control cells, on the other hand, harboring the pGREG576 cloning vector, displayed a slight and uniform distribution of fluorescence, like what can be observed as the host cells auto-fluorescence. Since CgFlr1 is predicted to be an integral membrane protein, these results strongly suggest a plasma membrane localization, like what was observed for its *S. cerevisiae* homolog Flr1.

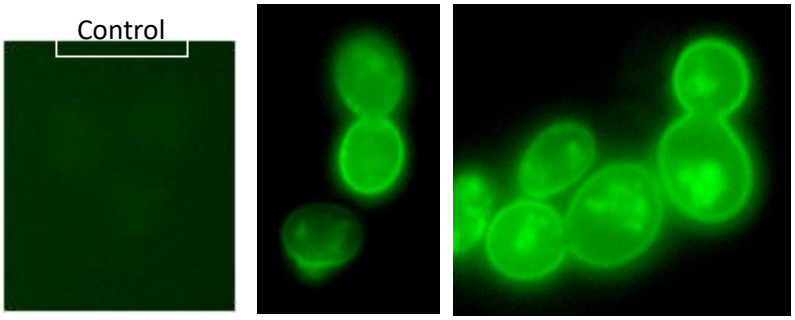


Figure 3.7 – Fluorescence of exponential phase BY4741 *S. cerevisiae* cells, harboring the expression plasmid pGREG576_*CgFLR1*. The expression and localization of CgFlr1 was analyzed through fluorescence microscopy and suggest a plasma membrane localization.

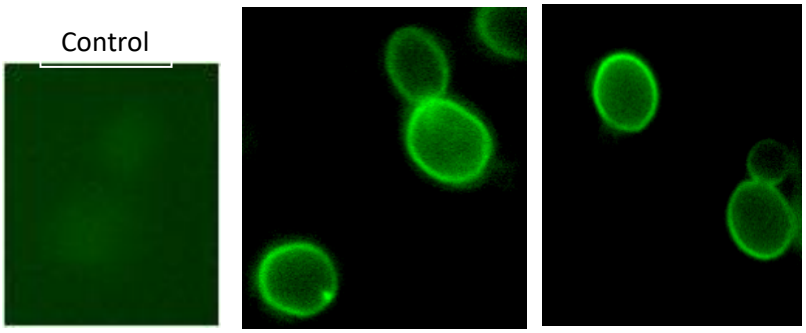


Figure 3.8 – Fluorescence of exponential phase KUE100 *C. glabrata* cells, harboring the expression plasmid pGREG576_MTI_*CgFLR1*. The expression and localization of CgFlr1 was analyzed through fluorescence microscopy and suggest a plasma membrane localization.

Based on spot assays, *CgFLR1* was found to confer resistance to 5-Flucytosine and mancozeb in *S. cerevisiae* (Figure 3.9). Significantly, *CgFLR1* expression appears to complement the 5-Flucytosine and mancozeb susceptibility phenotypes exhibited by the deletion of its *S. cerevisiae* homologue *FLR1* (Figure 3.9).

