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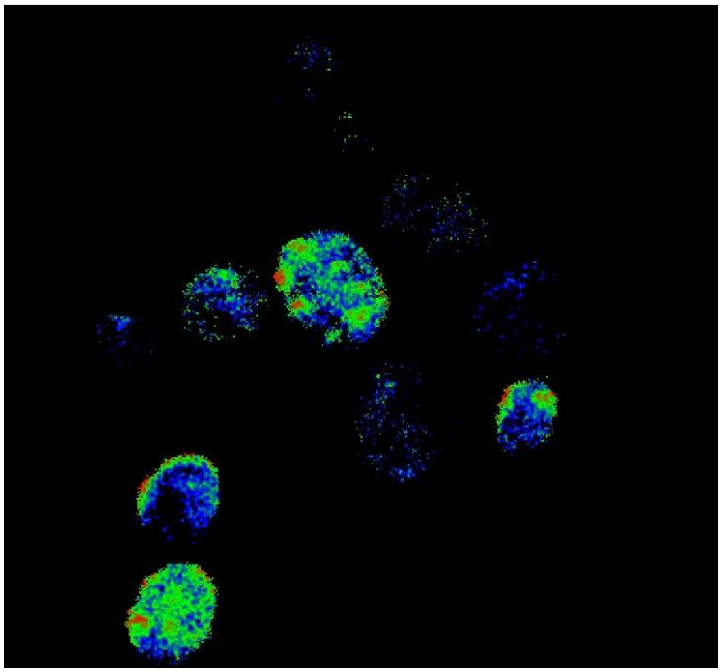

ABC and MFS transporters involved in multistress defense mechanisms in the model eukaryote *Saccharomyces cerevisiae*: roles of Pdr18 and Tpo1

Cláudia Sofia Pires Godinho

Supervisor: Doctor Isabel Maria de Sá-Correia Leite de Almeida

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Thesis approved in public session to obtain the PhD Degree in Biotechnology and Biosciences

Jury Final Classification: Pass with Distinction and Honour

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Jury

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ABSTRACT

The experimental model and industrially-relevant yeast species *Saccharomyces cerevisiae* provides a unique platform for the understanding of the mechanisms underlying yeast adaptation and tolerance to different stresses, which are frequently conserved among more complex eukaryotes. The action of transporters of the ATP-binding cassette (ABC) superfamily and the Major facilitator superfamily (MFS) is among those mechanisms, by presumably catalysing the extrusion of drugs and xenobiotic compounds. However, the action of these putative drug pumps is currently questioned and the notion that the role of these transporters may be indirect, and they may play endogenous biological roles that influence drug partition in the cell, is gaining momentum. The role that has been attributed to several plasma membrane ABC transporters in plasma membrane lipid homeostasis is one of those biological functions. This thesis work focuses on the mechanisms behind *S. cerevisiae* adaptation to stress induced by acetic and benzoic acid, two weak acids of different lipophilicity and thus different toxicity, both of high relevance in Biotechnology and the Food Industry.

The plasma membrane ABC transporter Pdr18 was found to be a determinant of yeast tolerance to acetic acid stress, and this phenotype was related with Pdr18 physiological role in ergosterol transport at yeast plasma membrane. Pdr18 was found to counteract acetic acid-stress induced decrease of plasma membrane lipid order, increase of the non-specific membrane permeability and decrease of transmembrane electrochemical potential. The coordinated transcriptional activation of *PDR18* and of several ergosterol biosynthetic genes during the response to acetic acid stress was demonstrated, providing additional support to the role of Pdr18 in ergosterol homeostasis by mediating ergosterol transport and plasma membrane lipid remodelling.

Pdr18 evolutionary history was also investigated in this work by examining 117 yeast strains genomes from 29 species across the Saccharomycetaceae family. The paralogous status of Pdr18 and Snq2 was confirmed and 214 Snq2/Pdr18 homologs were found in the genomes examined. Phylogenetic and neighbourhood analysis resulted in the proposal of the occurrence of a single duplication event in the common ancestral of the *Saccharomyces* genus yeasts to be on the origin of *PDR18* and *SNQ2*, followed by translocation of *PDR18* to chromosome XIV. Phenotypic profiling of the deletion mutants for *ScPDR18* and *ScSNQ2*, concerning susceptibility to a wide range of toxic compounds, indicated that ScPdr18 confers tolerance to almost all compounds tested (weak acids, herbicides, alcohols, clinical and agricultural fungicides, cations, mutagens, anti -arrythmic and -malarial compounds and polyamines), whereas ScSnq2

exhibits a narrower spectrum of compounds to which it confers tolerance (the herbicides barban, alachlor and metolachlor, the cations Li^{+} and $Mn^{2+,}$ the mutagen 4-NQO, the anti-malarial quinine, and the polyamine spermine). The species *Candida glabrata* that has diverged previously to the duplication event on the origin of Pdr18 and Snq2 only encodes one Pdr18/Snq2 homolog, the CgSnq2. Phenotypic profiling of *Cgsnq2*Δ indicates that although a major overlap in multidrug/multixenobiotic resistance profiling is found with ScSnq2, an overlap with ScPdr18 was observed concerning azole tolerance. These results provided insights into the functional divergence of ScPdr18, and ScSnq2 and the corresponding ancestral gene.

The MFS transporter Tpo1 was described in this work to be determinant of yeast tolerance to benzoic acid, a more hydrophobic weak acid than acetic acid. The transcript levels from the *TPO1* gene were found to increase 30-fold after one hour of exposure to benzoic acid stress, and this activation was found to be directly dependent on the amino acid-sensing transcription factors Gcn4 and Stp1. Benzoic acid-induced stress was found to induce a decrease in the intracellular levels of polyamines and of the amino acids glutamine, arginine and lysine, independently of *TPO1* expression. The decrease in the levels of nitrogenous compounds was considered to be the trigger of *TPO1* transcriptional activation registered under benzoic acid stress.

Altogether, the results of this thesis work provide insights and an integrative view on the role and evolution of plasma membrane ABC and MFS transporters, the regulatory networks behind their action, and of the importance of plasma membrane lipid composition and physical properties in yeast adaptation and tolerance to stress induced by the weak acids acetic and benzoic acids, of relevance in Biotechnology and Food Industry.

Keywords: *Saccharomyces cerevisiae*, plasma membrane ergosterol homeostasis, multidrug/multixenobiotic resistance transporters, multistress response, weak acids

RESUMO

A levedura modelo e microrganismo industrial *Saccharomyces cerevisiae* constitui uma plataforma de eleição para o estudo dos mecanismos de adaptação e tolerância de leveduras a diferentes agentes de stress, os quais são frequentemente conservados em eucariotas mais complexos. A ação dos transportadores de membrana das superfamílias ABC e MFS é um desses mecanismos, presumivelmente pela catálise da expulsão de drogas e compostos xenobióticos. Contudo, a ação destas putativas bombas de efluxo tem vindo a ser recentemente questionada, e a noção de que a ação destes transportadores pode ser indireta pelo desempenho de funções endógenas que influenciam a partição de drogas na célula, está a ganhar destaque. O papel atribuído a alguns transportadores ABC de membrana plasmática na homeostase de lípidos da mesma é um exemplo das funções biológicas endógenas desempenhadas por estes transportadores. O trabalho apresentado nesta tese está focado nos mecanismos de adaptação de *S. cerevisiae* a stress induzido pelos ácidos acético e benzoico, dois ácidos fracos com diferente lipofilicidade e, portanto, diferentes níveis de toxicidade para as células de levedura. Estes ácidos fracos são também relevantes na Biotecnologia e na Indústria Alimentar.

O transportador ABC de membrana plasmática Pdr18 foi identificado como determinante para a tolerância da levedura a stress induzido por ácido acético, e este fenótipo foi relacionado com o papel fisiológico deste transportador no transporte de ergosterol na membrana plasmática de levedura. A ação do Pdr18 demonstrou contrariar a diminuição da ordem lipídica da membrana plasmática, o aumento da permeabilidade inespecífica da membrana e a diminuição do potencial eletroquímico transmembranar, efeitos induzidos pelo stress gerado por ácido acético. Identificou-se uma ativação transcricional coordenada do gene *PDR18* e de vários genes da via de biossíntese do ergosterol durante a resposta ao stress induzido por ácido acético, o que suporta o papel do transportador Pdr18 na homeostase de ergosterol mediado pelo transporte de ergosterol e remodelação lipídica da membrana plasmática.

A história evolutiva do transportador Pdr18 foi investigada neste trabalho através da análise do genoma de 117 estirpes pertencentes a 29 espécies ao longo da família Saccharomycetaceae. A relação de paralogia dos transportadores Pdr18 e Snq2 foi confirmada e foram encontrados 214 homólogos do par Snq2/Pdr18 nos genomas examinados. A análise de filogenia e vizinhança genómica resultou na proposta da ocorrência de um evento de duplicação no ancestral comum às leveduras do género *Saccharomyces* como sendo a origem dos genes *PDR18* e *SNQ2*, seguida de

translocação do gene *PDR18* para o cromossoma XIV. O perfil fenotípico dos mutantes de eliminação para os genes *ScPDR18* e *ScSNQ2* na presença de um largo espetro de compostos tóxicos indicou que o ScPdr18 confere à levedura tolerância a quase todos os compostos testados (ácidos fracos, herbicidas, álcoois, fungicidas clínicos e agrícolas, catiões, compostos anti-arritmicos e anti-maláricos, e poliaminas). Por sua vez, o ScSnq2 demonstrou conferir tolerância a um espetro mais reduzido de compostos (os herbicidas barban, alachlor e metlachlor, os catiões Li^{+} e Mn^{2+}, o agente mutagénico 4-NQO, o composto antiarrítmico e antimalárico quinino e a poliamina espermina). A espécie *Candida glabrata*, cuja divergência ocorreu antes do evento de duplicação que originou os genes *PDR18* e *SNQ2*, apenas possui um homólogo Pdr18/Snq2, designado CgSnq2. A análise fenotípica do mutante *Cgsnq2*Δ indicou que apesar de haver uma grande sobreposição com ScSnq2 nos fenótipos de resistência a múltiplas drogas e xenobióticos, existe também uma sobreposição com ScPdr18 na tolerância a azóis. Estes resultados forneceram pistas sobre a divergência funcional entre ScPdr18, ScSnq2 e o gene ancestral correspondente.

O transportador MFS Tpo1 foi descrito neste trabalho como determinante para a tolerância da levedura a ácido benzoico, um ácido fraco mais hidrofóbico do que o anteriormente estudado ácido acético. Os níveis de transcritos do gene *TPO1* aumentaram 30 vezes após uma hora de exposição a stress por ácido benzoico, e esta ativação foi demonstrada ser diretamente dependente dos fatores de transcrição Gcn4 e Stp1, envolvidos na homeostase de amino ácidos. O stress induzido por ácido benzóico causa na célula uma descida nos níveis intracelulares de poliaminas e dos aminoácidos glutamina, arginina e lisina, independentemente da expressão do gene *TPO1*. A diminuição dos níveis de compostos azotados foi identificada como sendo o sinal que despoleta a ativação transcricional do gene *TPO1* registada na presença de ácido benzoico.

Os resultados apresentados nesta tese contribuem para o conhecimento e uma visão integrada do papel e da evolução dos transportadores de membrana plasmática das superfamílias ABC e MFS, bem como as redes regulatórias por detrás da sua ação, e da importância da composição lipídica da membrana plasmática e propriedades físicas na adaptação e tolerância da levedura aos ácidos fracos acético e benzoico, que têm relevância na Biotecnologia e Indústria Alimentar.

Palavras-chave: *Saccharomyces cerevisiae*, plasma membrane ergosterol homeostasis, multidrug/multixenobiotic resistance transporters, multistress response, weak acids.

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LIST OF ACRONYMS

2,4-D	2,4-dichlorophenoxyacetic acid
4-NQO	4-nitroquinoline 1-oxide
ABC	ATP-binding cassette
ATP	adenosine triphosphate
BLASTp	basic local alignment search tool protein
DHA	drug:H^+ antiporter family
DRM	detergent-resistant membrane
EDTA	ethylenediaminetetraacetic acid
GEX	glutathione exchangers
GP	generalized polarization
HUGO	human genome organization
MCPA	2-methyl-4-chlorophenoxyacetic acid
MDR	multidrug resistance
MXR	multixenobiotic resistance
MFS	major facilitator superfamily
MIC	minimum inhibitory concentration
MM	minimal medium
mRNA	messenger RNA
NBD	nucleotide binding domain
ORF	open reading frame
PBS	phosphate buffer saline
PDR	pleiotropic drug resistance
PDRE	pleiotropic drug responsive element
PI	propidium iodide
PM	plasma membrane
PMSF	phenylmethylsulfonyl fluoride
qRT-PCR	quantitative real-time polymerase chain reaction
TF	transcription factor
TMD	transmembrane domain
WGD	whole genome duplication
YEASTRACT	yeast search for transcriptional regulators and consensus tracking
YPD	yeast extract peptone dextrose

1 General Introduction

1.1 THESIS OUTLINE

Yeast plasma membrane represents the first contact between a microorganism and its environment, and as so, its composition and structure deeply affect the ability of the microorganism to restrain the entrance of toxic compounds (Bosmann, 1971; Rank, 1978; Mukhopadhyay et al., 2002; Peetla et al., 2013). Also, plasma membrane provides the lipidic environment for embedded proteins that catalyze the transport of drugs/xenobiotics or of other compounds that can affect the partition of the stressing agent and consequently, its toxicity (Rank, 1978; Kodedová and Sychrová, 2015; Mukhopadhyay et al., 2002; Peetla et al., 2013; Shahi and Moye-Rowley, 2009). Transporters from the ATP-binding cassette (ABC) and from the Major Facilitator Superfamilies (MFS) are among the plasma membrane embedded-proteins that can provide yeast with an improved tolerance to multiple drugs and xenobiotic compounds (dos Santos et al., 2014; Jungwirth and Kuchler, 2006; Piecuch and Obłak, 2014; Sá-Correia et al., 2009). Besides playing an important role in multidrug/multixenobiotic resistance (MDR/MXR), yeast plasma membrane is also the target of many compounds to which yeast cells are subjected in their natural environment. Together with ethanol and thermal stress, weak acids are one of the classes of compounds that can induce stress leading to plasma membrane damage and consequent disruption of its function as selective barrier, as well as dysfunction of plasma membrane-associated transporters (Booth and Stratford, 2003; Fernandes et al., 2005; Piper et al., 2001; Stratford and Anslow, 1996; Ullah et al., 2012).

The group led by Isabel Sá-Correia at the Biological Sciences Research Group (BSRG) of the Institute of Bioengineering and Biosciences (iBB) has been one of the leading groups with a focus on the study of toxicity, response and resistance to weak acid stress, both in the model yeast *Saccharomyces cerevisiae* and more recently in the highly weak acid tolerant food spoilage yeast *Zygosaccharomyces bailii* (Palma et al., 2018). This research topic is of paramount relevance as weak acids are used in the control of spoilage microorganisms in particular in the food industry, but also represent important inhibitors in industrial bioprocesses such as the production of wine and beer, and in the production of second-generation bioethanol from lignocellulosic hydrolysates, where the presence of acetic and formic acids is a problem (Palma et al., 2018). Different weak acids exhibit different hydrophobicity, and thus different ability to permeate yeast cells and lead to stress (Sikkema et al., 1995; Ullah et al., 2012). Also, although the plasma membrane is a known target, depending on the chain length, the accumulation of the anion inside the cell can induce different degrees of plasma membrane damage (Booth and Stratford, 2003; Piper et al., 2001; Ullah et al., 2012). Together with the plasma

membrane composition of yeast cells that is known to impact the rate of passive diffusion of weak acids, a more dense and compact plasma membrane is expected to be an advantage in industrial microorganisms that have to cope with this stress (Lindahl et al., 2016, 2017; Lindberg et al., 2013). The knowledge gathered on the mechanisms leading to yeast tolerance to stress induced by the different weak acids is expected to guide the engineering of more robust microbial strains capable to cope with the harsh environmental conditions to which they are subjected in fermentations-based industries. This knowledge is also critical to develop novel practices to prevent food spoilage, by guiding the development of new preservation techniques. The work described in this thesis is a contribute to the understanding of the molecular mechanisms underlying *S. cerevisiae* tolerance to the food preservatives acetic and benzoic acids. In addition, the results presented herein are expected to provide an integrative view on the relevance of the interaction between plasma membrane lipid composition and MDR/MXR transporters function and their impact in yeast tolerance to weak acid stress.

The first of the five chapters provides a literature review on plasma membrane lipidic composition, including plasma membrane-associated MDR/MXR transporters, as well as the contribution of their cross-talk in plasma membrane composition and structure. It is also reviewed the role of this interplay between the lipid composition and MDR/MXR transporters in yeast adaptive response to stress induced by industrially-relevant stresses, with a focus on weak acid stress.

In Chapter 2 the plasma membrane ABC transporter Pdr18 and modulation of plasma membrane composition and organization, in *S. cerevisiae* response to acetic acid-induced stress is studied. Pdr18 was previously found by our group to play a role in counteracting the decrease of ergosterol content in yeast plasma membrane under stress induced by the weak acid herbicide 2,4-D, a role proposed to be behind the MDR/MXR phenotype exhibited by cells expressing Pdr18 (Cabrito et al., 2011; Teixeira et al., 2012). This chapter takes benefit from the fluorescence probes available for studying membrane properties *in vivo*. This approach allows the evaluation of the impact of Pdr18-mediated transport of ergosterol in plasma membrane composition, lipidic order, non-specific permeability and transmembrane electrochemical potential in particular under acetic acid stress. Chapter 2 allows to detail the physiological role of Pdr18 in yeast cells, proposing a general model in which Pdr18 is a missing piece in the non-vesicular transport of ergosterol in the plasma membrane, thus completing the endoplasmic reticulum-to-plasma membrane route of ergosterol trafficking. The results described in this chapter have been published in the journal “Scientific Reports” (Godinho et al., 2018).

In Chapter 3, the evolutionary history of *S. cerevisiae* Pdr18 was reconstructed. Although the PDR transporters from the ABC superfamily have been on the focus of an earlier study on phylogeny and neighbourhood analysis (Seret et al., 2009), the duplication event that gave rise to the *PDR18* and its paralogue *SNQ2* genes was still a matter of debate when this thesis has started. This chapter arises from the opportunity that the extensive number of publicly available yeast genomes creates, allowing the examination of 117 strains' genomes from 29 yeast species belonging to the Saccharomycetacetaceae family for the presence of Pdr18 and Snq2 homologues, when compared to the 9 genomes from different yeast species analysed in the previous study. This allowed a proper view on the evolutionary history of *PDR18* and *SNQ2* genes across the Saccharomycetaceae family. Given that *PDR18* gene is in the sub-telomeric region of the chromosome XIV, and given its crucial physiological role in yeast cells' ability to respond to multiple stresses, the work presented in chapter 3 also aimed at getting more knowledge into the conservation of Pdr18 throughout the Saccharomyces genus' species analysed. This work also contributes to get more clues into the role of the ancestral gene on the origin of the paralogues *PDR18* and *SNQ2* and on the evolutionary processes acting on the gene copies originated on that duplication event, by studying the post-whole genome duplication species *Candida glabrata* member of the Pdr18/Snq2 homologues subfamily, the *CgSNQ2* gene. For that, the role of ScPdr18, ScSnq2 and CgSnq2 in MDR/MXR is examined by profiling the susceptibility of the corresponding deletion mutants to a wide range of toxic compounds. The results presented in this chapter are included in a manuscript in preparation for submission in the research topic of the Evolutionary and Genomic Microbiology section of Frontiers in Genetics.