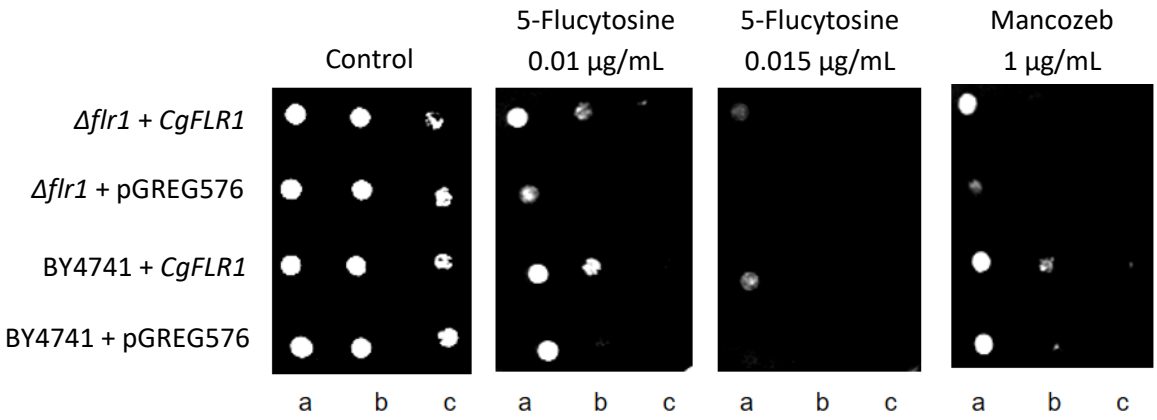


Figure 3.9 - Spot assays comparing the growth susceptibility to antifungal drugs 5-Flucytosine and mancozeb, at the indicated concentrations, of the *Δflr1* and BY4741 with *CgFLR1* or pGREG576, in MMB agar plates. Results of assays performed in the absence of drugs are presented as controls. Cell suspensions used to prepare the spots were grown in the absence of antifungal drugs, until an $OD_{600\ nm}$ of 0.4 ± 0.04 was attained (exponential phase). Cell suspension used to prepare the spots in (b) and (c) were 1:5 and 1:25 dilutions of the cell suspension used in (a) ($OD_{600\ nm}$ of 0.05 ± 0.005) respectively.

3.5.3 Assessment of the contribution of *CgFLR1, CgFLR2, CgTPO1_1* and *CgTPO1_2* to 5-Flucytosine accumulation

After verifying the involvement of *CgFLR1*, *CgFLR2*, *CgTPO1_1* and *CgTPO1_2* in the resistance to 5-FC, the possible involvement in 5-FC accumulation of *C. glabrata* cells was assessed.

From the obtained results it is visible that there is an increased intracellular accumulation of 5-FC in cells devoid of CgFlr1 or CgFlr2, but not in the absence of CgTpo1_1 or CgTpo1_2 (Figures 3.10 and 3.11).

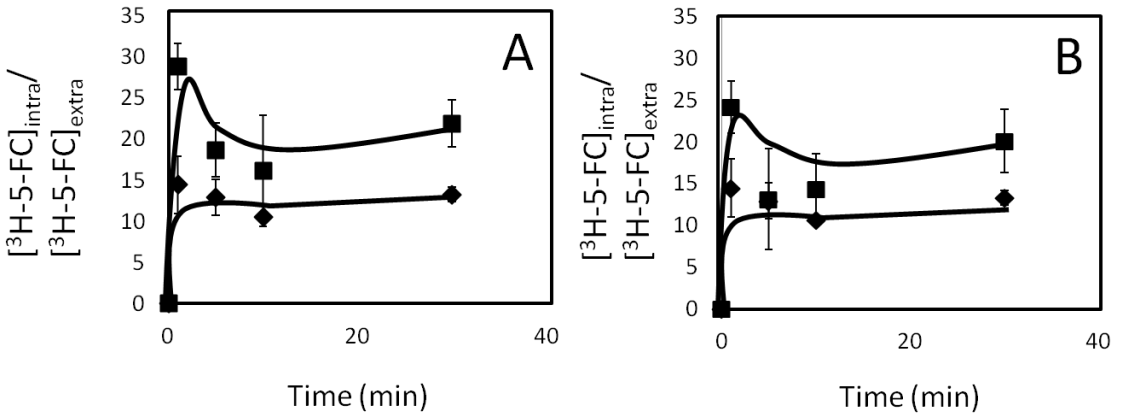


Figure 3.10 - Time-course accumulation of 5-FC in strains Kchr606 (♦) wild-type and *Δcgflr1* (■) (A) and Kchr606 (♦) and *Δcgflr2* (■) (B), in the presence of radiolabelled ^{3}H-5-Flucytosine. Error bars were calculated based on the standard deviation for each sample.

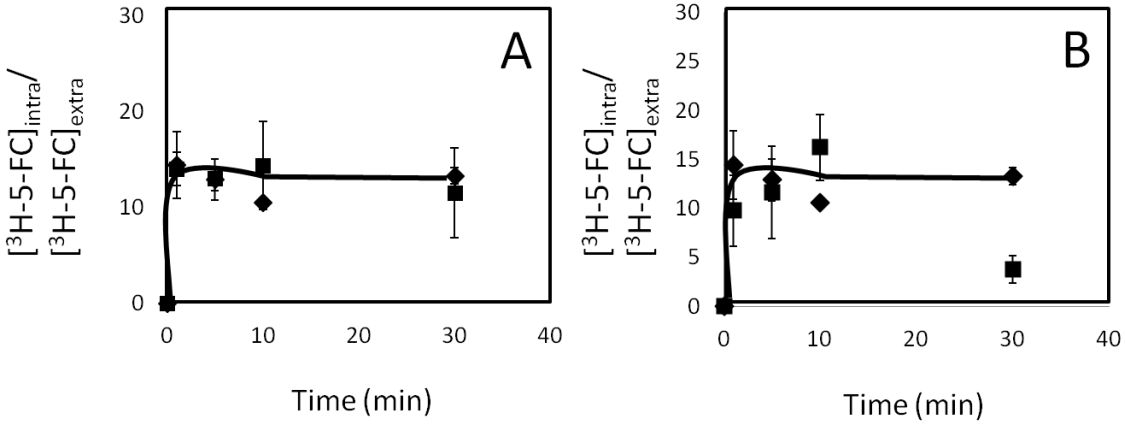


Figure 3.11 - Time-course accumulation of 5-FC in strains Kchr606 (♦) wild-type and *ΔCgtpo1_1* (■) (A) and Kchr606 (♦) and *ΔCgtpo1_2* (■) (B), in the presence of radiolabelled ^{3}H-5-Flucytosine. Error bars were calculated based on the standard deviation for each sample.

This observation may imply that CgFlr1 and CgFlr2 exert their action in 5-FC resistance directly by affecting its transport across the plasma membrane, while CgTpo1_1 and CgTpo1_2 appear to affect 5-FC resistance indirectly.

3.5.4 Transcript levels of genes *CgFLR1, CgFLR2, CgTPO1_1* and *CgTPO1_2*

When cells are subject to stress, they often induce the expression of the genes required for the resistance to that stress. RT-PCR was used to study the effect of 5-FC stress exposure and of the transcription factor CgPdr1 on the transcript levels of the genes *CgFLR1*, *CgFLR2*, *CgTPO1_1* and *CgTPO1_2*, in control conditions and upon exposure to 5-FC.

From the obtained results, there appears to be a 1.8-fold up-regulation of *CgFLR1* gene upon exposure to 5-FC, which is consistent with the results obtained in the expression proteomics analysis. No change in the expression of the remaining genes could be observed in wild-type cells upon exposure to 5-FC (Figure 3.12). The expression of the four genes appears to be constitutively higher in the *Δcgpdr1* deletion mutant cells which is most likely due to an indirect effect.

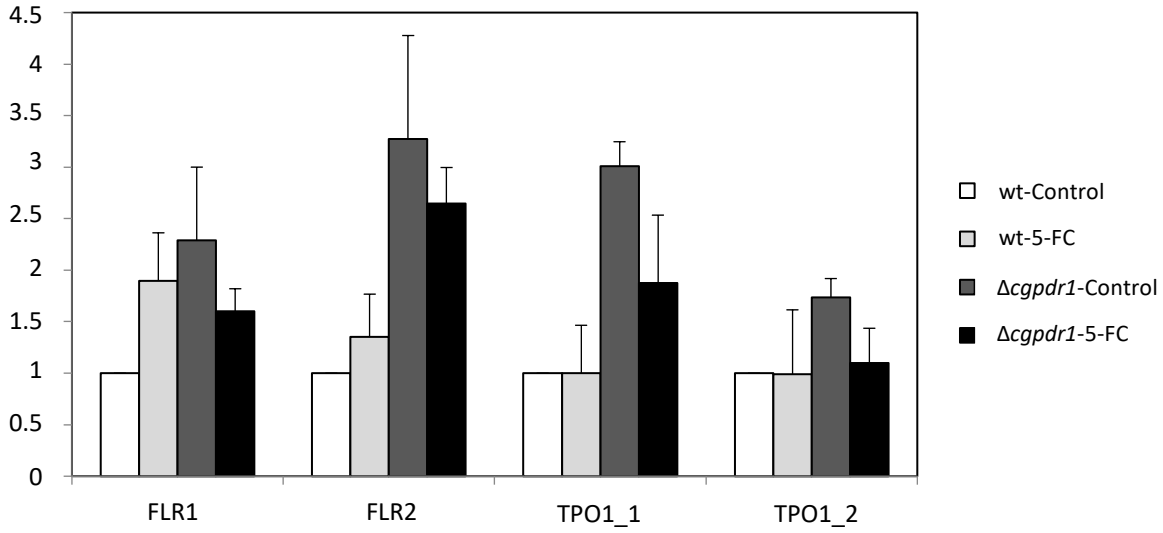


Figure 3.12 – Comparison of the variation of the expression of *CgFLR1, CgFLR2, CgTPO1_1* and *CgTPO1_2* by real-time analysis, in the 66032 *C. glabrata* wild-type strain (wt) and in the 66032u_ Δ*cgpdr1* deletion mutant *Δpdr1* strains, after exposure to 5-Flucytosine 5 µg/mL. Expression of *CgACT1* gene was used as internal control. The presented transcript levels were obtained by quantitative RT-PCR and are relative *CgFLR1/CgACT1, CgFLR2/CgACT1, CgTPO1_1/CgACT1* or *CgTPO1_2/CgACT1* mRNA,relative to the values registered in the 66032 parental strain in control conditions. The indicated values are averages of at least two independent experiments. Error bars show standard deviation (*p < 0.5)*.