Chapter 4 focuses on the role and regulation of the MFS-MDR/MXR transporter Tpo1 in yeast tolerance to the more liposoluble weak acid benzoic acid, compared with the short-chain acetic acid studied in Chapter 2. The plasma membrane transporter Tpo1 was previously found to be a determinant of yeast resistance to a multitude of drugs/xenobiotics, and has a described physiological role in polyamine export, constituting another example in which a physiological role in the cell can be behind a role in MDR/MXR (dos Santos et al., 2014; Sá-Correia et al., 2009). This study aimed at understanding the regulatory mechanisms triggered by benzoic acid stress that activate Tpo1. This knowledge not only may provide clues into the physiological role of Tpo1 but also contribute to extend the knowledge on the complex regulatory network behind TPO1 regulation. A link between Tpo1 physiological role in polyamine export and alleviation of benzoic acid toxicity was hypothesized and investigated. The results obtained in this

work were published in the journal “Applied Microbiology and Biotechnology” (Godinho et al., 2017).

At last, Chapter 5 includes an integrated discussion of the results obtained and on future perspectives taking into consideration all the previous chapters in the state of the art context.

The contribution of the candidate for the experimental and bioinformatic work presented in each chapter in this thesis is as follows. In Chapter 2, the candidate performed most of the experimental work with the collaboration of the co-authors Catarina S. Prata (figure 2-2), Carlos Cardoso and Narcisa M. Bandarra (figure 2-4, ergosterol quantification), Sandra N. Pinto and Fábio Fernandes (experiments involving fluorescence probes). The candidate also collaborated in the preparation of the corresponding article. In Chapter3, the candidate carried out the phylogenetic and gene neighbourhood analysis in collaboration with Paulo J. Dias, performed the phenotypic analysis of *C. glabrata* strains, and performed the phenotypic analysis in *S. cerevisiae* strains in collaboration with Elise Ponçot. The candidate also contributed to the preparation of the manuscript. Chapter 4 was essentially the result of the work performed by the candidate, who also contributed to the writing of the corresponding article.

1.2 PLASMA MEMBRANE MDR/MXR TRANSPORTERS AND LIPID HOMEOSTASIS: CROSS-TALKS IN *SACCHAROMYCES CEREVISIAE* MULTISTRESS RESISTANCE

1.2.1 Yeast as an experimental model to study stress response and resistance mechanisms

Saccharomyces cerevisiae is a well-established experimental eukaryotic model and the mechanisms underlying this yeast species resistance to different stresses are conserved among more complex eukaryotes (Simon and Bedalov, 2004; Steinmetz et al., 2002). This fact makes the study of the adaptive response and resistance to cytotoxic compounds in yeast of major importance. Indeed, yeast not only possesses the advantages of an experimental model, such as being generally recognized as safe (GRAS) and easy cultivation, but also benefits from the extensive post-genomic analyses performed since the first release of the full genome sequence (dos Santos and Sá-Correia, 2015; Goffeau, 2000). Strain collections of single deletion mutants for all (or all nonessential) genes have been largely applied to toxicogenomics/chemogenomics studies to identify large sets of proteins responsible for conferring tolerance to a large number of stressing conditions in yeast (dos Santos et al., 2012, 2014; dos Santos and Sá-Correia, 2015; Hillenmeyer et al., 2008).

Current knowledge on the stress resistance mechanisms found in yeast considers those related with plasma membrane composition as essential, as plasma membrane represents an active interface between the cell interior and its environment. The lipid composition and organization of the plasma membrane greatly impact yeast resistance to stress as these parameters influence plasma membrane role as selective barrier to the entrance of compounds by passive diffusion (Bosmann, 1971; Mukhopadhyay et al., 2002; Peetla et al., 2013; Rank et al., 1978). Also, yeast plasma membrane is a lipid matrix for several embedded proteins, and thus its composition and structure can influence the transport of nutrients, the extrusion of toxic compounds and the maintenance of the transmembrane electrochemical potential (Kodedová and Sychrová, 2015; Mukhopadhyay et al., 2002; Peetla et al., 2013; Rank et al., 1978; Shahi and Moye-Rowley, 2009). Among plasma membrane transporters are those commonly associated to the acquisition of multidrug/multixenobiotic resistance (MDR/MXR) in yeast, that belong mainly to the ATP-binding cassette (ABC) superfamily (Balzi and Goffeau, 1995; Gulshan and Moye-Rowley, 2007; Higgins, 2007; Jungwirth and Kuchler, 2006), and to the Major Facilitator Superfamily (MFS) (dos Santos et al., 2014; Gbelska et al., 2006; Sá-Correia et al., 2009). This general introduction will focus on plasma membrane lipid

composition, the biological role of integral plasma membrane transporters from those superfamilies, and their regulation and interplay in yeast response to stress.

1.2.2 *Saccharomyces cerevisiae* plasma membrane

Yeast plasma membrane plays crucial biological roles in the cells, such as physical protection and control of osmotic stability, control of the entrance and exit of soluble compounds, the anchoring of the cytoskeleton, the functioning of endocytosis and exocytosis processes, signal transduction, cell to cell recognition, and adhesion (van der Rest et al., 1995). Yeast plasma membrane is composed of a lipid bilayer of a mix of polar lipids, and proteins. The most well-accepted model for plasma membrane structure is the fluid mosaic model proposed by Singer and Nicolson (Singer and Nicolson, 1972), which considers that lipids can diffuse freely within the plasma membrane plane and also exhibit rotational and transversal motions, named flip-flop. Although this model considers both lipids and embedded proteins to diffuse freely, it is now known that the membrane components have restrictions to their movements such as association with proteins or cytoskeleton and cell matrix elements (Trimble and Grinstein, 2015). A more recent addition to the fluid mosaic model proposes that plasma membrane lipids are unequally distributed between the two leaflets, causing lipid asymmetry, or, within the same leaflet, forming microdomains, both of which are needed for mechanical stability, vesicular transport and apoptosis (Ikeda et al., 2006). This patchwork organization of the lipid components was also found to influence the localization and activity of plasma membrane proteins (Spira et al., 2012).

1.2.2.1 Lipid composition

The main lipids present in yeast plasma membrane are glycerophospholipids (70%), sphingolipids (15%) and sterols (15%) (Klose et al., 2012) and their structure, synthesis and regulation are shortly described below. A simplified scheme of the synthesis of the major classes of plasma membrane lipids and how their pathways are interconnected is schematized in figure 1-1.

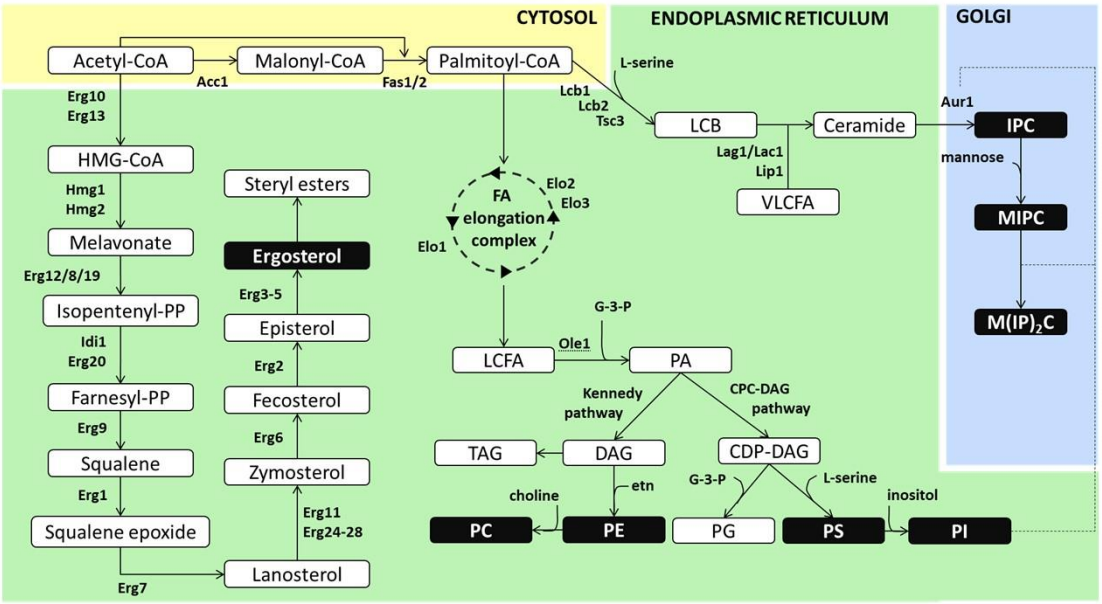
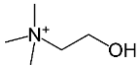
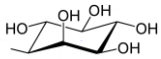
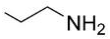
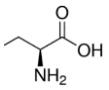
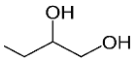
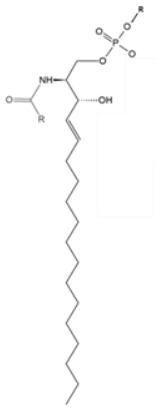
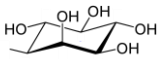
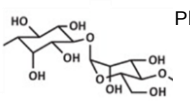
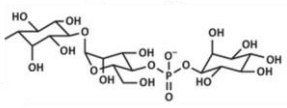
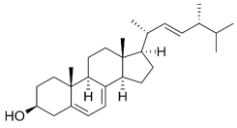


Figure 1-1. Glycerophospholipids, sphingolipid and sterols biosynthesis in *S. cerevisiae*.
Schematic representation of the synthesis of the main plasma membrane lipid species. The background colours indicate different cell compartments for each step and the main lipid species present in the plasma membrane of *S. cerevisiae* are shown in black boxes. Abbreviations: CDP-DAG cytidine diphosphatediacylglycerol; DAG diacylglycerol; Etn ethanolamine; G-3-P glycerol-3-phosphate; IPC inositol phosphate ceramide; LCB long chain base; LCFA long-chain fatty acid; MIPC mannosyl-inositol phosphate-ceramide; M(IP)2C mannosyl-di-inositol phosphate-ceramide; PA phosphatidic acid; PC phosphatidylcholine; PE phosphatidylethanolamine; PG phosphatidylglycerol; PI phosphatidylinositol; PS phosphatidylserine; TAG triacylglycerol; VLCFA very long-chain fatty acid. Adapted from Lindberg et al., 2013.

1.2.2.1.1 Glycerophospholipds

Glycerophospholipids are the most abundant lipids composing yeast plasma membrane (Daum et al., 1998; Klose et al., 2012). The structure of a glycerophospholipid consists of a glycerol backbone linked by ester bonds to a phosphate group and to two fatty acid acyl chains (Klug and Daum, 2014; van der Rest et al., 1995). The fatty acid chains present in *S. cerevisiae* plasma membrane are C_{14}-C_{18} and can be unsaturated at the Δ^9 position only, given that the Δ^9 desaturase Ole1 is the only active desaturase in this yeast species (Martin et al., 2007; Stukey et al., 1989). Glycerophospholipids are classified according to the head group that is linked to the phosphate group and the structure and nomenclature of glycerophospholipid species found in yeast are described in table 1-1 and their prevalence in the cell membranes in table 1-2.

Table 1-1. Glycerophospholipid, sphingolipid and sterols structure, subclass, head group structure and main location in yeast cells.
Information gathered from van der Rest et al., 1995.

Lipid class and backbone structure	Lipid subclass	Head group structure	Main location
Glycerophospholipids 	Phophatidylcholine (PC)		All membranes
	Phophatidylinositol (PI)		All membranes
	Phosphatidylethanolamine (PE)		All membranes
	Phosphatidylserine (PS)		All membranes
	Phosphatidylglycerol (PG)		(-) intermediate
	Phosphatidic acid (PA)		(-) intermediate
Sphingolipids 	Ceramide		(-) intermediate
	Inositol phosphoryl ceramide (IPC)		Plasma membrane
	Mannosyl-inositol phosphoryl ceramide (MIPC)		Plasma membrane
	Mannosyl-di-inositol phosphoryl ceramide		Plasma membrane
Sterols	Ergosterol		Plasma membrane

De novo glycerophospholipids and sphingolipids have in common the synthesis of fatty acyl chains. Fatty acid composition in yeast is rather simple, consisting mainly of palmitoleic acid (C16:1), oleic acid (C18:1), palmitic acid (C16:0), stearic acid (C18:0) and residual levels of myristic acid (C14:0) and cerotic acid (C26:0) (Tuller et al., 1999; Viljoen et al., 1986). For the fatty acid synthesis, acetyl-CoA is carboxylated by Acc1 carboxylase to malonyl-CoA (Hasslacher et al., 1993) from which fatty acid chains up to C_{18} are formed by fatty acid synthase (FAS) complex composed by synthases Fas1 and Fas2, catalyzed in the cytosol (Leibundgut et al., 2008; Stoops and Wakil, 1978). C_{14}-C_{18} fatty acids serve as building blocks for the synthesis of phosphatidic acid (PA) in the endoplasmic reticulum (ER) and this molecule is converted to cytidine diphosphate-diacylglycerol (CDP-DAG) by Cds1 (Shen et al., 1996). Cytidine diphosphate-diacylglycerol is the precursor for all phospholipids synthesized in the cell and can be combined with glycerol-3-phosphate to yield phosphatidylglycerol (PG), with serine, yielding phosphatidylserine (PS) or with inositol, to give phosphatidylinositol (PI). Phosphatidylserine can also be decarboxylated to originate phosphatidylethanolamine (PE), which can be sequentially methylated to yield phosphatidylcholine (PC). Phosphatidylethanolamine and phosphatidylcholine can also be produced directly from diphosphatediacylglycerol (DAG) by combination with ethanolamine or choline, respectively, through the Kennedy pathway (Bürgermeister et al., 2004; van der Rest et al., 1995). The storage lipid triacylglycerol (TAG) is formed by the linkage of an additional fatty acyl chain to DAG and is stored at lipid droplets (Athenstaedt and Daum, 2011).

Table 1-2. Glycerophospholipid composition of *S. cerevisiae* organelles.
Adapted from Zinser et al., 1991. Abbreviations: PC phosphatidylcholine; PE phosphatidylethanolamine; PG phosphatidylglycerol; PI phosphatidylinositol; PS phosphatidylserine; PA phosphatidic acid.

	Mol% of total phospholipids					
	PC	**PE**	**PI**	**PS**	**PA**	**Others**
Homogenate	51.0	25.0	11.4	5.1	1.1	6.4
Plasma membrane	11.3	24.6	27.2	32.2	3.3	1.4
Endoplasmic reticulum	38.9	18.6	22.4	6.4	3.4	10.3
Mitochondria	33.4	22.7	20.6	3.3	1.7	17.3
Peroxisomes	39.8	17.4	22.0	2.5	6.1	13.2

Glycerophospholipid synthesis is essentially regulated by three key players: the transcription factors Ino1 and Ino2 and the suppressor Opi1. Ino1 and Ino2 bind directly to a common region (called the inositol-responsive element – UAS_{INO}) in the promoter region of the above referred genes encoding glycerophospholipid biosynthetic pathway enzymes, and activate their expression (Carman and Han, 2011). Opi1 is an endoplasmic reticulum protein that associates with the membrane of this organelle by

interacting with the integral membrane protein Scs2 (Loewen et al., 2003), and this association depends on the stabilization by adequate levels of phosphatidic acid in the membrane (Carman and Han, 2011; Loewen et al., 2004; White et al., 1991). When phosphatidic acid levels are low, this association of Opi1 with Scs2 is unstable and this repressor is therefore able to translocate to the nucleus and block the transcription of the glycerophospholipid biosynthetic enzyme genes by binding to the inositol-responsive element region. Opi1 role as a repressor was also found to be regulated post-translationally by phosphorylation, occurring at multiple serines, by protein kinase A (Sreenivas and Carman, 2003), protein kinase C (Sreenivas et al., 2001), and casein kinase II (Chang and Carman, 2006). Phosphorylation at Ser^{10}, Ser^{31}, and Ser^{251} was found to stimulate Opi1 repressor function, whereas phosphorylation at Ser^{26} attenuates its repressor function (Chang and Carman, 2006).

1.2.2.1.2 Sphingolipids

The structure of sphingolipids consists of one long chain base (LCB) originating from a C_{16}-C_{18} fatty acid linked by a serine amide linkage to a very long chain fatty acid (VLCFA), commonly a C_{24}-C_{26} (Klug and Daum, 2014; van der Rest et al., 1995). Sphingolipids also contain a substituent group linked to a phosphate group and the nature of this group leads to the sphingolipid classification (table 1-1). The acyl chains of sphingolipids are saturated and can be modified by hydroxylation modification (Megyeri et al., 2016).

The synthesis of C_{14}-C_{18} that was described in the previous section is important because they are building blocks for long chain base synthesis by the condensation in the endoplasmic reticulum of fatty acyl-CoA with serine, by the action of the three enzymes Lcb1-3 (Dickson et al., 2006). Fatty acids up to C_{26} constitute the very long chain fatty acid and result from the action of the fatty acid elongation complex composed by Elo1-3 on the Acc1-resultant malonyl-CoA (Leonard et al., 2004; Oh et al., 1997). The linkage of a long chain base with a very long chain fatty acid to form ceramides occurs in the endoplasmic reticulum and is catalyzed by the action of two ceramide synthase paralogs (Lag1 and Lac1) together with Lip1 (Schorling et al., 2001). Sphingolipid synthesis proceeds in the Golgi apparatus where Aur1 catalyzes the attachment of inositol phosphate to the ceramides to form inositol-phosphoceramide (IPC) (Nagiec et al., 1997). Inositol-phosphoceramide can be converted to mannose-inositol-phosphoceramide (MIPC) by the addition of mannose in a step catalyzed by the enzyme complex Csg1, Csg2 and Csh1 (Uemura et al., 2003). Mannose-(inositol-$P)_2$-ceramide [$M(IP)_2C$] is formed by the addition of another inositol phosphate to mannose-inositol-phosphoceramide by Ipt1 (Dickson et al., 1997).

Regulation of sphingolipid synthesis is still poorly characterized. Orm1 and Orm2 were found to physically interact with Lcb1 and Lcb2, avoiding their association and consequently their role as long-chain base synthases (Han et al., 2010). Also, sphingolipid synthesis is regulated by Sac1 which blocks the substrate feeding of Aur1, thus avoiding inositol-phosphoceramide synthesis (Brice et al., 2009).

1.2.2.1.3 Sterols

The main yeast sterol present in the plasma membrane is ergosterol. It is composed of a hydroxyl head group, an acyl side chain and a four-ring structure.

Sterol synthesis occurs by a cascade of almost 30 enzymatic reactions starting with acetyl-CoA, essentially taking place in the endoplasmic reticulum (Klug and Daum, 2014). Evidences support the localization of late enzymes of the sterol synthesis in lipid particles, secretory vesicles and plasma membrane (Zinser et al., 1993). The initial steps of the sterol biosynthetic pathway involve the condensation of three acetyl-CoA molecules to form 3-hydroxy-3-methylglutaryl-CoA by Erg10 and Erg13, which is reduced to mevalonate by Hmg1 and Hmg2 (Basson et al., 1986; Hiser et al., 1994; Miziorko, 2011) and sequentially phosphorylated and decarboxylated to isopentenyl pyrophosphate by the action of Erg12, Erg8 and Erg19 (Bergès et al., 1997; Oulmouden and Karst, 1991; Tsay and Robinson, 1991). Geranyl pyrophosphate is then formed by isomerization and combination of two molecules of isopentenyl pyrophosphate (Anderson et al., 1989). The chemical linkage with a third molecule yields farnesyl pyrophosphate that is sequentially converted into squalene, lanosterol, zymosterol, fecosterol, episterol, the final product being ergosterol (figure 1-1). In addition, sterols can also be converted into steryl esters through esterification with long chain fatty acids, which are stored in lipid droplets (Fei et al., 2008).

Ergosterol production is tightly regulated in order to avoid accumulation of free sterols which become toxic for yeast cells. This regulation is mainly attained by feedback mechanisms such as the degradation in the proteosome of 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase required for the early steps of the pathway, when yeast cells have an intracellular excess of sterols (Burg and Espenshade, 2011). The isozymes Hmg1 and Hmg2 are also a step of tight regulation. Evidences support that regulation of Hmg1 is post-translational and points mevalonate as the molecular signal, although the regulatory mechanism is still unclear (Burg and Espenshade, 2011). Hmg2 is regulated by protein turnover following recognition and ubiquitination by the multi-subunit, membrane-associated HMG-CoA reductase degradation complex (Burg and Espenshade, 2011). Upon sterol depletion, however, the transcription factors Upc2 and

Ecm22 bind to the sterol regulatory element present in the promoter region of most of the ergosterol biosynthetic genes, thus activating their transcription (Davies and Rine, 2006). Upc2 was also proved to regulate the transcriptional activation of *AUS1* and *PDR11* genes, that encode ABC transporters involved in the uptake of exogenous sterols (Zavrel et al., 2013).

1.2.2.2 Plasma membrane transporters involved in *S. cerevisiae* stress resistance

Although there are many mechanisms known to mediate acquisition of resistance to stress induced by certain drugs and xenobiotic compounds, a special attention will be given here to the action of plasma membrane transporters involved in MDR/MXR. In yeast, they belong to two superfamilies: the ATP-binding cassette (ABC) superfamily, and the Major Facilitator Superfamily of transporters.

1.2.2.2.1 ABC transporters in yeast stress resistance

ABC transporters constitute one of the largest classes of transporters and are widespread among prokaryotes and eukaryotes (Higgins, 2001; Holland and A. Blight, 1999). These transporters are responsible for catalyzing the ATP-dependent transport of a diverse range of solutes across cell membranes against the concentration gradient. ABC transporters share a common architecture of two hydrophobic regions each comprising six transmembrane domains (TMD), and two hydrophilic domains including a conserved cassette with ATP-binding motifs (nucleotide binding domain – NBD) (Linton, 2007; Locher, 2009; Paumi et al., 2009). Although the most common ABC transporter architecture is $[TMD\text{-}NBD]^2$, some variations occur. For example, some yeast and plant ABC transporters that confer MDR/MXR are known to possess the reverse topology $[NBD\text{-}TMD]^2$ (Lamping et al., 2010; Van Den Brûle and Smart, 2002) The functioning of ABC transporters is nowadays considered to obey to the "ATP switch model" that proposes that the binding of a given substrate to the TMD open to the cytosol triggers a conformational change in the NBDs that lead to the opening of the substrate binding pocket to release the substrate to the extracellular media (Higgins and Linton, 2004). According to this model, the conformation of the ABC transporter is recovered by the hydrolysis of the ATP molecule (Higgins and Linton, 2004).

Complete inventories of the ABC transporters in *S. cerevisiae* identified 30 members of this superfamily (figure 1-2), of which only 22 have predicted transmembrane spans and are therefore considered true ABC transporters (Paumi et al., 2009). The true ABC transporters were found to cluster into 6 phylogenetic subfamilies (Decottignies and

Goffeau, 1997; Paumi et al., 2009). These subfamilies have recently adopted the now commonly accepted nomenclature proposed by Human Genome Organization (HUGO) classification and therefore are classified from ABCB to ABCG, although two transporters (*CAF16* and *YDR061w*) cannot be classified into any of the HUGO subfamilies (figure 1-2). Transporters from the ABCG subfamily are one of the most thoroughly studied in yeast. They present a characteristic reverse topology structure and are commonly associated with the MDR/MXR phenomenon, being therefore denominated Pleiotropic Drug Resistance (PDR) transporters (Balzi and Goffeau, 1995; Jungwirth and Kuchler, 2006).

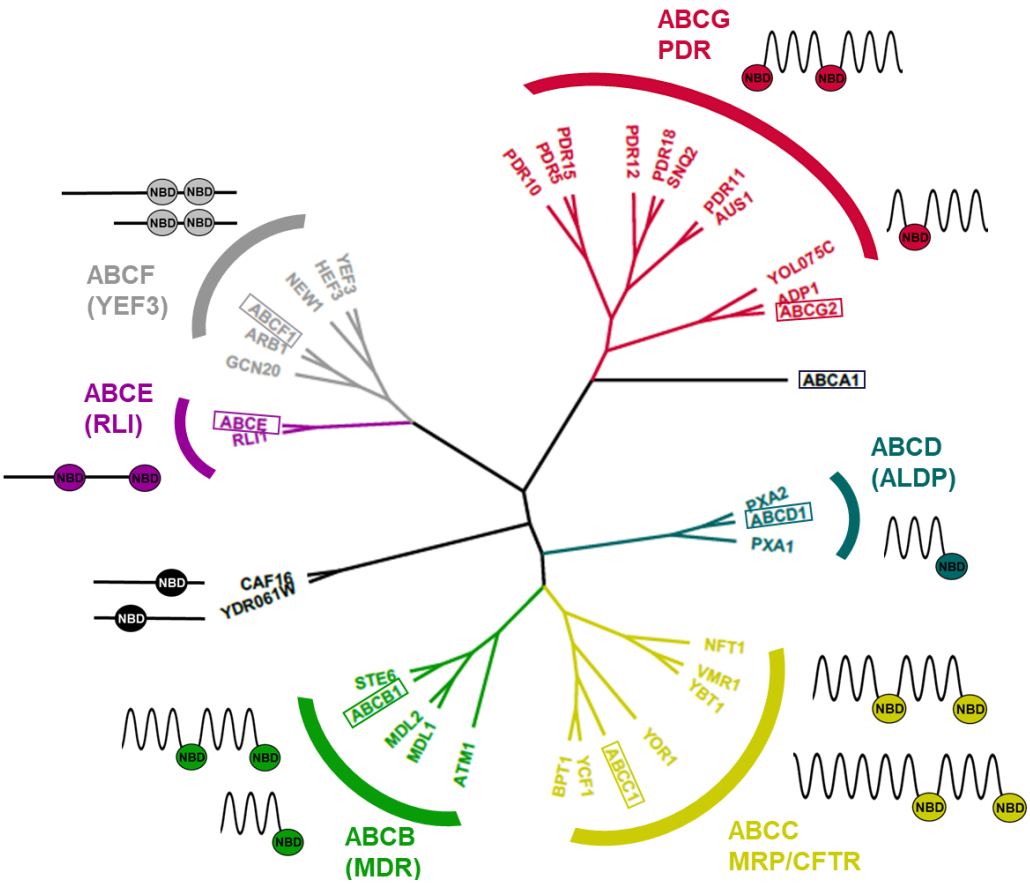


Figure 1-2. Phylogenetic tree of yeast ABC transporters and schematic representation of their architecture.
The protein sequences of the complete set of 30 ABC transporters identified in *S. cerevisiae* were retrieved from SGD. An element of each family of ABC transporters from *Homo sapiens* was retrieved from Uniprot and included in the phylogenetic analysis (marked in the tree with a square). Alignment and phylogenetic reconstructions were performed using the function "build" of ETE3 v3.0.0b32 (Huerta-Cepas et al., 2016) as implemented on the GenomeNet (http://www.genome.jp/tools/ete/). Alignment was performed with MUSCLE v3.8.31 with the default options (Edgar, 2004). ML tree was inferred using PhyML v20160115 ran with model JTT and parameters: -f m --pinv e -o tlr --alpha e --nclasses 4 --bootstrap -2 (Guindon et al., 2010). Branch supports are the Chi^2-based parametric values return by the approximate likelihood ratio test. Tree output visual formatting was performed using FigTree v1.4.3. Each cluster is identified by the name of the corresponding family in both nomenclatures currently in use. Information on ABC transporters architecture is schematically represented with information gathered from Paumi et al., 2009. Abbreviations: NBD nucleotide binding domain.

The Pleiotropic drug resistance (PDR/ABCG) transporters of *S. cerevisiae*

The inventory of all PDR transporters in *S. cerevisiae* considers six PDR *sensu stricto* transporters (Pdr5, Snq2, Pdr12, Pdr15, Pdr10 and Pdr18) based on the fact that these transporters possess the $[NBF\text{-}TMD]^2$ topology, the presence of a cysteine residue instead of the lysine residue in N-terminal Walker A motifs, a specific NVEQ motif in the C-terminal ABC signature and a documented phenotype of multidrug resistance (Seret et al., 2009). The Pdr11, Aus1 and YOL075c are considered members of the PDR family as they share the characteristic $[NBF\text{-}TMD]^2$ topology, but since no role in MDR/MXR was found and they do not present the K/C substitution in the Walker A motif, they were considered as Pdrp *sensu lato* (Seret et al., 2009). Transporters from the PDR subfamily and documented function are described in table 1-3.

Table 1-3. PDR proteins involved in MDR/MXR in *S. cerevisiae*.

ORF	PDR protein	Documented function	References
YOR153W	Pdr5	Multidrug/multixenobiotic resistance; Glycerophospholipid translocation; Quorum sensing	Decottignies et al., 1998; Ernst et al., 2005; Hlaváček et al., 2009; Kihara and Igarashi, 2004; Mahé et al., 1996; Miyahara et al., 1996a
YDR011W	Snq2	Multidrug/multixenobiotic resistance; Quorum sensing	Hlaváček et al., 2009; Servos et al., 1993
YOR328W	Pdr10	Multidrug/multixenobiotic resistance; Regulation of plasma membrane microdomain formation	Nishida et al., 2013 Rockwell et al., 2009
YDR406W	Pdr15	Multidrug/multixenobiotic resistance	Wolfgert et al., 2004
YPL058C	Pdr12	Weak acid resistance	Hatzixanthis et al., 2003; Piper et al., 1998, 2001
YNR070W	Pdr18	Multidrug/multixenobiotic resistance; Active transport of ergosterol in the plasma membrane	Cabrito et al., 2011; Teixeira et al., 2012; this work

Pdr5 and Snq2 are MDR/MXR transporters and their roles were thoroughly investigated as they are proposed to extrude several structurally and functionally unrelated xenobiotic compounds across plasma membrane, based on the susceptibility phenotypes of the individual and double deletion mutants for the two genes, *PDR5* and *SNQ2* (Ernst et al., 2005; Hlaváček et al., 2009; Kihara and Igarashi, 2004; Mahé et al., 1996a; Miyahara et al., 1996b; Servos et al., 1993). Despite being close homologues, Pdr5 and Snq2 have both overlapping and specific substrate profiles (Decottignies et al., 1994, 1995). The substrate profile of Pdr5 ranges from clinical antifungals, herbicides, and agricultural fungicides to anticancer drugs and human steroid hormones (Balzi and Goffeau, 1994; Bissinger and Kuchler, 1994; Emter et al., 2002; Kolaczkowski et al., 1998; Mahé et al., 1996a; Mamnun et al., 2004; Miyahara et al., 1996b; Rogers et al., 2001). Snq2 was first

identified because of its role in yeast resistance to the chemical mutagens 4-nitroquinoline 1-oxide (4-NQO) and triaziquone (Servos et al., 1993) and, later, several publications contributed to extend the range of compounds to which *SNQ2* expression confers increased tolerance in yeast (Hirata et al., 1994; Kolaczkowska et al., 2008; Kolaczkowski et al., 1998; Ling et al., 2013; Mahé et al., 1996a, 1996b; Miyahara et al., 1996b; Nishida et al., 2013; Piper et al., 2003; Servos et al., 1993; Snider et al., 2013; Tsujimoto et al., 2015; van Leeuwen et al., 2012; Ververidis et al., 2001; Wehrschütz-Sigl et al., 2004). Pdr5 is implicated in the translocation of phospholipids between the two plasma membrane monolayers, thus contributing to plasma membrane asymmetry (Decottignies et al., 1998). Although no role in lipid homeostasis was demonstrated for Snq2 transporter, it was shown that Snq2 is involved in alleviation of estradiol toxicity in *S. cerevisiae* (Mahé et al., 1996a), which led to some speculation on a possible role in lipid translocation (Kuchler et al., 1997; Mahé et al., 1996a).

Differently from Pdr5 and Snq2, Pdr12 is characterized by its high substrate specificity (Piper et al., 1998), conferring resistance to weak acids like sorbate, benzoate, acetate and propionate usually used as food preservatives (Hatzixanthis et al., 2003; Piper et al., 1998, 2001).

Pdr15 is a known determinant of resistance to chloramphenicol and polyoxyethylene9-laurylether (Wolfgert et al., 2004).

Pdr10 was found to confer resistance to the anionic dyes calcofluor white and congo red (Rockwell et al., 2009), and to the organic solvents n-decane and n-undecane (Nishida et al., 2013). Interestingly, Pdr10 is another example of a PDR transporter involved in plasma membrane composition homeostasis, as it was found to control the plasma membrane microenvironment of Pdr12 although the precise mechanisms are unclear (Rockwell et al., 2009).

Pdr18 was first identified by its role in yeast tolerance to the herbicide 2,4-dichlorophenoxyacetic acid (2,4-D) and the range of compounds towards which it exerts a protective effect was expanded to the herbicides 2-methyl-4-chlorophenoxyaceticacid (MCPA) and barban, the agricultural fungicide mancozeb, the metal cations Zn^{2+}, Mn^{2+}, Cu^{2+} and Cd^{2+} (Cabrito et al., 2011), and ethanol (Teixeira et al., 2012). Stress induced by exposure to 2,4-D was found to lead to decreased plasma membrane ergosterol content and this deleterious effect was found to be counteracted by Pdr18 expression (Cabrito et al., 2011). In fact, cells expressing *PDR18* gene were found to have a higher transmembrane electrochemical potential even in the absence of any stressing agent, consistent with the proposed function of this transporter in increasing plasma membrane

ergosterol content (Cabrito et al., 2011). A *PDR18*-overexpressing strain, in which the *PDR18* promoter was replaced in the genome by the stronger *PDR5* promoter, showed increased ethanol tolerance and was able to attain higher ethanol production yield than the parental strain in high gravity fermentation conditions (Teixeira et al., 2012). This improved performance was found to correlate with the ability of the *PDR18*-overexpressing strain to restrain plasma membrane permeabilization induced throughout high gravity fermentation (Teixeira et al., 2012).

Regulation of *S. cerevisiae* PDR transporters

Regulation of PDR transporters entails a regulatory network of TFs, centered in the homologous $Zn(II)_2Cys_6$ zinc cluster paralogue transcription factors Pdr1 and Pdr3 (Akache and Turcotte, 2002; Balzi et al., 1987; Delaveau et al., 1994; Kolaczkowska and Goffeau, 1999). These TFs form homo- or heterodimers (Mamnun et al., 2004) and recognize the same nucleotide sequence in the promoter region of target genes, the so called Pdr1/Pdr3 pleiotropic drug responsive element (PDRE) (Katzmann et al., 1996; Mahé et al., 1996b; Wolfger et al., 1997). It is considered that there are additional transcription regulators modulating Pdr1/Pdr3 activity, such as the repressor Rdr1 that forms heterodimers with Pdr1 or Pdr3 competing with the Pdr1/Pdr3 heterodimers for the binding in PDRE locations, documented to repress the transcriptional activation of Pdr5 (Hellauer et al., 2002). Stb5 is documented to form heterodimers with Pdr1 following the same mode of repression of transcriptional activation of the PDR genes *PDR5* and *SNQ2* as described for Rdr1 (Akache and Turcotte, 2002). Besides the action of Pdr1 and Pdr3, other transcription factors are involved in the activation of PDR genes' expression. Yap1 is a zinc-finger transcription factor that is known to be the master regulator of oxidative stress response in *S. cerevisiae*, and was found to directly activate the transcription levels from *PDR5* and *SNQ2* genes (Miyahara et al., 1996a). Expression of Snq2 is known to depend not only on the activity of Pdr1 and Pdr3, but also on Yrr1 (Le Crom et al., 2002). The general stress response transcription activator Msn2 also seems to play a role in the regulation of the PDR network, as it was found to transcriptionally activate the expression of *PDR15* in response to high osmolarity, heat shock, low pH, weak acids, and starvation (Wolfgert et al., 2004). The very well-defined role of Pdr12 in yeast tolerance to weak acid stress was found to depend on the transcription factor War1 that is activated by phosphorylation in response to the decrease of intracellular pH and accumulation of the anions resulting from the entrance and dissociation of the weak acid (Kren et al., 2003; Schüller et al., 2004). The PDR *sensu lato* transporters Pdr11 and Aus1 that are involved in sterol uptake in the case of blockage of the ergosterol biosynthesis pathway, have no documented regulation by Pdr1 or Pdr3. Instead, Pdr11

and Aus1 seem to be regulated by Ecm22 and Umc2, involved in regulation of membrane sterol homeostasis (Crowley et al., 1998; Vik A and Rine, 2001). A scheme of the regulatory network acting on the activation of the PDR transporters' genes is presented in figure 1-3. This network scheme was prepared based on the information gathered in the YEASTRACT database (Teixeira et al., 2017).

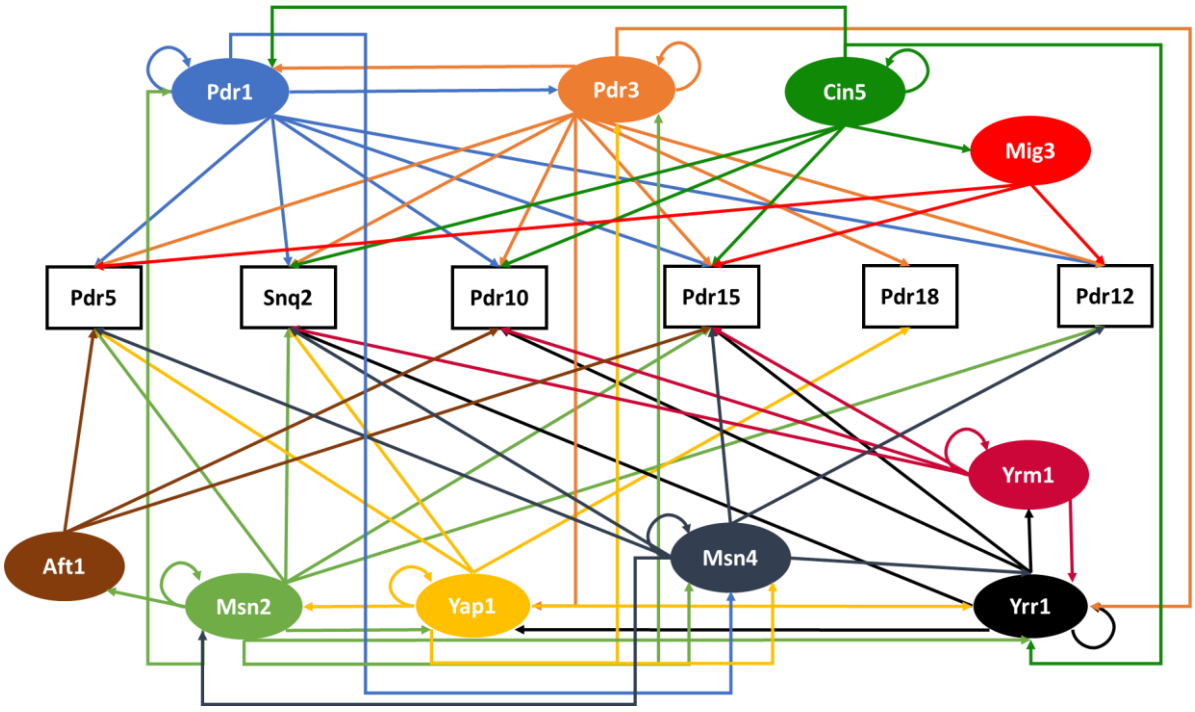


Figure 1-3. Transcriptional regulatory network controlling the expression of *S. cerevisiae* PDR transporters genes under stress.
The regulatory network was built based on information gathered from YEASTRACT database. Only documented regulations based on DNA binding or expression evidence are considered and the stress environmental condition was selected. Due to extensive complexity of the regulatory associations under the control of PDR genes under stress, the dataset was reduced to transcription factors regulating at least 50% of the PDR genes. The original network obtained from YEASTRACT database is provided as supplementary figure S1-1.