4 Discussion

The antifungal drug 5-FC is a drug that has fallen into disuse due to the rapid acquisition of resistance by fungal pathogens and to its moderate toxicity in humans, limiting the administration of higher dosages. The identification of the mechanisms underlying these phenomena are thus, crucial to consider the reintroduction of 5-FC as a therapeutic approach.

In this work, the changes that occur at the level of the membrane proteome in *C. glabrata* cells exposed to 5-FC were characterized, leading to the identification of new mechanisms of resistance to this antifungal (summarized in Figure 4.1, adapted from [93, 97]).

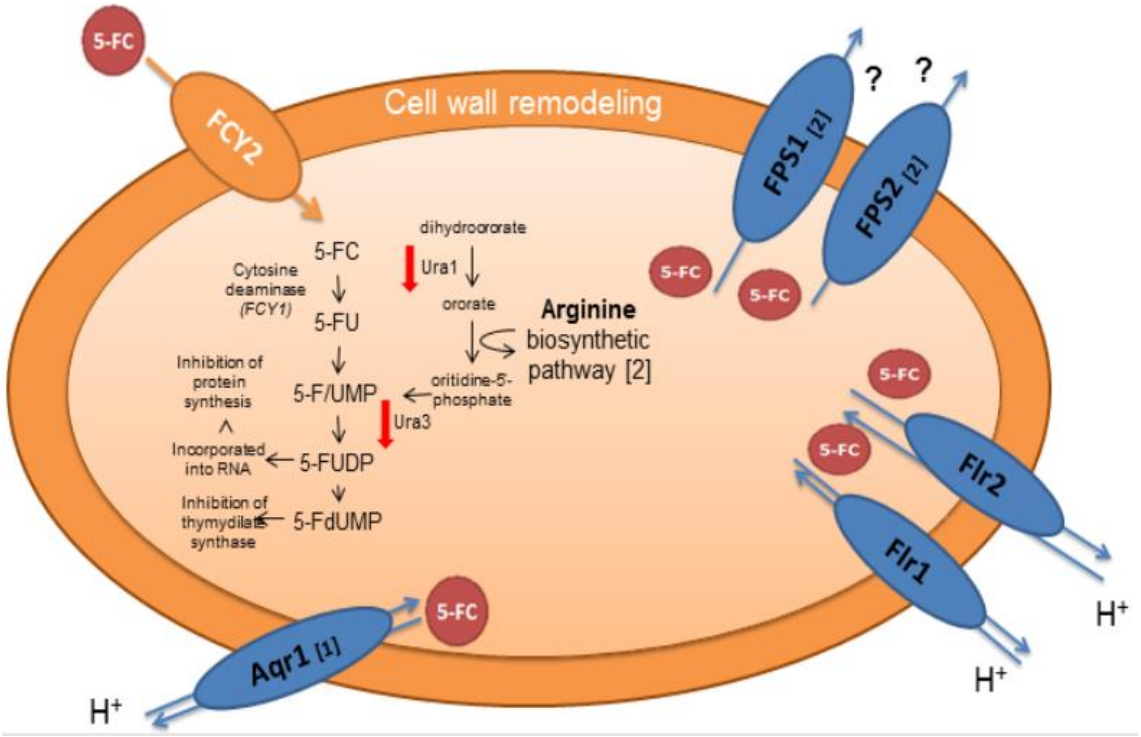


Figure 4.1 - Current model of the mechanisms of resistance to 5-FC in *C. glabrata*, including the new data generated in this study [93 97].

One of the most interesting aspects of this work concerns the identification of CgPdr1 transcription factor as a determinant of resistance to 5-FC. This transcription factor is considered a major cause of resistance to azoles [99], but, based on the results of this study, it becomes clear that its activity may underlie the simultaneous acquisition of multiple antifungal drugs. The role of transcription factor CgPdr1 in 5-FC resistance probably relates to the fact it regulates about 50 % of the membrane proteome response to 5-FC. Interestingly, the traditional targets of CgPdr1, including the ABC drug efflux pumps CgCdr1, CgYor1 and CgSnq2

are suppressed in response to 5-FC, which suggests that, although being active in the response to azoles and to 5-FC, the action of Pdr1 at the level of transcriptional control appears to be different. It will be interesting to test whether this differential outcome of CgPdr1 activity is linked to differences in terms of the conformation of this transcription factor, whose activation is known to occur by the direct binding to the drug molecule [101]. It appears reasonable to hypothesize that binding to different compounds are likely to affect differently the final conformation of the transcription factor altering the final result of its action.