Although the main first described targets of the PDR regulatory network are the PDR encoding transporters, other genes were found to be regulated by Pdr1 and Pdr3, such as *TPO1*, that encodes a MDR/MXR transporter belonging to the Major Facilitator Superfamily, and *IPT1* which encodes an enzyme performing a key step of the sphingolipid biosynthetic pathway. Also, Pdr1 and Pdr3 seem to impact plasma membrane composition by controlling the expression of Pdr16, a phosphatidylinositol transfer protein (Li et al., 2000; van den Hazel et al., 1999). This suggests that the PDR network not only influences yeast MDR/MXR by controlling the expression of plasma membrane transporters but may also affect structure and function of plasma membrane itself at the level of the lipid environment.

As an example of the complexity of the network behind the activation of PDR transporters in response to a given stress is the activation of *PDR18* transcription in response to 2,4-D-imposed stress which was found to be dependent on Nrg1, Yap1 and Pdr3 (Cabrito et al., 2011). Deletion of *PDR3* or *YAP1* genes lead to a reduction of Pdr18 activation under 2,4-D stress, which is consistent with the fact that these transcription factors are key players in broad stress defense responses (Cabrito et al., 2011). Deletion of Nrg1 abolished completely the transcriptional activation registered for *PDR18*, a role that was proven to be indirect, as the abrogation of the Nrg1-binding site in the *PDR18* promoter region only led to a slight change in herbicide-dependent transcriptional up-regulation of *PDR18* (Cabrito et al., 2011).

1.2.2.2.2 MFS transporters in yeast stress resistance

Transporters from the Major Facilitator Superfamily (MFS) are secondary carriers of small solutes, involved in uniport, symport and antiport transport processes in response to a chemiosmotic gradient (dos Santos et al., 2014; Pao et al., 1998; Sá-Correia and Tenreiro, 2002). It is proposed that MFS transporters involved in multidrug resistance (MFS-MDR) perform the antiport of drugs/xenobiotics with protons (dos Santos et al., 2014). Structurally, MFS-MDR transporters are single-polypeptide transporters, with approximately 500-600 amino acids, with two structural units of six or seven transmembrane spanning α-helical domains (TMD), connected by a cytoplasmatic loop (Marger and Saier, 1993). MFS transporters were therefore classified into two sub-families, depending on whether their protein products contained 12 or 14 transmembrane segments: the 12-spanner drug:H^+ antiporter family 1 (DHA1) and the 14-spanner drug:H^+ antiporter family 2 (DHA2) (Nelissen et al., 1995, 1997) (figure 1-4). MFS protein classification comprised two more families, the siderophore transporters (ARN) and the glutathione exchangers (GEX) (Dhaoui et al., 2011; Haas et al., 2008; Heymann et al., 1999, 2000b, 2000a; Lesuisse et al., 1998; Yun et al., 2000). However, recent phylogenetic and neighbourhood analysis revealed that DHA2, ARN and GEX share a common root, and therefore it was proposed the inclusion of these three families in the new MFS gene family (DAG) (Dias and Sá-Correia, 2013) (figure 1-4). Although members from both the DHA1 and DAG families have been proved to confer yeast with a MDR/MXR phenotype, this introductory chapter will focus on the extensively studied transporters from the DHA1 family, in which the Tpo1 transporter, the focus of Chapter 4, is included.

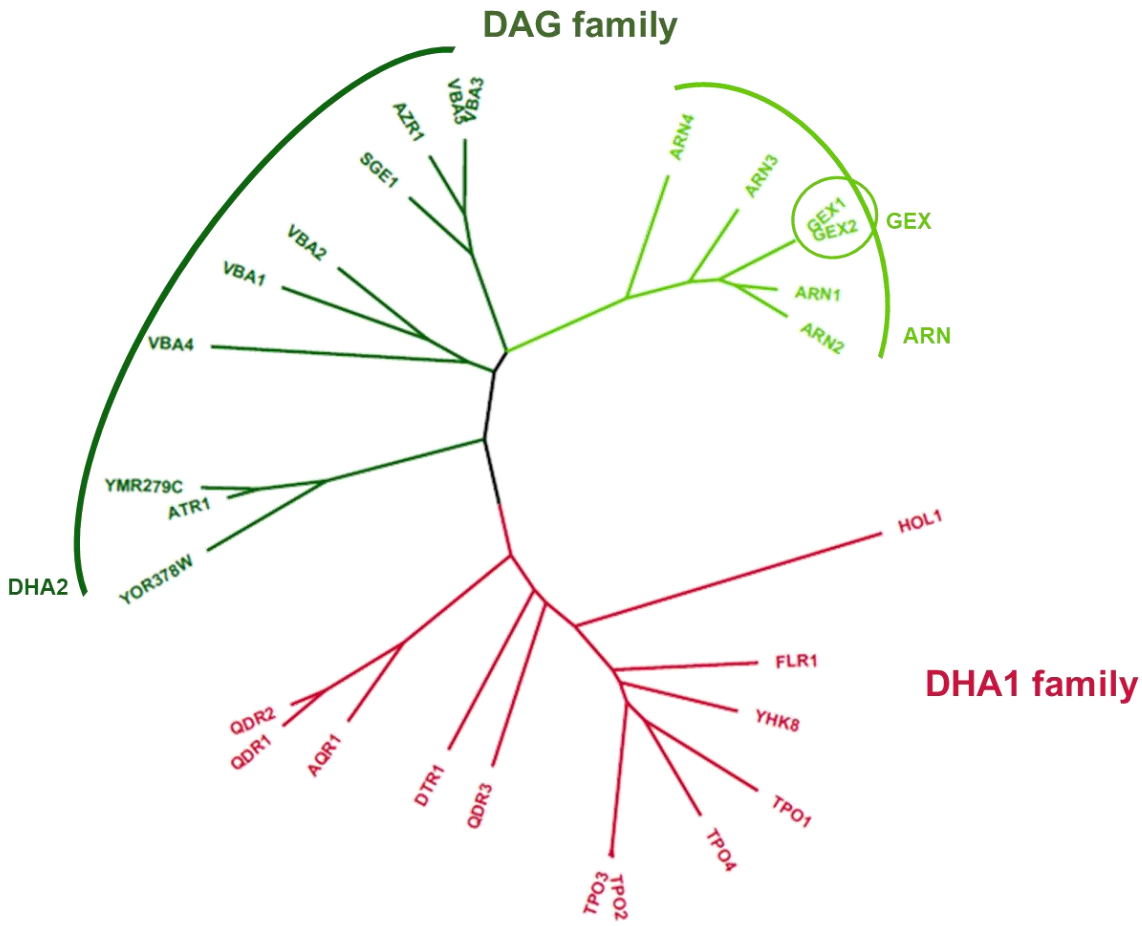


Figure 1-4. Phylogenetic tree of yeast MFS-MDR/MXR transporters.
The protein sequences of the MFS transporters involved in *S. cerevisiae* MDR/MXR were retrieved from SGD. The alignment, phylogenetic analysis and tree building were performed using the same tools as figure 1-2.

The *S. cerevisiae* DHA1 family of transporters

The DHA1 family of transporters in *S. cerevisiae* is composed by 12 proteins, encoded by *AQR1*, *DTR1*, *FLR1*, *QDR1*, *QDR2*, *QDR3*, *TPO1*, *TPO2*, *TPO3*, *TPO4*, *YHK8* and *HOL1* genes (Dias et al., 2010; Sá-Correia et al., 2009). Transporters from the DHA1 family and documented function described below are summarized in Table 1-4.

Table 1-4. MFS-MDR/MXR transporters from the DHA1 family and documented functions in *S. cerevisiae*.

ORF	PDR protein	Documented function	References
YNL065W	Aqr1	Multidrug/multixenobiotic resistance; Excretion of excess amino acids; Possible role in DNA replication during stress response	Velasco et al., 2004; Tkach et al., 2012
YBR180W	Dtr1	Multidrug/multixenobiotic resistance; Translocation of bisformyl dityrosine during spore wall maturation	Felder et al., 2002
YBR008C	Flr1	Multidrug/multixenobiotic resistance	Alarco et al., 1997
YIL120W	Qdr1	Multidrug/multixenobiotic resistance; Involved in spore wall assembly	Lin et al., 2013
YIL121W	Qdr2	Multidrug/multixenobiotic resistance; Potassium and copper homeostasis	Vargas et al., 2007; Ríos et al., 2013
YBR043C	Qdr3	Multidrug/multixenobiotic resistance; Involved in spore wall assembly; Polyamine homeostasis	Lin et al., 2013; Teixeira et al., 2011
YLL028W	Tpo1	Multidrug/multixenobiotic resistance; Hypothesized role in lipid homeostasis; Polyamine homeostasis	Albertsen et al., 2003; Tomitori et al., 1999, 2001; Uemura et al., 2005; Kennedy and Bard, 2001
YGR138C	Tpo2	Multidrug/multixenobiotic resistance; Polyamine homeostasis	Albertsen et al., 2003; Tomitori et al., 1999, 2001; Uemura et al., 2005
YPR156C	Tpo3	Multidrug/multixenobiotic resistance; Polyamine homeostasis	Albertsen et al., 2003; Tomitori et al., 1999, 2001; Uemura et al., 2005
YOR273C	Tpo4	Multidrug/multixenobiotic resistance; Polyamine homeostasis	Albertsen et al., 2003; Tomitori et al., 1999, 2001; Uemura et al., 2005
YHR048W	Yhk8	Up-regulated in response to azole drugs	Barker et al., 2003
YNR055C	Hol1	Import of histidinol and cations	Wright et al., 1996

Aqr1 is one of these transporters with a physiological function assigned, the catalysis of the excretion of amino acids (Velasco et al., 2004) that can accumulate in the cytosol under specific stress conditions. Also, Aqr1 has been found to relocalize from the plasma membrane to the cytoplasm when cells are challenged with the replication inhibitor hydroxyurea, suggesting a possible role for Aqr1 in DNA replication during stress response (Tkach et al., 2012). Aqr1 has also a described role in MDR/MXR, as it was found to confer resistance to a wide range of chemical compounds such as the azole drug ketoconazole, the cationic dye crystal violet, the food preservatives acetic and propionic acid, the antiarrhytmic and antimalarial drugs quinidine and, less significantly, to quinine (Tenreiro et al., 2002).

Dtr1 also has a physiological function assigned, it plays a role in facilitating the translocation of bisformyl dityrosine, the major building block of the spore surface, during

spore wall maturation (Felder et al., 2002). Also, Dtr1 was found to confer improved tolerance to quinine, quinidine and propionic acid in yeast (Felder et al., 2002).

Flr1 has also been on the focus of several studies but no physiological role in the cell other than being a MDR/MXR determinant was attributed to this transporter. Flr1 (fluconazole resistance protein 1) was first identified by its role in conferring resistance to fluconazole (Alarco et al., 1997). However, the range of compounds to which it confers resistance is much larger, including cycloheximide, 4-nitroquinoline 1- oxide (4- NQO), benomyl, methotrexate, diazoborine, cerulenin, diamide, diethylmaleate, menadione, mancozeb, and paracetamol (Alarco et al., 1997; Brôco et al., 1999; Jungwirth et al., 2000; Nguyên et al., 2001; Oskouian and Saba, 1999; Srikanth et al., 2005; Teixeira et al., 2008).

Qdr1 also has no physiological role assigned, although it was recently identified as part of a complex gene network controlling spore wall assembly (Lin et al., 2013). Its role in MDR/MXR was described based on the improved resistance of yeast cells expressing *QDR1* gene to ketoconazole and quinidine, barban, cisplatin and bleomycin (Nunes et al., 2001; Tenreiro et al., 2005; Vargas et al., 2004).

The *qdr2*Δ deletion mutant exhibits a susceptibility profile similar to *qdr1*Δ profile (Tenreiro et al., 2005; Vargas et al., 2004), plus an increased susceptibility to lithium and sodium (Ríos et al., 2013). However, a physiological role was first assigned to Qdr2 as it was proposed to contribute to potassium homeostasis in conditions of limiting K^+, due either to a limited availability of K^+ in the growth medium or to the presence of quinidine, known to decrease the uptake rate of K^+ (Vargas et al., 2007). Later, Qdr2 was also found to play a role in copper homeostasis, contributing to oxidative stress response in yeast (Ríos et al., 2013).

Qdr3 seems to have a multiple role in yeast cell physiology, as it is involved in spore wall assembly, together with its close homologues Dtr1 and Qdr1 (Lin et al., 2013), and also deletion mutant for *QDR3* gene shows a respiratory deficiency under glucose depletion, although the concrete role of this transporter in respiration is not clear (Dikicioglu et al., 2008). A recent work has shown that cells devoid of Qdr3 rearrange their metabolism and accumulate intracellular glucose, glycerol, and inorganic phosphate when growing under conditions of limiting glucose or ammonium (Dikicioglu et al., 2014). Another physiological role attributed to Qdr3 is the export of the polyamines spermine and spermidine, thus alleviating the polyamine-induced disruption of the plasma membrane potential (Teixeira et al., 2011a).

Tpo1 (transport of polyamines protein 1) was first described as a transport protein specific for polyamines, enabling cell detoxification from these cations, and Tpo2, Tpo3 and Tpo4 were found later to have a similar role (Albertsen et al., 2003; Tomitori et al., 1999, 2001; Uemura et al., 2005). Although Tpo2-4 do not show extensive roles in MDR/MXR, Tpo1 is the DHA1 transporter associated so far with yeast marked resistance to a broader range of drugs/xenobiotics (Sá-Correia et al., 2009). *TPO1* expression was found to alleviate the toxicity of the metal ions cadmium and aluminium (Cabrito et al. 2009), the antimalarial drugs quinidine and artesunate (Alenquer et al. 2006; do Valle Matta et al. 2001), the immunosuppressant mycophenolic acid (Desmoucelles et al. 2002), the herbicides 2,4-dichlorophenoxyacetic acid (2,4-D) and barban (Cabrito et al. 2009; Teixeira and Sá-Correia 2002), the anticancer agent bleomycine (Berra et al. 2014; Hillenmeyer et al. 2008), the antifungals nodoconazole and mancozeb (Dias et al. 2010; Hillenmeyer et al. 2008), the nonsteroidal anti-inflammatory drug diclofenac (Mima et al. 2007) and the weak acids acetic, propionic, decanoic and octanoic acids (Borrull et al. 2015; Legras et al. 2010; Mira et al. 2009). A link between Tpo1 and lipid homeostasis was also previously proposed, as the deletion of *TPO1* gene leads to increased sensitivity to nystatin, and to accumulation of ergosterol in the membrane (Kennedy and Bard, 2001). This was correlated with a role for Tpo1 in activating the expression of the squalene synthase (*ERG9*) gene (Kennedy and Bard, 2001). Although the question to whether such a broad role of Tpo1 in MDR/MXR is related to its physiological role on polyamine extrusion is not answered yet, evidence has shown that Tpo1-mediated export of spermine and spermidine is required for the response to oxidative stress by interfering with the timing of those response mechanisms such as the induction of antioxidant proteins and cell cycle delay (Krüger et al., 2013).

Yhk8 and Hol1 proteins have no reported role in MDR/MXR until now, however, *YHK8* transcription was found to be up-regulated in cells exhibiting reduced susceptibility to azole drugs (Barker et al., 2003) and mutations in *HOL1* have been shown to enhance the ability of yeast cells to import histidinol (a precursor of histidine) as well as mono- and divalent cations (Wright et al., 1996).

Regulation of *S. cerevisiae* DHA1 transporters in response to chemical stress

Beyond the expected regulation of DHA1 transporters by transcription factors associated to MDR/MXR, most of the regulatory associations described so far imply transcription factors not necessarily associated with drug/xenobiotic resistance but also with oxidative stress, general stress response and nutrient levels (figure 1-5). Considering documented DNA binding plus expression evidences without imposing any environmental condition

restriction, the pleiotropic drug resistance-associated transcription factors Pdr1 and Pdr3 were only found to regulate 9 and 7, respectively, out of the 12 DHA1 transporters described herein. Msn2 and Ms4, the key regulators of the general stress response, were found to control the expression of 10 and 9 of the 12 DHA1 transporters, respectively. The key transcription factor controlling oxidative stress response in yeast, Yap1, was found to regulate 9 DHA1 transporters, although in fact all 12 DHA1 transporters possess a Yap1-responsive element in their promoter region and consequently, further studies could unveil a role in Yap1 in the regulation of the remaining 3 transporters: Hol1, Qdr2 and Tpo3. Gcn4 in another transcription factor shown to regulate DHA1 members of the MFS transporters. This activator of amino acid biosynthetic genes, in response to amino acid starvation, was found to regulate 8 out of the 12 transporters under analysis.

The involvement of ABC transporters in lipid transport has called recent attention to the role of lipid homeostasis in MDR/MXR (Jungwirth and Kuchler, 2006; Prasad et al., 2016). Although no demonstrated role for MFS-MDR/MXR in lipid transport has been found so far, transcription factors involved in the regulation of lipid metabolism are documented to regulate the expression of MFS transporters. This is the case of Spt23, involved in the regulation of the Δ9 desaturase *OLE1* gene (Zhang et al., 1999), that regulates 6 members of the DHA1 family. Cst6 and Pip2, that are regulators of oleate responsive genes, were also found to regulate 4 DHA1 transporters each.

As an example of the regulatory network acting on the activation of DHA1 genes, *TPO1* gene expression was found to be transcriptionally activated by the PDR network regulators Pdr1 and Pdr3 in conditions of stress imposed by a wide range of drugs/xenobiotics such as: artesunate (Alenquer et al., 2006), fluphenazine (Fardeau et al., 2007), benomyl (Lucau-Danila et al., 2005), and the chlorinated phenoxyacetic acid herbicides MCPA and 2,4-D (Teixeira and Sá-Correia, 2002). However, transcriptional activation of *TPO1* was also found to be mediated by the oxidative stress response transcription factor Yap1, in the presence of the anticancer drug bleomycin (Berra et al., 2014). Also, *TPO1* expression was found to be regulated by the multidrug resistance transcription factor paralogues Yrr1 and Yrm1, in the presence of hop iso-α-acids (Hazelwood et al., 2010).

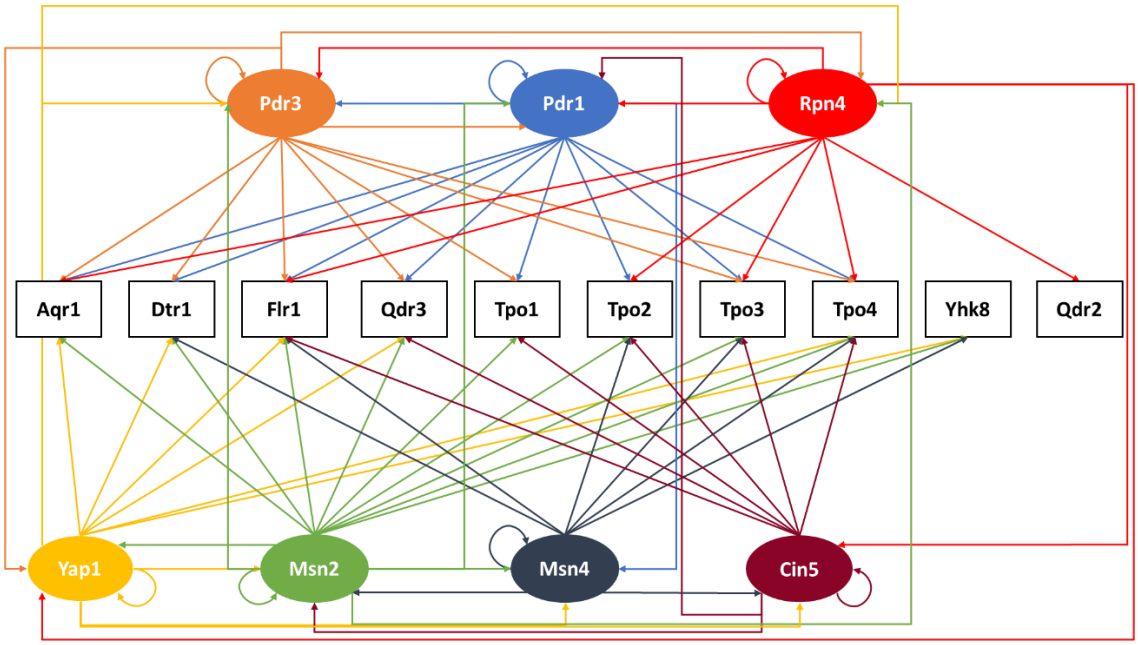


Figure 1-5. Transcriptional regulatory network controlling the expression of *S. cerevisiae* MFS-MDR/MXR transporters genes from the DHA1 family, under stress.
The regulatory network was built based on information gathered from YEASTRACT database. Only documented regulations based on DNA binding or expression evidence are considered and the stress environmental condition was selected. Due to extensive complexity of the regulatory associations under the control of MFS-MDR/MXR genes under stress, the dataset was reduced to transcription factors regulating at least 50% of these genes. The original network obtained from YEASTRACT database is provided as supplementary figure S1-2.

An incredible amount of information suggestive of the transcriptional regulation of MFS-MDR/MXR transporters has been gathered in *S. cerevisiae*, especially since the boost of the genome-wide approaches. The availability of databases such as YEASTRACT (www.yeastract.com) (Teixeira et al., 2017) is a precious aid in unveiling the complex regulatory networks acting on the activation of MDR/MXR transporters both in the context of yeast stress response, but also in understanding the mechanisms behind the action of this proteins in yeast cell physiology in the absence of any toxic compound.

1.2.3 Plasma membrane lipid-protein cross talks and *S. cerevisiae* response to stress

1.2.3.1 The importance of lipid-protein interaction for plasma membrane structure and organization

Associations between lipids and proteins in the plasma membrane are responsible for the formation of micro and macro domains. Although glycerophospholipids allow a well distributed plasma membrane matrix, sterols and sphingolipids do not mix randomly within this matrix and are known to pack together and cluster into microdomains called lipid rafts or detergent-resistant membrane (DRM) fractions (Mouritsen and Jørgensen, 1997). Lipid rafts/DRM microdomains are compacted zones where the bilayer is condensed (Alvarez et al., 2007), and have been implicated as preferential plasma membrane zones for a number of processes such as protein sorting, secretion, and endocytosis (Wachtler and Balasubramanian, 2006). Not only the content of ergosterol is crucial for plasma membrane stability and adequate selective permeability to avoid the passive diffuse of toxic compounds into the cell (Eisenkolb et al., 2002; Parks and Casey, 1995), but also its availability to form microdomains such as lipid rafts is of paramount importance in MDR/MXR. Lack of ergosterol limits the formation of lipid rafts, which reduces the correct delivery of certain plasma membrane transporters and the required organization for the optimal activity of membrane embedded drug pumps (Bagnat et al., 2001). It is quite clear nowadays that proteins self-organize in domains of the membrane conferring a “patchwork” organization to the plasma membrane (Spira et al., 2012). The raft-associated arginine permease Can1 is a very well-studied example, as its correct trafficking to the plasma membrane was found to depend on the non-raft lipid phosphatidylethanolamine (Opekarová and Tanner, 2003) and on ergosterol (Malinska et al., 2004). However, the raft-associated plasma membrane H^+-ATPase Pma1 (Bagnat et al., 2001) is not associated with the Can1-raft domains, as these proteins occupy two different nonoverlapping membrane microdomains, reflecting that more factors contribute to plasma membrane protein localization (Malinska et al., 2004). Some ABC transporters whose activity was found to depend on its location in the plasma membrane, localize within lipid rafts (Gulati et al., 2015). Also, the *Candida albicans* ABC transporter CaCdr1, the ScPdr5 orthologue, was found to localize to raft domains and its trafficking was proved to be hindered by defects on sphingolipid and sterol synthesis (Pasrija et al., 2008).

Not only lipid organization and content can modulate the fate of plasma membrane proteins, but also MDR transporters of the ABC superfamily were found to have a role in

lipid bilayer homeostasis (Jungwirth and Kuchler, 2006; Prasad et al., 2016). *Saccharomyces cerevisiae* Pdr11 and Aus1 are responsible for the uptake of exogenously supplied ergosterol in anaerobic conditions when ergosterol biosynthesis is impaired, and are considered to play a role in the transport of exogenously supplied ergosterol to the Golgi apparatus, where esterification occurs (Gulati et al., 2015; Li and Prinz, 2004; Wilcox et al., 2002). Also, as summarized in the "The Pleiotropic drug resistance (PDR/ABCG) transporters of *S. cerevisiae"* section, the ABC efflux pumps Pdr5 and Yor1 are implicated in the translocation of phospholipids between the two plasma membrane monolayers, thus contributing to plasma membrane asymmetry (Decottignies et al., 1998; Kaur and Bachhawat, 1999). Pdr18 mediates ergosterol transport and was found to be essential to reduce toxicity induced by the herbicide 2,4-D that negatively impacts ergosterol plasma membrane levels, and by membrane-permeabilizing agents such as ethanol (Cabrito et al., 2011; Teixeira et al., 2012). Pdr10 contributes to the normal expression, and/or sorting and trafficking, and/or catalytic competence of Chs3 and Pdr12 (Rockwell et al., 2009). Ste6 is a demonstrated phospholipid transporter (Jungwirth and Kuchler, 2006; Schuller et al., 2007). Pdr16 and Pdr17 expression was also proposed to regulate lipid metabolic pathways (Li et al., 2000; Van Den Hazel et al., 1999) but the underlying mechanisms were not detailed. Another relevant fact that raises the importance of the PDR network in lipid composition of membranes is that some sphingolipids synthesizing enzymes such as Ipt1, Lcb2, Sur2, and Lac1 contain a pleiotropic drug responsive element (PDRE) within the promoter regions of the encoding genes, that have their expression induced by Pdr1 and Pdr3 upon environmental stimulus (Han et al., 2010). All these facts indicate that the PDR pathway has a role in phospholipid and sterol composition of the plasma membrane, controlling in part the distribution of lipid components in inner and outer leaflets of the plasma membrane (Gulshan and Moye-Rowley, 2007).

1.2.3.2 Lipid-protein cross-talk in yeast stress response to ethanol and thermal stresses

As a widely-used industrial microorganism, *S. cerevisiae* primary sources of inhibitory stress include the accumulation of high concentration of ethanol and of undesirable by-products, the need of high operating temperatures in certain fermentations, and suboptimal pH levels (Cheng and Kao, 2014). Tolerance to high concentrations of ethanol and temperature are among the most desired phenotypes and consequently, among the most studied stress responses in yeast (Cheng and Kao, 2014). Interestingly, ethanol and growth at supraoptimal temperatures both impact plasma membrane structure and stability, and thus the mechanisms underlying yeast resistance to these

two stressful conditions are somehow similar (Adachi et al., 1995; Barry and Gawrisch, 1994; Feller et al., 2002; Kranenburg and Smit, 2004; Piper, 1995; Verghese et al., 2012). Moreover, ethanol and thermal stress have synergistic effects, with the maximum, minimum and optimal temperature for yeast growth decreasing with increasing ethanol concentration (van Uden and da Cruz Duarte, 1981). As so, when the ethanol concentration becomes high enough to decrease these temperature parameters of yeast growth below the process temperature, the yeast population becomes increasingly subject to ethanol-enhanced thermal death (van Uden and da Cruz Duarte, 1981).

Exposure of the yeast cell to toxic concentration of ethanol and supraoptimal growth temperatures, individually or combined, is known to increase plasma membrane fluidity, decreasing plasma membrane thickness and consequently decreasing its integrity (Adachi et al., 1995; Barry and Gawrisch, 1994; Feller et al., 2002; Kranenburg and Smit, 2004; Piper, 1995; Verghese et al., 2012). Loss of plasma membrane integrity reflects in higher levels of plasma membrane permeability resulting in loss of function as a selective barrier to the entrance of solutes by passive diffusion, and dissipation of the proton motive force that drives secondary transport of solutes (Aguilera et al., 2006; Alexandre et al., 1994b). Membrane disorganization and reduced thickness under ethanol-induced stress was found to induce the impairment of sorting of glycosylphosphatidylinositol-linked proteins (Schiavone et al., 2016), commonly associated with lipid rafts and predicted to play different roles in cell wall biogenesis or remodeling (Pittet and Conzelmann, 2007; Richard and Plaine, 2007). Changes in membrane thickness can also result in exposure of hydrophobic amino acid residues of integral membrane proteins, leading to membrane protein aggregation to minimize the exposure of hydrophobic portions (Killian, 1998; Lee, 2004).

An early study was able to correlate growth in the presence of increasing concentrations of ethanol, with the increase in ergosterol levels in yeast cells and a role for ergosterol incorporation was proposed in maintaining yeast plasma membrane fluidity (del Castillo Agudo, 1992). Further studies confirmed that increased sterol incorporation, alone or combined with increased content of unsaturated lipids, in fact counteracts the ethanol-induced formation of the interdigitation of the plasma membrane (Vanegas et al., 2010, 2012; You et al., 2003). Genes involved in lipid membrane composition such as *ERG2* and *ERG24,* involved in ergosterol biosynthesis, and *KCS1*, *LIP5*, *OPI3*, *PDX3*, and *IDI1*, involved in phospholipid biosynthesis, were identified as conferring resistance to high ethanol concentrations in a genome-wide survey, thus contributing to the concept that ethanol stress response includes the remodeling of the plasma membrane (Teixeira et al., 2009). A study based on adaptive laboratory evolution selecting for thermotolerant

mutants came to the conclusion that all isolates exhibiting improved tolerance contained nonsense mutations in the *ERG3* gene (Caspeta et al., 2014a). Although these mutations did not change the total sterol content, they render the intermediate bended-sterol fecosterol to be the main sterol present, instead of the common flat-sterol ergosterol (Caspeta et al., 2014a). Previous reports already have related the incorporation of branched sterols such as sitosterol and bended sterol-like lipids such as bacteriohopanetetrol in protection of *Archaea* and plant cells' membranes from high temperatures (Dufourc, 2008). This calls attention to the fact that not only improved sterol content but also sterol structure can impact plasma membrane organization, providing improved tolerance to membrane-perturbing agents. Small variations in the sterol structure in fact were already found to drastically alter the ability of the sterol to induce domain formation *in vitro* and *in vivo* (Orädd et al., 2009; Ranadive and Lala, 1987; Shahedi et al., 2006).

The action of plasma membrane associated proteins also contributes to alleviate ethanol and thermal-induced stress, and most of the times is dependent on the remodeling of the plasma membrane lipid composition proposed by the studies mentioned above. Plasma membrane H^+-ATPase counteracts the dissipation of plasma membrane electrochemical potential and was found in many studies to be strongly dependent on the lipid environment of the plasma membrane (Aguilera et al., 2006; Serrano, 1988; Wach et al., 1990). Early studies showed that in ethanol-adapted cells, the impact of ethanol supplementation on the inhibition of H^+-ATPase activity is much lower (Alexandre et al., 1994a), probably reflecting the adaptation of plasma membrane lipid composition to ethanol. The aquaglyceroprotein Fps1 is a plasma membrane glycerol channel that was found to be a determinant for maximum ethanol tolerance in a genome-wide survey (Teixeira et al., 2009). *FPS1* gene expression was found to decrease the intracellular accumulation of radiolabeled ethanol, thus alleviating its toxicity to the cell (Teixeira et al., 2009). Although the exact mechanism behind this is not clarified, Fps1 expression is described to increase ergosterol content in yeast plasma membrane, which can contribute to a less permeable plasma membrane therefore altering drug partition and influencing its toxicity (Toh et al., 2001). Also, the ABC transporter Pdr18 was found to be a determinant of yeast tolerance to ethanol and as observed for Fps1, Pdr18 was found to contribute to decreased intracellular accumulation of ethanol (Teixeira et al., 2012). Further analysis showed that, in fact, the role of Pdr18 in ethanol tolerance is related to its physiological role in ergosterol transport at the plasma membrane level, thus contributing to higher ergosterol content and consequently, lower ethanol-induced permeability (Cabrito et al., 2011; Teixeira et al., 2012). The overexpression of *PDR18*

gene was found to enable a better performance of yeast cells in high gravity fermentation, by greatly restricting non-specific permeabilization induced by the increasing concentration of ethanol in the medium, thus allowing higher ethanol production (Teixeira et al., 2012). The examples presented herein indicate not only the important role of plasma membrane lipid composition for the action of membrane-embedded proteins but also suggest an interconnection among plasma membrane transporters and membrane lipid homeostasis in stress resistance.

1.2.3.3 Lipid-protein cross-talk in yeast stress response to weak acid stress

S. cerevisiae has a predominant role in the food industry, such as in the production of bread and the alcoholic beverages wine and beer. However, food-spoilage yeast species can be a major threat to the food industry due to their ability to overcome the toxicity imposed by weak acid food preservatives, perturbing the stability of the products and causing organoleptic changes, thus generating drastic economic losses (James and Stratford, 2003). As most food spoilage filamentous fungi and yeasts (*Zygsaccharomyces bailii*) are closely related to the unicellular model yeast *S. cerevisiae* (Palma et al., 2018), the knowledge gathered on the mechanisms underlying *S. cerevisiae* adaptation and resistance to weak acid food preservatives is important to guide preservation practices in the food industry. Yeast ability to overcome weak acid stress can also be beneficial as acetic acid is an important by-product of alcoholic fermentation that together with the increasing concentration of ethanol can lead to stuck or sluggish fermentations (Graves et al., 2006; Rasmussen et al., 1995). Also, the future of bioethanol production partially relies on the use of lignocellulosic materials as a substrate, that require a pre-treatment for the release of fermentable sugars (Almeida et al., 2007; Jönsson and Martín, 2016; Koppram et al., 2014; Olofsson et al., 2008). This pre-treatment often leads to the release of many inhibitory compounds, a mixture in which acetic acid has a great prevalence (Jönsson and Martín, 2016). Therefore, the ability of microbial strains to perform profitable bioethanol production depends on their ability to overcome stress induced by this weak acid.

Weak acid food preservatives widely used include sorbic, benzoic, propionic, and acetic acids (Booth et al., 1989; Booth and Stratford, 2003), but this Introductory chapter will focus on acetic and benzoic acids, considering that these are the weak acids under study in Chapter 2 and 4, respectively. A schematic model summarizing the toxicity and documented responses of yeast cell in weak acid induced stress is presented in figure 1-6. In acidic environments, extracellular pH is lower than the pKa of the weak acid, and

the undissociated uncharged form of the acid is the dominant, making it possible for the weak acid to permeate the cell. Weak acid toxicity depends on their structure, increasing with carbon chain length, a parameter that affects their lipophilicity. It is nowadays known that the weak acid toxicity correlates with its partition coefficients, that is, the ability of a weak acids to permeate yeast plasma membrane is an important determinant of the efficacy of growth inhibition (Sikkema et al., 1995; Ullah et al., 2012). It can therefore be concluded that the more hydrophobic benzoic acid has higher ability of diffusing across the plasma membrane, and therefore a lower concentration is needed to induce yeast toxicity, when compared to the more lipophilic acetic acid. Intracellularly, these weak acids encounter a near-to-neutral pH and dissociate, which leads to the accumulation of the counterion and to the release of protons contributing to intracellular acidification (Mira et al., 2010d; Palma et al., 2018; Russell, 1992). Among the numerous deleterious effects of weak acid stress in yeast, the impact in plasma membrane is quite severe. The drop in the intracellular pH caused by proton release leads to dissipation of the transmembrane electrochemical potential compromising its role as a selective barrier and the functioning of membrane-embedded proteins. Low intracellular pH affects trafficking mechanisms responsible for plasma membrane recycling and domain formation, and can cause the blocking of enzymatic reactions of the central metabolism that, among other roles, produce the building blocks for lipid synthesis (Mira et al., 2010d; Orij et al., 2012; Pampulha and Loureiro-Dias, 1989, 1990). Also, the inhibition caused by the accumulation of the counterion can lead to plasma membrane damage, increase of turgor pressure and oxidative stress (Fernandes et al., 2005; Piper et al., 2001; Stratford and Anslow, 1996), being more drastic in more hydrophobic anions such as benzoate (Booth and Stratford, 2003; Piper et al., 2001; Ullah et al., 2012). Consistently, benzoic acid was found to perturb intracellular membrane dynamics, by affecting ER-to-Golgi trafficking (Hazan et al., 2004).

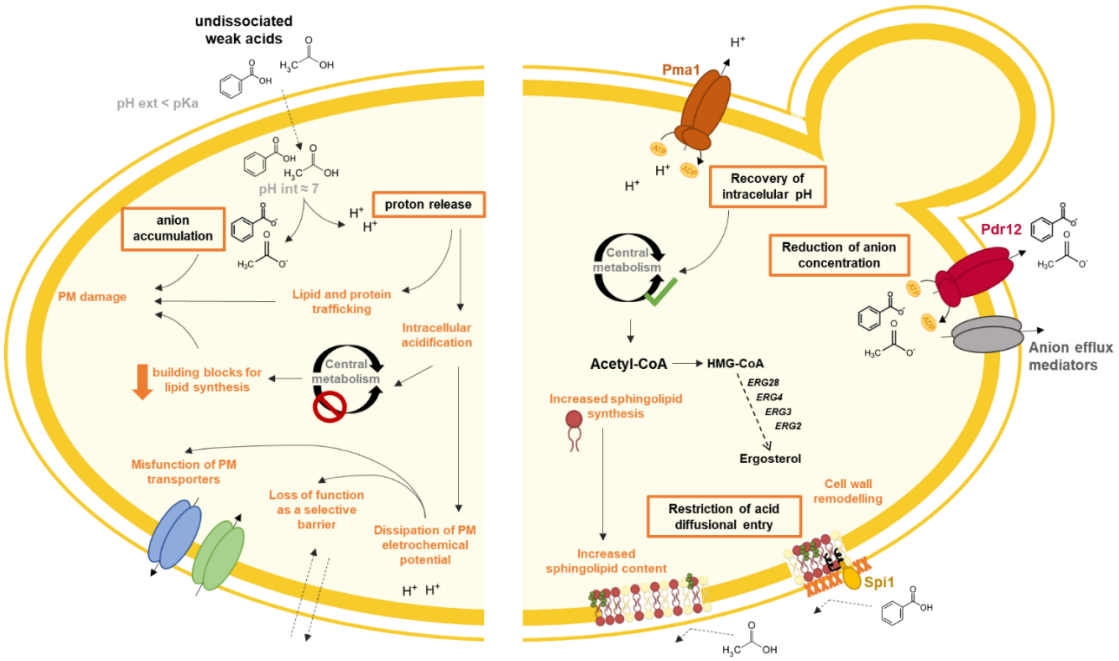


Figure 1-6. Schematic model for toxicity and adaptive response mechanisms induced by weak-acid stress, focusing on *S. cerevisiae* plasma membrane.
Mechanisms and deleterious effects of weak acid-induced stress in *S. cerevisiae* are represented on the left, whereas the adaptive response mechanisms are presented on the right (see text for further details and references). Abbreviations: pHext external pH; pHint intracelular pH; PM plasma membrane.