A large proportion of the 5-FC response, at the membrane proteome level, was found to be related to RNA and protein metabolism. Interestingly, an overall increased expression of ribosome and translation associated genes was observed, which is exactly the opposite of the typical general stress response [102]. This unusual behavior may be related to the specific mechanism of action of 5-FC. In both *S. cerevisiae* and *Candida* yeast cells, 5-FC is converted to 5-fluorouracil, which is then converted into phosphorylated 5-fluorouridylic acid and incorporated into RNA, resulting in the perturbation of both RNA metabolism and in the disruption of protein synthesis [87]. It is thus reasonable to assume that the RNA- a protein-metabolism-related genes identified herein as responding to 5-FC challenge. This is also consistent with the results from previous microarray analyses of the transcriptome-wide *S. cerevisiae* [103] or *C. glabrata* [104] response to 5-FC, which highlighted the relevance of RNA metabolism. It is also in agreement with a previous chemogenomic analysis of the determinants of 5-FC resistance in the model yeast *S. cerevisiae*, in which about one fourth of the determinants of resistance to this drug were found to be related to RNA and protein metabolism [97]. Overall, it appears that *C. glabrata* cells try to compensate, with the increased expression of translation associated proteins, the detrimental effect that 5-FC exerts in this process.

Another interesting feature of the proteomics response includes the down-regulation of the nucleotide biosynthesis related proteins Ura1 and Ura3. Ura1 catalyses the synthesis of ororate, which is, upon conversion to oritidine-5-phosphate, funneled into the production of UMP (Uridine Monophosphate), while Ura3 catalyses the conversion of UMP to UDP (Uridine Diphosphate), which is then used for RNA synthesis. This same pathway is used to process 5-FC into its toxic products, including 5F-UDP, which upon incorporation in RNA molecules will inhibit protein synthesis. It appears, thus, that the cell responds to 5-FC induced stress by decreasing the expression of enzymes required for its conversion to toxic 5-FC products. In this context, it is also interesting to point out that the *Δcgpdr1* mutant exhibits very high levels of

Ura1 and Ura3, when compared to the parental strain, which may contribute to the susceptibility phenotype displayed by this mutant strain.

Finally, among the results obtained from the membrane proteomics analysis, the role of the MDR transporter CgFlr1, and of its homologs, in 5-FC response was further analysed. Both CgFlr1 and CgFlr2 were found to confer resistance to 5-FC, apparently due to their role in controlling the levels of 5-FC accumulation within *C. glabrata* cells. Interestingly, CgFlr2 was further found to confer resistance to azoles and amphotericin B placing this transporter at the intersection of multiple antifungal resistance mechanisms. These two transporters, belonging to the DHA family in *C. glabrata*, constitute, thus, two additional players in the antifungal drug resistance phenomenon. Our group had previously shown that the acquaglyceroporins CgFps1 and CgFps2 [97], as well as the DHA transporters Aqr1 [93] are determinants of 5-FC resistance as well, suggesting that 5-FC extrusion is an important mechanism of resistance against this antifungal drug and showing this phenomenon to be the consequence of the additive contribution of several players. Surprisingly, in the case of CgTpo1_1 and CgTpo1_2 this study shows that their contribution does not affect directly the intracellular concentration of the drug. It will be interesting to see what is the role played by Tpo1_1 and Tpo1_2 transporters, indirectly leading to the acquisition of resistance to 5-FC.

In conclusion, the results described in this study highlight the importance of MDR transporters from the MFS in antifungal resistance phenotypes, particularly concerning 5-FC. The characterization of *C. glabrata* CgFlr1 and CgFlr2 MDR transporters involved in 5-FC drug resistance reinforces the need to study the remaining members of this family in this increasingly relevant pathogenic yeast, given that these transporters are likely to have clinical impact [32]. This work further highlights the importance of genome/proteome-wide approaches in the identification of new antifungal resistance mechanisms. The newly identified processes stand out as promising targets for the development of new 5-FC chemosensitizers, which would expectedly allow for the use of decreased therapeutic dosages of 5-FC, limiting the development of 5-FC resistance which usually happens very fast and enabling a more extensive (re)use of this antifungal drug.

5 References

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