Yeast adaptation to weak acids comprises a number of molecular responses that aim to return pH to levels near to neutral pH, to reduce the concentration of the counterions, and to restrict the diffusional entry of weak acids. The plasma membrane H^+-ATPase Pma1 is described to play an important role in weak acid adaptation period, as its activity was found to restore the plasma membrane electrochemical potential by more actively extruding protons to the external media (Makrantoni et al., 2007; Mira et al., 2010d; Piper et al., 2001; Stratford et al., 2013). However, recent studies report that Pma1 activity is essential in response to acetic acid stress (and not as important under benzoic acid stress which is inhibitory for much lower concentration levels), because intracellular acidification and acidification-related deleterious effects are the major cause for growth inhibition in acetic acid stress (Ullah et al., 2012). This introductory chapter will focus mainly on the weak acid stress response mechanisms at the level at the plasma membrane that aim to restore plasma membrane function and its remodeling to restrict the diffusional entry of the acid. The comparison between the complete lipidomic profiling of acetic acid adapted and unadapted cells of *S. cerevisiae* and of the highly acetic acid resistant yeast *Zygosaccharomyces bailii*, indicated that incorporation of complex sphingolipid occurs in response to acetic acid stress in both species (Lindberg et al., 2013). Also in the same study, the higher fraction of sphingolipids incorporated in *Z. bailii* when compared to *S. cerevisiae* confirmed the correlation between sphingolipid content

and acetic acid tolerance. *Z. bailli* was found to reduce the content of the glycerophospholipids phosphatidylethanolamine, phosphatidylcholine and phosphatidylserine, possibly a mechanism for enriching sphingolipid content, as the fatty acids constituting these glycerophospholipids serve as supply for the synthesis of sphingolipids (Lindberg et al., 2013). The reduction of the content of glycerophospholipids was not found to be significant in *S. cerevisiae* adaptation to acetic acid (Lindberg et al., 2013). Studies on model membranes confirmed that the higher incorporation of sphingolipids results in thicker and more dense membranes, with lower permeability to acetic acid passive diffusion (Lindahl et al., 2016). Consistently, it was proved that inhibition of sphingolipid biosynthesis decreases acetic acid tolerance by increasing its passive diffusion, in the highly resistant yeast species *Zygosaccharomyces bailii* (Lindahl et al., 2016). Interestingly, the inhibition of sphingolipid biosynthesis did not decrease *Z. bailii* tolerance to the more hydrophobic benzoic acid, probably because the passive diffusion rate of benzoic acid is high and the changes in sphingolipid content exerted in the study by myriocin were not drastic enough to significantly reduce passive diffusion of this weak acid (Lindahl et al., 2016). Although no report previous to the work presented in this thesis was found to assign a role for ergosterol in acetic acid stress response, several genes encoding ergosterol biosynthetic enzymes were previously found to be determinants of acetic acid resistance (Mira et al., 2010c).

The mechanisms of yeast adaptation to benzoic acid-induced stress are less known. A study from 1997 reported for the first time the role of benzoate extrusion in alleviating benzoic acid stress, although the determinant for this extrusion was not clarified (Henriques et al., 1997). Later, benzoate extrusion was attributed to the ABC transporter Pdr12 (Piper et al., 1998). The cell wall-anchored protein Spi1 was found to modulate cell wall structure in response to benzoic acid-induced stress to avoid plasma membrane damage (Simões et al., 2006). Interestingly, a decrease in membrane permeability was reported as a response mechanism to overcome benzoic acid-induced stress (Kresnowati et al., 2008).

1.2.4 MDR/MXR transporters: are they really drug/xenobiotic pumps?

Although MDR/MXR transporters have traditionally been considered as drug efflux pumps, contributing to decrease intracellular concentration of a given toxic compound by pumping it out of the cell, a new light has been shed into the mechanisms through which these proteins alleviate yeast stress (Cabrito et al., 2011; dos Santos et al., 2014; Panwar et al., 2008; Roepe, 2000; Sherlach and Roepe, 2014). In fact, a more broader view proposes that MDR/MXR transporters act by altering drug transport therefore promoting the decrease of the drug to drug target exposure (Sherlach and Roepe, 2014). Whether this is a result of direct translocation of drugs/xenobiotics out of the cell, or indirect influence on drugs/xenobiotics accumulation by physical or chemical changes in plasma membrane (Sherlach and Roepe, 2014), requires more detailed experimental approaches. An extremely high number of MDR/MXR transporters, both from ABC and MF Superfamilies are encoded in the genome of *S. cerevisiae*, which also seems to indicate that MDR/MXR mediated by transporters is not an exceptional phenomenon, but a very conserved mechanism through which yeast can respond to environmental challenges and thrive (dos Santos et al., 2014; Jungwirth and Kuchler, 2006). This raises the question of what is the role of these MDR/MXR determinants in the absence of any environmental challenge. Most likely, the understanding of the physiological function of the MDR/MXR transporters can unveil some clues on the mechanisms behind their action in stress resistance.

1.2.5 Yeast drug pumps and resistance to multistresses of agronomical relevance in plants.

The studies performed on *S. cerevisiae* MDR/MXR transporters are useful to guide promising strategies for innovation in different organisms. An example is the heterologous expression of yeast MDR/MXR determinants in the plant model *Arabidopsis thaliana*. A recent study from our laboratory reports the impact in *Arabidopsis thaliana* of the heterologous expression of the ABC and MFS transporters ScPdr5 and ScTpo1, respectively, found to confer tolerance to the herbicides 2,4-D in *S. cerevisiae* (Remy et al., 2017). Expression of these transporters in *Arabidopsis thaliana* led to increased tolerance to 2,4-D and barban in the model plant. Moreover, correlating with the described phenotypes for these transporters in *S. cerevisiae*, ScTpo1 confers tolerance to the herbicides alachlor and metolachlor, the fungicide mancozeb and the cations Co^{2+}, Cu^{2+}, Ni^{2+}, Al^{3+} and Cd^{2+}, in planta whereas expression of ScPdr5 led to increased cycloheximide tolerance *in planta* (Remy et al., 2017). This kind of study can contribute to plant biotechnology, since the identification of mechanisms of tolerance allowing plants to resist to the action of agrochemicals and other agricultural relevant stresses can open new paths for the development of efficient strategies to improve crop productivity.

Also, the expression of MDR/MXR transporters from more complex organisms in *S. cerevisiae* is successfully allowing the study of the MDR/MXR phenomenon in complex eukaryotes. For example, in a study from our laboratory, *A. thaliana* homologues of the *S. cerevisiae* Tpo1 were screened for a role in 2,4-D tolerance (Cabrito et al., 2009). The ORF *At5g13750* was chosen and its expression in yeast was optimized and the correct localization at the plasma membrane was confirmed. This *A. thaliana* transporter, named ZIFL1 (zinc-induced facilitator-like 1), was found to complement the absence of the *ScTPO1* gene and improved the parental strain resistance to 2,4-D. The expression of both *ScTPO1* and *At5g13750* was found to further decrease intracellular accumulation of 2,4-D in yeast cells. Expression of *At5g13750* also rendered yeast cells with increased tolerance to Indole-3-acetic acid, Al3+ and Tl^{3+}(Cabrito et al., 2009). Other subsequent studies unveiled the physiological role of a number of *A. thaliana* MFS transporters of unknown function: *AtZIFL2* was found to modulate cesium and potassium homeostasis in Arabidopsis (Remy et al., 2015); *AtPHT1;9* and *AtPHT1;8* transporters mediate inorganic phosphate acquisition by the *A. thaliana* root during phosphorus starvation (Remy et al., 2012).

2 Pdr18 is involved in yeast response to acetic acid stress counteracting the decrease of plasma membrane ergosterol content and order

This Chapter contains results published in:

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2.1 ABSTRACT

Saccharomyces cerevisiae has the ability to become less sensitive to a broad range of chemically and functionally unrelated cytotoxic compounds. Among multistress resistance mechanisms is the one mediated by plasma membrane efflux pump proteins belonging to the ABC superfamily, questionably proposed to enhance the kinetics of extrusion of all these compounds. This study provides new insights into the biological role and impact in yeast response to acetic acid stress of the multistress resistance determinant Pdr18 proposed to mediate ergosterol incorporation in plasma membrane. The described coordinated activation of the transcription of *PDR18* and of several ergosterol biosynthetic genes (*ERG2-4*, *ERG6*, *ERG24*) during the period of adaptation to acetic acid inhibited growth provides further support to the involvement of Pdr18 in yeast response to maintain plasma membrane ergosterol content in stressed cells. Pdr18 role in ergosterol homeostasis helps the cell to counteract acetic acid-induced decrease of plasma membrane lipid order, increase of the non-specific membrane permeability and decrease of transmembrane electrochemical potential. Collectively, our results support the notion that Pdr18-mediated multistress resistance is closely linked to the status of plasma membrane lipid environment related with ergosterol content and the associated plasma membrane properties.

2.2 INTRODUCTION

The acquisition of multidrug/multixenobiotic resistance (MDR/MXR) is widespread in nature and has clinical, agricultural and biotechnological implications. MDR/MXR transporters that are presumably able to catalyze the efflux of multiple cytotoxic compounds play a role in this phenomenon (dos Santos et al., 2014; Jungwirth and Kuchler, 2006; Monk and Goffeau, 2008; Piecuch and Obłak, 2014; Sá-Correia et al., 2009). Two of the described MDR/MXR transporter families occur in all classes of organisms and belong to the ATP binding cassette (ABC) superfamily, which uses the hydrolysis of ATP to translocate a variety of solutes across biological membranes (Jungwirth and Kuchler, 2006; Piecuch and Obłak, 2014), or to the Major Facilitator Superfamily (MFS) which energetically drive their transport utilizing the transmembrane electrochemical gradient (dos Santos et al., 2014; Sá-Correia et al., 2009). Although those proteins have been traditionally considered drug exporters, their physiological function and the exact mechanism of their involvement in resistance to cytotoxic compounds are still unclear and it is puzzling that a wide range of structurally and functionally unrelated substrates may be exported by a specific transporter. Moreover, numerous ATP-binding cassette (ABC) transporters implicated in MDR/MXR are present in yeast genomes, strongly suggesting that they might have important physiological roles even in the absence of drugs/xenobiotics (Piecuch and Obłak, 2014). In fact, recent studies support the concept that at least some MDR/MXR transporters exert their effect as the result of a natural physiological role in the cell, rather than through the direct export of cytotoxic compounds (Cabrito et al., 2011; dos Santos et al., 2014; Prasad and Panwar, 2004; Teixeira et al., 2012). In particular, the role of ABC-MDR/MXR transporters in yeast plasma membrane lipid homeostasis is gaining increasing attention (Prasad et al., 2016) and can also explain the MDR/MXR phenotype exhibited by cells expressing these transporters. Indeed, the maintenance of plasma membrane integrity is crucial in yeast cell tolerance to stress and lipids play a critical role in determining membrane physical properties and regulating the function of membrane associated proteins (Beney and Gervais, 2001; Caspeta et al., 2015; Dupont et al., 2011; Henderson and Block, 2014; Singh, 2017; Ziółkowska et al., 2012). The *Saccharomyces cerevisiae* ABC transporters demonstrated in the scientific literature as involved in lipid trafficking and membrane lipid homeostasis are shown in figure 2-1. For example, Pdr11 and Aus1 are responsible for the uptake of exogenously supplied ergosterol in anaerobic conditions when ergosterol biosynthesis is impaired (Gulati et al., 2015; Li and Prinz, 2004; Wilcox et al., 2002). Also, the ABC efflux pumps Pdr5, Yor1 and Ste6 are implicated in the translocation of phospholipids between the two plasma membrane

monolayers, thus contributing to plasma membrane asymmetry (Decottignies et al., 1998; Ruetz et al., 1997). Pdr10 is associated with detergent-resistant domains in the plasma membrane and affects lipid distribution and maintenance of membrane micro-environment for the adequate functioning of membrane embedded proteins including the ABC-MDR transporter Pdr12 (Rockwell et al., 2009). Pdr16 and Pdr17 do not localize to the plasma membrane and their expression was proposed to regulate lipid metabolic pathways (Holič et al., 2014; Li et al., 2000; Van Den Hazel et al., 1999) but the underlying mechanisms were not detailed.

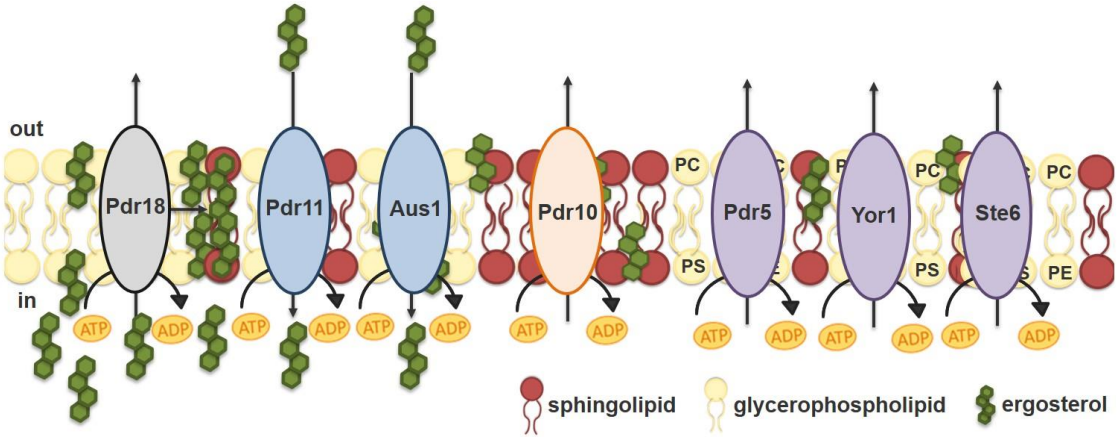


Figure 2-1. Plasma membrane ABC transporters of S. cerevisiae involved in lipid trafficking.
Schematic representation of a set of ABC transporters that localize to the plasma membrane and are documented to play physiological roles in lipid trafficking a and homeostasis in *S. cerevisiae* (Cabrito et al., 2011; Decottignies et al., 1998; Gulati et al., 2015; Li and Prinz, 2004; Rockwell et al., 2009; Ruetz et al., 1997; Teixeira et al., 2012; Wilcox et al., 2002). Pdr18, Pdr11, Aus1, Pdr10 and Pdr5 belong to the pleiotropic drug resistance (PDR) subfamily, Yor1 belongs to the multidrug resistance-associated protein (MRP) subfamily and Ste6 belongs to the multidrug resistance (MDR) subfamily of ABC transporters. The proposed biological functions are detailed in the Introduction section. Ergosterol transport, from its site of synthesis to the plasma membrane, is considered to occur by equilibration ($t_{1/2} \sim$ 10−15 min) of endoplasmic reticulum and plasma membrane ergosterol pools via a bidirectional, nonvesicular process (Baumann et al., 2005). Abbreviations: phosphatidylcholine (PC), phosphatidylserine (PS), phosphatidylethanolamine (PE).

The present work focuses on the yeast ABC transporter Pdr18, previously described as a MDR/MXR determinant and proposed to mediate ergosterol incorporation in yeast plasma membrane (Cabrito et al., 2011; Teixeira et al., 2012), Pdr18 expression confers increased yeast resistance to the herbicides 2,4-dichlorophenoxyacetic acid (2,4-D), 2-methyl-4-chlorophenoxyaceticacid (MCPA), and barban, the agricultural fungicide mancozeb, the metal cations Zn^{2+}, Mn^{2+}, Cu^{2+} and Cd^{2+} (Cabrito et al., 2011), and to ethanol(Teixeira et al., 2012). *PDR18* expression was found to contribute to reduce [^{14}C]-2,4-D intracellular accumulation but its action may be indirect since 2,4-D induces the decrease of plasma membrane ergosterol content, this deleterious effect being much stronger in the *pdr18*Δ background (Cabrito et al., 2011).

In this article, we describe Pdr18 role in yeast adaptation and tolerance to acetic acid stress. Acetic acid is widely used by the food industry as preservative of acidic food and beverages but there are spoilage yeasts that are able to cope with standard levels causing severe economic losses (Fleet, 2007). Acetic acid tolerance may however be a highly desirable trait in industrial yeasts, since this weak acid is an industrial fermentation byproduct, its accumulation in the medium contributing to reduced fermentation performance (Gibson et al., 2007; Lu et al., 2012). Moreover, acetic acid is present in lignocellulosic biomass hydrolysates for the production of bioethanol and other bio-based chemicals (Almeida et al., 2007; Koppram et al., 2014). Therefore, the understanding of the mechanisms underlying the toxic effects and adaptation and tolerance to acetic acid in the biotechnologically-relevant yeast species *S. cerevisiae* is essential to guide more efficient strategies either for food spoilage control or for the development of more robust industrial strains (dos Santos et al., 2012; dos Santos and Sá-Correia, 2015; Geng et al., 2017; Mira et al., 2010c, 2010d; Mollapour et al., 2008; Palma et al., 2018).

Yeast adaptation to weak acids is a complex multifactorial process considered to include mechanisms leading to the reduction of the intracellular concentration of the counter-ions through their increased efflux from the cell interior (Mira et al., 2010d) and restriction of the diffusional entry of the liposoluble acid form into the cell (Lindahl et al., 2016; Mira et al., 2010d; Palma et al., 2018). In particular, incorporation of a higher fraction of sphingolipids was found to improve membrane bilayer thickness and density, leading to the decrease of plasma membrane non-specific permeability in the highly acetic acid resistant species *Zygosaccharomyces bailii* (Lindahl et al., 2016). Interestingly, the sphingolipid biosynthetic pathway was found to be up-regulated in response to acetic acid-induced stress (Guerreiro et al., 2016). Also, plasma membrane enrichment in saturated acyl chains arising from glycerophospholipids and complex sphingolipids was also found to occur in response to acetic acid stress and lead to reduced cell permeability and increased membrane order (Lindberg et al., 2013; Simons and Sampaio, 2011). A chemogenomic study has identified in *S. cerevisiae* several acetic acid resistance determinant genes encoding ergosterol biosynthetic enzymes (Mira et al., 2010c) and the inclusion of a higher content of sterols in yeast plasma membrane demonstrated to counteract the deleterious effects of ethanol (Aguilera et al., 2006; Chi and Arneborg, 1999; del Castillo Agudo, 1992; Vanegas et al., 2010, 2012) and heat shock (Caspeta et al., 2014b; Swan and Watson, 1998). The content of ergosterol is not only crucial for plasma membrane stability and adequate selective permeability barrier to avoid the passive diffusion of toxic compounds into the cell (Eisenkolb et al., 2002; Parks and Casey, 1995), but is also related with the formation of lipid-raft domains that

may modulate the activity of membrane-embedded pumps (Bagnat et al., 2001; Pasrija et al., 2008).

The present study provides new insights into the biological role and impact of Pdr18 in yeast cell response under stress, with a focus on acetic acid stress. The observed coordinated activation of the transcription of *PDR18* and of several ergosterol biosynthetic genes during the period of adaptation to acetic acid inhibited growth provides further support to the involvement of Pdr18 in yeast response to maintain ergosterol content in plasma membrane of yeast stressed cells. The observed impact that such biological role has in counteracting acetic acid-induced decrease of plasma membrane lipid order, increase of the non-specific permeability and decrease of the transmembrane electrochemical potential establishes a link between this ABC transporter biological activity and its involvement in multistress resistance.

2.3 MATERIALS AND METHODS

2.3.1 Strains, plasmids and growth conditions

Saccharomyces cerevisiae parental strain BY4741 (*MATa*, *his3Δ1*, *leu2Δ0*, *met15Δ0*, *ura3Δ0*) and the derived deletion mutant *pdr18*Δ were obtained from the EUROSCARF collection (http://web.uni-frankfurt.de/fb15/mikro/euroscarf), as well as plasmid pYCG_*PDR18*, expressing the *PDR18* gene from its natural promoter, and the corresponding cloning vector, pRS416, used for phenotypic complementation assays.

Yeast cells were cultivated at 30°C with orbital agitation (250 rpm) in liquid minimal growth medium supplemented with the amino acids and the nucleotide to support growth of the auxotrophic strains (MM4). MM4 contained 1.7 g/L yeast nitrogen base without amino acids and ammonium sulphate (Difco, Michigan, USA), 20 g/L glucose (Merck, Darmstadt, Germany), 2.65 g/L $(NH_4)_2SO_4$ (Panreac AppliChem, Connecticut, USA), 20 mg/L L-methionine, 20 mg/L L-histidine (both from Merck, Darmstadt, Germany), 60 mg/L L-leucine and 20 mg/L L-uracil (both from Sigma, Missouri, USA), adjusted to pH 4.0 with HCl. Solid medium was prepared by the addition of 20g/L agar (Iberagar, Barreiro, Portugal) and the pH was set to 4.5 with HCl. Cells harboring the cloning vector pRS416 or derived plasmids were grown in the same medium lacking uracil supplementation (MM4-U medium) to maintain selective pressure.

2.3.2 Susceptibility assays

Growth curves were obtained by inoculating a mid-exponential cell suspension in 100 mL flasks containing 50 mL MM4 pH 4.0 supplemented or not with acetic acid pH 4.0 (set with NaOH) at the desired concentration. The starting OD_{600nm} of the cultivation media was standardized at 0.1±0.05. Growth with orbital agitation was followed at 30°C by measuring OD_{600nm}.

Yeast cell suspensions used for the spot assays were prepared from a mid-exponential cell culture grown in MM4 medium, diluted to an OD_{600nm} of 0.05 (lane a in the figures), followed by two serial dilutions of 1:5 each (lanes b and c). These cell suspensions were plated as 4 µL spots onto the surface of MM4 pH 4.5 solid medium, supplemented or not with acetic acid. Complementation tests were perfomed with yeast cells harboring either the empty vector pRS416 or the recombinant plasmid pYCG_*PDR18*. For the complementation tests, spot assays were prepared as described before and cell suspensions were spotted onto MM4-U agar plates that were incubated at 30°C for 72 hours.

2.3.3 Time-course transcription analysis of *PDR18* and *ERG*-genes

For gene transcription assays, BY4741 cells were harvested at adequate time points from liquid medium cultivations performed in the presence and absence of 60 mM acetic acid, as described above. Total RNA was extracted by the hot phenol method(Collart and Oliviero, 1993). The real-time Reverse Transcription–PCR protocol used followed the manufacturer's instructions (Applied Biosystems, California, USA) and the primers used for the amplification of each target cDNA were designed using the Primer Express software (Applied Biosystems, California, USA) (supplementary table S2-1). The RT–PCR reaction was carried out using a thermal cycler block (7500 Real-Time PCR System; Applied Biosystems, California, USA). The *ACT1* mRNA level was used as the internal control. The relative value obtained for each target gene at the initial time point (15 minutes of incubation following inoculation) under unstressed conditions was set as 1 and the remaining values are relative to that value.

2.3.4 Ergosterol quantification in yeast cell membranes

For quantification of ergosterol in yeast cell membranes, the parental and *pdr18*Δ strains were cultivated in MM4 either in the presence or absence of acetic acid stress (60 mM at pH 4.0) as described above. Exponentially-growing cells were harvested by centrifugation (5000 *g*, 5 minutes, 4°C), resuspended in homogenization buffer containing 50 mM Tris/HCl (pH 7.5), 2.5 mM EDTA and a protease inhibitor cocktail (1 mM PMSF and 1 µg/mL each of leupeptin, pepstatin A and aprotinin). Cells were broken by vortex-mixing with glass beads (Glaperlon 0.40–0.60 mm). The cell membranes were recovered by centrifugation (1000 *g*, 10 minutes, 4°C) to remove unbroken cells and finally the cell membranes were pelleted by ultracentifugation at 25 000 rev./min for 1 hour (rotor type SW41Ti, Beckman Coulter). The pelleted cell membranes were resuspended in a buffer containing 20 mM Tris/HCl (pH 7.5), 150 mM NaCl, 20% glycerol and protease inhibitors at the concentrations mentioned above, stored frozen and freeze-dried until sterol extraction. Sterol extraction involved addition of 250 µL 5-□-cholestane (internal standard, Sigma) in dichloromethane and a lipid extraction using 600 µL methanol (Merck) and 300 µL dichloromethane (Merck). Samples were homogenized for 60 seconds at 2-4 °C in a homogenizer (polytron 10-35 GT, Kinematica) at 10,000 rpm. Afterwards, 300 µL ultra-pure water and 300 µL dichloromethane were added. Samples were centrifuged (3000 *g*, 10 minutes, 4°C). The lower phase was filtered through an anhydrous sodium sulfate (Merck) layer and solvent evaporated under N_2 at 20 °C. Lipids were saponified with 500 µL of 2.5 N KOH (Merck) at 80 °C for 1 hour. Saponified lipids

were extracted with 1 ml of saturated NaCl (Merck) and 5 ml hexane (Merck). Organic phase was evaporated under N_2 at 20°C. Derivatization was done with 100 µL N,O-bis(trimethylsilyl)trifluoroacetamide with trimethylchlorosilane (Sigma) at 80°C for 12 hours. Derivatized sterols were analyzed using GC-MS (Scion 456 GC-SQ, Bruker) with a DB5 column (30 m×0.2 mm, 0.20 µm). The carrier gas was helium (flow rate of 1 mL/min). Oven temperature was held at 120°C for 1 min, increased to 300°C at a rate of 2°C/min, and held at 300°C for 20 min. Injection and detector temperatures were 300°C. Peak identification was based on retention time and comparison with external standards in the spectral database. Quantification was achieved through the internal standard peak area. Sterol concentration was normalized taking into account phosphate content in the freeze-dried samples.

2.3.5 Assessement of membrane lipid order by Laurdan fluorescence

Membrane lipid order was estimated in exponentially-growing cells of parental and *pdr18*Δ strains either in the absence or presence of acetic acid, by the normalized ratio of Laurdan fluorescence emission measured in two channels. Growth curves were performed as described above and cells at mid-exponential phase (OD_{600nm}=0.5) were centrifuged (6000 *g*, 5 minutes), washed three times and resuspended in 200 µL PBS buffer (137 mM NaCl; 2.7 mM KCl; 10 mM Na_2HPO_4; 1.8 mM KH_2PO_4 in 1 L distilled water, pH adjusted to 7.4 with HCl; filtered). These cell suspensions were deposited in a glass 8-wells chamber (Ibidi, Martinsried, Germany) treated with poly-L-lysine (Sigma, Missouri, USA) for better adherence of living yeast cells. The incubation with 5 µM Laurdan (Sigma, Missouri, USA) was performed at room temperature for 1 hour followed by two washing steps with PBS. Images were obtained by two-photon excitation fluorescence microscopy on a Leica TCS SP5 (Leica Microsystems CMS GmbH, Manheim, Germany) inverted confocal microscope (DMI600), using a Ti:sapphire laser (Mai Tai, Spectra-Physics, Darmstadt, Germany) as the excitation source, and an apochromatic water immersion objective (63×, NA 1.2; Zeiss, Jena, Germany). The excitation wavelength was set to 780 nm and the fluorescence emission of Laurdan was collected at 400–460 nm and 470–530 nm. Laurdan exhibits shifts in its fluorescence emission maximum in response to changes in membrane hydration and solvent relaxation and this shift can be estimated through a normalized ratiometric measurement of the fluorescence intensity recorded in the two spectral channels, the generalized polarization (GP) value (Parasassi and Krasnowska, 1998; Sýkora et al., 2002). GP images were obtained and analysed using homemade software developed in a MATLAB

environment (Mathworks, Natick, MA), with the GP value defined as $GP=(I_{400–460} - G \cdot I_{470–530})/(I_{400–460} + G \cdot I_{470–530})$. Both channel intensities are corrected for background contributions, and the calibration factor G was obtained from imaging Laurdan in DMSO using the same experimental conditions as those set for the samples under study (Owen et al., 2012).

2.3.6 Permeability and plasma membrane potential estimation

Differences in the plasma membrane potential of the parental strain BY4741 and the derived deletion mutant *pdr18*Δ grown in the presence or absence of acetic acid were estimated based on the shift in the maximum emission wavelength of the fluorescent probe DiS-C_3(3) (3-3'-Dipropylthiacarbocyanine iodide), as described before(Gaskova et al., 1998). For the timepoints tested, yeast cells were harvested as described above for other assessments and resuspended in citrate-phosphate (CP) buffer to a final OD_{600nm} of 0.5. 5-mL aliquots of cell suspensions were labelled with DiS-C_3(3) (Sigma-Aldrich) at a final concentration of 10^{-7} M, for 30 minutes at room temperature with orbital agitation of 70 rpm. Carbonyl cyanide *p*-chlorophenylhydrazone (CCCP) was added to an exponentially-growing culture of parental strain cells, to a final concentration of 5 µM, and this condition was used as a positive control for full membrane depolarization(Höfer and Novacky, 1986). Fluorescence emission spectra of the cell suspensions were collected from 555 to 790 nm (at wavelength excitation of 531 nm) in 0.5 cm x 0.5 cm quartz cuvettes using a SLM-Aminco 8110 Series 2 spectrofluorometer (Rochester, New York, USA).

Plasma membrane permeability was assessed by the passive uptake of propidium iodide (PI; 20 mM in DMSO, Invitrogen), from cells harvested at adequate time points of the cultivation of parental strain and *pdr18*Δ cells either in the presence of absence of 60 mM acetic acid. PI was added to 1 mL of 4 x 10^7 cells/mL to a final concentration of 20 µM, cell suspensions and incubated in the dark with orbital agitation (15 minutes, 250 rpm). Cells exposed to PI were centrifuged (17,500 x g for 5 minutes), washed twice and ressuspended in PBS buffer to a final 10^7 cells/mL aliquots. PI-fluorescence emission spectrum (collected in the range 563-700 nm, at an excitation wavelength of 535 nm) were obtained using a SLM-Aminco 8100 series 2 spectrofluorometer with double excitation and emission monochromators MC400 (Spectronic Instruments, Inc., Rochester, NY), in a right-angle geometry. The light source was a 450 W Xe arc lamp and the reference a Rhodamine B quantum counter solution. All measurements were performed in 0.5 cm × 0.5 cm quartz cuvettes and under a constant temperature of 30ºC by using a thermostated sample holder with a Julabo circulating water bath (Houston,

Texas, USA). Gain adjustment of the apparatus was performed using a positive control for full permeabilization (exponentially-growing parental strain cells exposed during 30 minutes to 50% ethanol in PBS). The spectra obtained were integrated and the area under the curve was calculated.

To evaluate the population heterogeneity for the cell permeability trait, at adequate time points the cell population strained with PI was observed using an Axioplan microscope equipped with adequate epifluorescence interface filters (BP450-490 and LP520; Zeiss). Fluorescence images were obtained with a cooled charge-coupled device camera (Cool SNAPFX; Roper Scientific Photometrics), and the images were analyzed with MetaMorph, version 3.5. Cell-to-cell fluorescence intensity was defined as the average of pixel by pixel intensity in the selected region of interest and a minimum of 50 cells/experiment were used. The fluorescence images were background corrected by using dark-current images.

2.4 RESULTS

2.4.1 *PDR18* gene expression is required for maximum tolerance to acetic acid

The expression of the ATP-binding cassette plasma membrane transporter encoding gene *PDR18* was found to be required to partially overcome the effects of acetic acid induced stress in yeast growth (figures 2-2 and 2-3). Susceptibility towards this weak acid was assessed based on yeast growth in liquid medium by comparing the growth of a parental strain *Saccharomyces cerevisiae* BY4741 and the derived deletion mutant *pdr18*Δ exposed to a wide range of acetic acid concentrations and determining the minimum inhibitory concentration (MIC) (figure 2-2). While the parental strain was able to grow in the presence of concentrations equal or below 95 mM, but not in the presence of 100 mM acetic acid (the calculated MIC for this strain), the MIC for the deletion mutant *pdr18*Δ was 75 mM (figure 2-2). Moreover, the final biomass attained after yeast cultivation under acetic acid stress showed a dose-dependent decrease and was more evident for the *pdr18*Δ mutant (figure 2-2).

parental strain

Acetic acid (mM) \ Time (h)	0	24	48	72	96	120	144	168	192	216	240
0	0.19	3.48	3.59	3.32	3.16	3.15	2.90	3.15	3.20	3.19	3.22
50	0.20	3.38	3.44	3.34	3.06	3.20	2.89	2.83	2.85	2.88	2.86
60	0.16	3.42	3.27	3.28	3.08	3.30	2.89	2.86	2.89	2.88	2.90
65	0.15	0.31	3.10	2.78	2.75	2.64	2.70	2.68	2.73	2.72	2.83
70	0.16	0.21	2.98	2.76	2.68	2.76	2.79	2.78	2.74	2.73	2.71
75	0.15	0.20	1.36	2.71	2.58	2.57	2.53	2.53	2.52	2.53	2.51
80	0.15	0.20	0.23	0.26	0.26	2.72	2.70	2.65	2.61	2.56	2.50
85	0.15	0.19	0.26	0.25	0.30	2.62	2.57	2.45	2.39	2.37	2.38
90	0.15	0.19	0.19	0.11	0.23	2.65	2.60	2.54	2.49	2.40	2.24
95	0.14	0.20	0.20	0.20	0.22	0.74	2.14	2.20	2.19	2.21	2.30
100	0.14	0.20	0.20	0.20	0.19	0.15	0.15	0.15	0.15	0.15	0.18

***pdr18*Δ**

Acetic acid (mM) \ Time (h)	0	24	48	72	96	120	144	168	192	216	240
0	0.18	2.98	3.28	3.20	3.54	3.20	2.98	2.99	2.95	2.97	2.94
50	0.13	0.17	0.96	2.82	2.86	3.00	2.88	2.86	2.85	2.84	2.84
60	0.13	0.16	0.16	0.35	2.71	2.88	2.79	2.75	2.70	2.65	2.60
65	0.16	0.21	0.22	0.24	2.57	2.57	2.57	2.58	2.60	2.56	2.54
70	0.12	0.15	0.16	0.15	0.18	2.66	2.71	2.66	2.63	2.60	2.56
75	0.16	0.19	0.20	0.19	0.15	0.17	0.16	0.16	0.16	0.16	0.16
80	0.13	0.17	0.17	0.16	0.16	0.16	0.15	0.16	0.16	0.16	0.16
85	0.14	0.15	0.16	0.17	0.16	0.16	0.16	0.15	0.15	0.15	0.15
90	0.14	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.14
95	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.14	0.15
100	0.15	0.15	0.16	0.16	0.16	0.17	0.16	0.16	0.16	0.16	0.16

OD_{600nm}

0.1 → 3.6

Figure 2-2. Inhibitory effect of acetic acid in the parental strain and *pdr18*Δ mutant growth. Growth was assessed based on culture OD_{600nm} during 240 hours of incubation in liquid MM4 supplemented with increasing concentrations of acetic acid (at pH 4.0). The minimum inhibitory concentration (MIC) found for the parental strain and *pdr18*Δ mutant (100 or 75 mM acetic acid, respectively) is underlined. Results are representative of three independent growth experiments.

Spot assays in agar plates of MM4 medium supplemented with 60 mM of acetic acid (at pH 4.5) confirmed the role of Pdr18 as an acetic acid resistance determinant (figure 2-3A). In fact, the susceptibility phenotype of the *pdr18*Δ mutant under acetic acid was rescued by the insertion of a centromeric plasmid expressing the *PDR18* gene under the

control of its natural promoter. Moreover, the expression of Pdr18 from the recombinant plasmid in the parental strain improved its resistance to acetic acid (figure 2-3B).

Most of the experiments described in this work were performed using the standardized concentration of acetic acid (60 mM, at pH 4.0). Under these conditions, growth curve of the parental strain in acetic acid supplemented MM4 medium exhibit a lag-phase of approximately 10 hours that was not detectable in the absence of acetic acid stress, while the duration of the acetic acid-induced lag-phase was extended to approximately 40 hours for the *pdr18*Δ mutant (figure 2-3C; supplementary figure S2-1). Also, the maximum specific growth rates and the final biomass concentrations attained were more drastically reduced on the mutant compared to the parental strain under acetic acid stress (figure 2-3C).

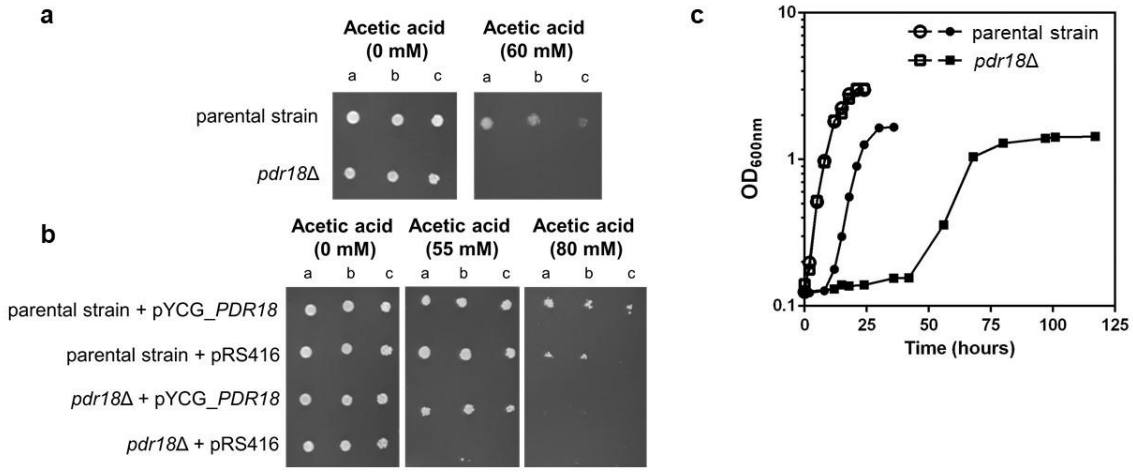


Figure 2-3. *PDR18* gene is a determinant of acetic acid tolerance in yeast.
(a) Comparison of growth by spot assays of the parental strain and *pdr18*Δ mutant cell suspensions plated in solid MM4 media supplemented or not with acetic acid (at pH 4.5); **(b)** Complementation assays of growth in solid MM4-U media of the parental and *pdr18*Δ strains, harboring plasmid pRS416 (cloning vector) or the recombinant vector expressing *PDR18* from its natural promoter. In **(a)** and **(b)** the cell suspensions used to prepare the spots in lanes **b**) and **c**) were 1:5 and 1:25 serial dilutions, respectively, of the cell suspensions with an OD_{600nm}=0.05±0.005 in lane **a**) prepared from an exponentially growing culture of each strain. **(c)** Growth curves of the parental (○, ●) and *pdr18*Δ (□, ■) strains in MM4 liquid medium supplemented (●, ■) or not (○, □) with 60 mM acetic acid at pH 4.0 based on culture OD_{600nm}. Results of all panels of the figure are representative of at least three independent growth experiments.

2.4.2 *PDR18* and *ERG*-genes transcription is up-regulated in response to acetic acid stress contributing to the maintenance of plasma membrane ergosterol content

Levels of mRNA from *PDR18* and selected *ERG*-genes in *S. cerevisiae* BY4741 cells were compared during cultivation of cells not previously adapted to acetic acid in MM4

or MM4 supplemented with 60 mM acetic acid at several time-points (figure 2-4). Results show that *PDR18* transcription is activated in response to acetic acid stress. Maximum activation levels occur when the cell population resumes growth after an extended period of adaptation to growth under acetic acid stress and these levels were 3-fold higher than in unstressed cells. However, during exponential growth in the presence of acetic acid, mRNA levels from *PDR18* decreased to values close to those in unstressed cells. In the absence of stress, the transcription profile of *PDR18* is also dependent on the growth phase, mRNA levels peaking during exponential growth (figure 2-4).

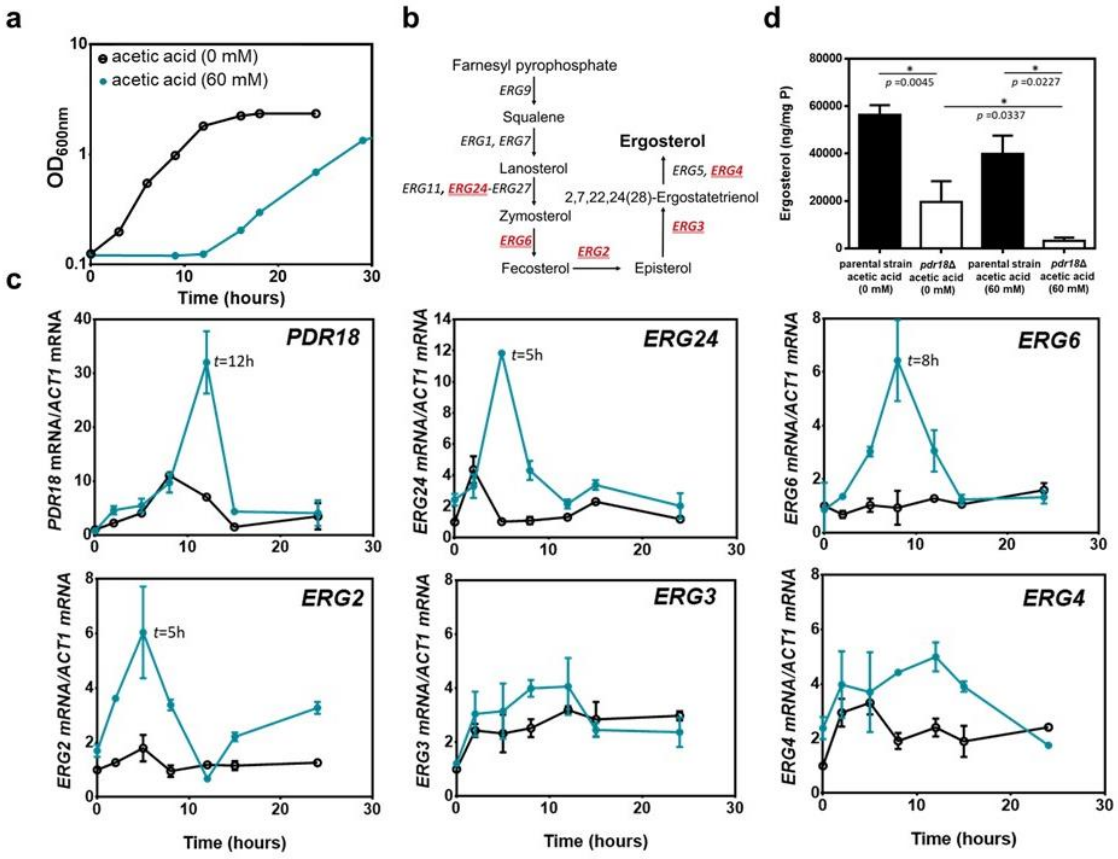


Figure 2-4. Levels of mRNA from *PDR18* and from several ergosterol biosynthetic pathway genes during cultivation in absence or presence of acetic acid and comparison of ergosterol levels.
(a) Growth curve of *S. cerevisiae* BY4741 in absence (○) or presence (●) of 60 mM acetic acid (at pH 4.0) based on culture OD_{600nm}; **(b)** Ergosterol biosynthetic pathway [adapted from KEGG (http://www.genome.jp/kegg/pathway.html)]; the *ERG*-genes under study are highlighted in red; **(c)** mRNA levels from the indicated genes by qRT-PCR, using *ACT1* as the reference gene. **(d)** Ergosterol content in cell membranes from exponentially-growing cells of the parental and *pdr18*Δ strains in the absence or presence of 60 mM acetic acid (60 mM). Error bars represent standard deviation resultant from at least two biological replicates with three technical replicates each.

Since several enzymes catalyzing post-squalene steps of the ergosterol biosynthetic pathway have been related with acetic acid stress response(Kawahata et al., 2006; Mira et al., 2010c), the transcription profiles of *ERG2-4*, *ERG6*, and *ERG24* genes were obtained. These genes are known determinants of resistance to acetic acid/weak acid

stress in yeast(Mira et al., 2010c, 2010d; Schüller et al., 2004). The transcription levels from those *ERG* genes during acetic acid stressed growth show two profiles: *ERG24*, *ERG6* and *ERG2* have a peak of activation (6-12 fold) after 5-8 hours, by the mid- to end of the phase of latency, while for *ERG3* and *ERG4* genes the activation levels are lower (2-5-fold) and were maintained throughout cultivation (figure 2-4). Ergosterol quantification confirms its lower content in membranes of exponentially-growing unstressed cells of the deletion mutant *pdr18*Δ compared to the parental strain. The ergosterol content in *pdr18*Δ cells exponentially-growing under acetic acid stress was found to be dramatically reduced (approximately 10-fold), compared to parental strain levels cells growing in the same conditions (figure 2-4). These results confirm the need of Pdr18 expression to maintain ergosterol content in plasma membrane especially under acetic acid stress. Collectively, these results are consistent with the biological function attributed to Pdr18 in ergosterol homeostasis and indicate that under acetic acid stress *PDR18* gene expression regulation is part of the yeast response to acetic acid stress together with the activation of ergosterol biosynthesis.

2.4.3 *PDR18* expression leads to a more ordered plasma membrane especially under acetic acid stress

As ergosterol content is an important factor affecting yeast plasma membrane order(Abe and Hiraki, 2009; Nes et al., 1978; Vanegas et al., 2012), the next step was to assess plasma membrane order in the parental and *pdr18*Δ cell strains grown either unstressed or in the presence of acetic acid stress to further understand the impact that *PDR18* expression has at the level of plasma membrane properties.

Laurdan is a membrane dye whose fluorescence emission spectrum depends on lipid packing. For more ordered membranes, the emission spectrum of Laurdan is shifted to lower wavelengths relative to the spectrum of the same probe in disordered membranes. This spectral shift can be quantified through the calculation of a generalized polarization (GP) factor from the fluorescence intensity of two spectral channels of Laurdan emission spectra(Parasassi and Krasnowska, 1998). The GP value of Laurdan is a convenient and quantitative parameter to evaluate membrane packing, with higher values reflecting higher ordering.

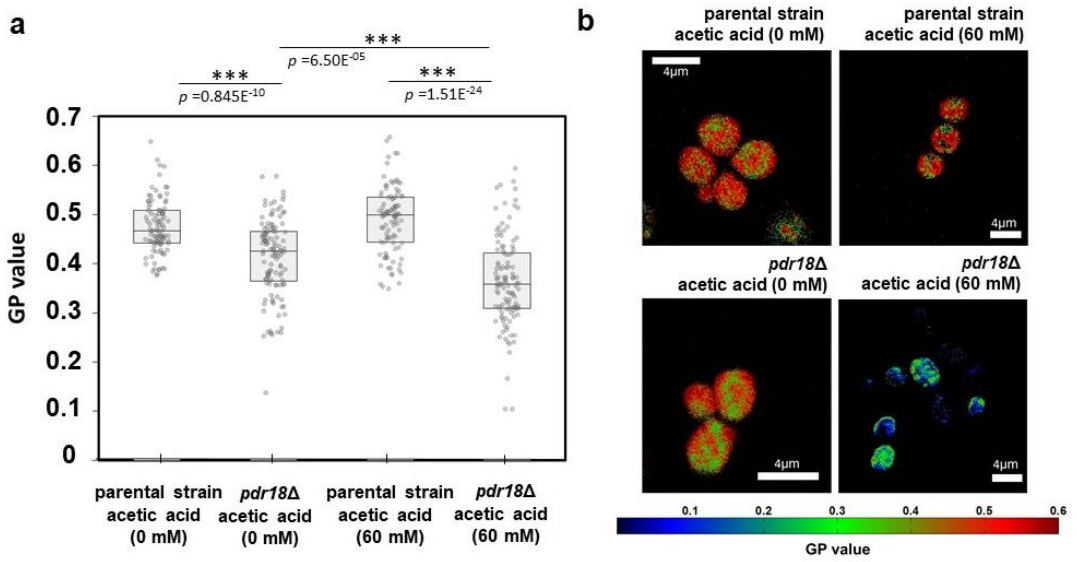


Figure 2-5. Plasma membrane order of the parental and *pdr18*Δ strains growing in absence or presence of acetic acid, as reported by Laurdan Generalized Polarization (GP) values.
(a) GP values from individual exponentially-growing cells of the parental and *pdr18*Δ strains cultivated either in the presence or absence of 60 mM acetic acid at pH 4.0. The boxplots represent the first and third quartiles and median for each cell population; **(b)** Examples of Laurdan Generalized Polarization images for the parental strain and *pdr18*Δ cells grown in absence or presence of acetic acid stress. Results arise from the analysis of at least 100 cells obtained from three independent assays. Asterisks represent statistical significant differences, based on t-student tests.

The distribution of Laurdan Generalized Polarization (GP) values for each individual cell is shown in figure 2-5A and illustrative examples of GP images are shown in figure 2-5B. The parental strain cell population harvested in the exponential phase of growth show a significantly higher plasma membrane order than the population of cells lacking *PDR18* gene, when both strains were grown either in the absence (p=0.845E^{-10}) or in presence (p =1.51E^{-24}) of 60 mM acetic acid at pH 4.0 (figure 2-5A). Parental cells harvested in the exponential phase of growth without acetic acid or adapted to that concentration of acetic acid show no significant difference in plasma membrane order, as assessed by the GP mean value (figure 2-5A). However, Laurdan GP values, reflecting plasma membrane order, of *pdr18*Δ cells decreased by 12% when cultivated in the presence of acetic acid (figure 2-5A). Also noteworthy is that Laurdan GP values for the *pdr18*Δ cell population exhibit an interquartile range (Q3-Q1) 53% larger than the parental strain population, both grown in the absence of acetic acid, which indicates a higher cell heterogeneity for plasma membrane order within the *pdr18*Δ cell population (figure 2-5A). Adapted cells exponentially-growing in the presence of acetic acid that lack the *PDR18* gene, exhibit a lower plasma membrane order (25% lower GP mean value) and a more heterogeneous profile for this trait (25% larger interquartile range), compared with the stressed parental strain population (figure 2-5A).

2.4.4 Acetic acid stress induces an increase in plasma membrane permeability when yeast cells lack *PDR18*

The less ordered plasma membrane lipid environment of the *pdr18*Δ deletion mutant assessed before was found to have a considerable impact on the physiological function of yeast plasma membrane as a selective barrier in the presence of acetic acid, with plasma membrane permeability being inferred based on propidium iodide (PI) passive uptake (figures 2-6 and 2-7). PI is a cell-impermeant dye with a high affinity for nucleic acids. The dye only penetrates cells with compromised plasma membranes; the subsequent binding to intracellular nucleic acids increases PI fluorescence up to 30-fold(Stiefel et al., 2015). Therefore, the PI fluorescence intensity parameter can be correlated with the proportion of permeabilized cells.

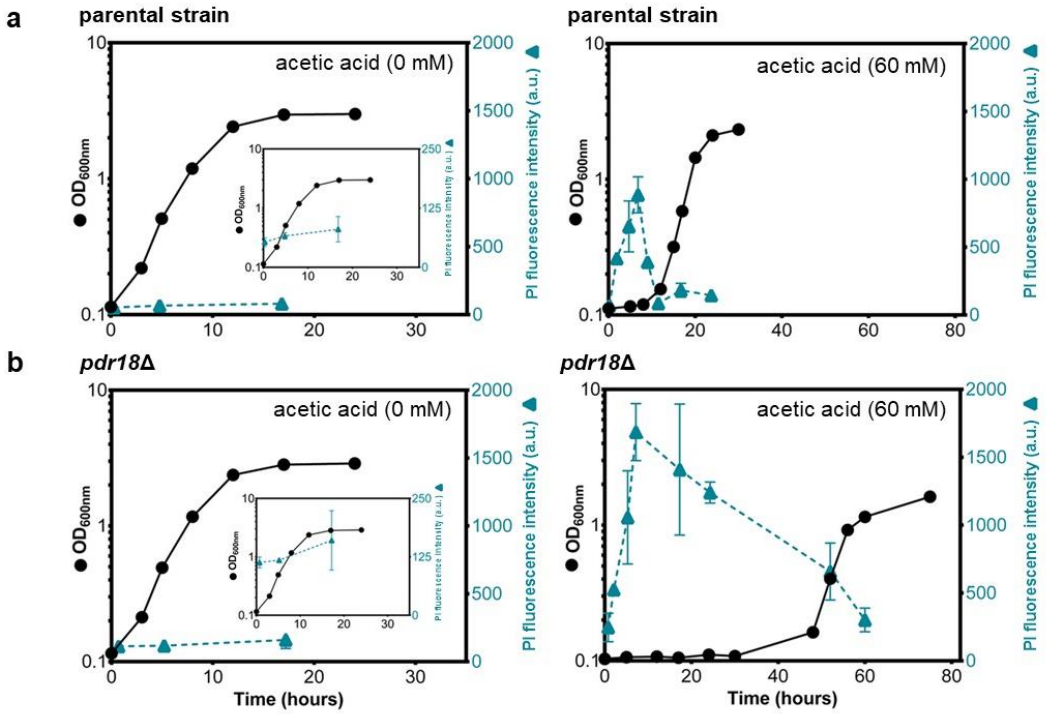
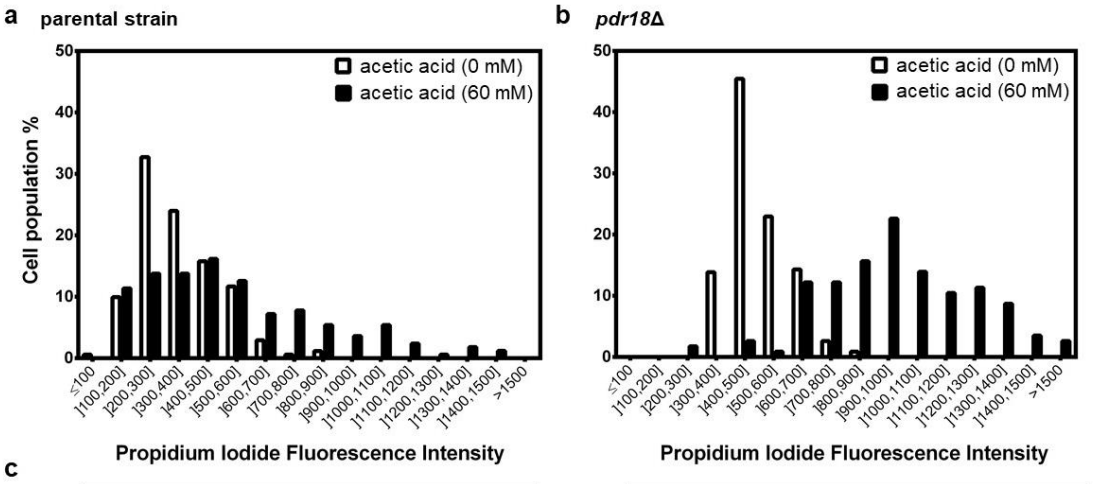


Figure 2-6. Plasma membrane permeability of the parental strain and *pdr18*Δ cells during the growth curve in the presence or absence of acetic acid.
Comparison of plasma membrane permeability (▲; arbitrary units) during cultivation of the parental **(a)** or *pdr18Δ* **(a)** strains in the presence or absence of 60 mM acetic acid at pH 4.0. The estimation of plasma membrane permeability is based on the fluorescence intensity values exhibited by yeast cells upon passive accumulation of propidium iodide. Graphs with a more appropriate fluorescence intensity scale are shown to allow comparison between **(a)** and **(b)** in the absence of acetic acid. Error bars represent standard deviation (n=3).

Even in the absence of any stress, cells deleted for *PDR18* are more permeable than cells of the parental strain harvested during the growth curve (25% higher permeability; figure 2-6). Acetic acid supplementation of the growth medium (60 mM at pH 4.0) leads

to an increased plasma membrane permeability in parental strain cells during the growth curve, PI uptake values peaking at 7 hours (10-fold the levels in absence of acetic acid) by the time when cell population resumes growth after the adaptation phase (figure 2-6A). Remarkably, during exponential growth under acetic acid stress, yeast cell permeability is reduced, and cells harvested at mid-exponential phase exhibit a permeability value similar (only 1.3-fold higher) to unstressed cells (figure 2-6A). In *pdr18*Δ mutant cells, plasma membrane permeability peaks during acetic-acid induced lag-phase, reaching values above those registered for the parental strain and much higher (15-fold) than those registered for unstressed cells (figure 2-6B). Even during exponential growth adapted to acetic acid, the permeability of cells deleted for *PDR18* gene is above the values for unstressed cells (4-fold higher) and this cell population was unable to recover from acetic acid-induced permeabilization during lag-phase to levels similar to unstressed cells (figure 2-6B).



	acetic acid (0 mM)	acetic acid (60 mM)
Amplitude	29,31	14,61
Mean	315,6	431,7
SD	132,4	277,6

	acetic acid (0 mM)	acetic acid (60 mM)
Amplitude	43,53	17,95
Mean	469,4	962,7
SD	84,95	261,9

Figure 2-7. Cell-by-cell propidium iodide fluorescence in exponentially-growing cell populations of parental or pdr18Δ strain in the presence or absence of acetic acid.
Distribution of propidium iodide fluorescence intensity classes of membrane permeability of the parental strain (**a**) and *pdr18Δ* (**b**) cell population harvested in the exponential phase in absence (□) or presence (■) of 60 mM acetic acid; **(c)** Statistical parameters of a Gaussian curve fit to the respective data shown in **(a)** and **(b)**.

Analysis of fluorescence levels of individual cells allow the comparison of exponentially-growing cell populations fluorescence distributions (figures 2-7A and 2-7B). The distributions fit to a gaussian curve (figure 2-7C) and the acetic acid-adapted parental cell population exhibits a higher average PI fluorescence intensity and more heterogeneous cell-to-cell permeability levels [higher standard deviation (SD) and lower

amplitude] than the unstressed cell population (figure 2-7). Deletion of *PDR18* gene leads to a fluorescence distribution in the cell population with a higher average value of plasma membrane permeability even for cells cultivated in the absence of acetic acid. Such heterogeneity increases under acetic acid stress (figure 2-7).

2.4.5 *PDR18* gene expression is essential to maintain plasma membrane potential in absence or presence of acetic acid stress

The effect of *PDR18* expression in plasma membrane electrochemical potential when yeast cells were cultivated in presence or absence of acetic acid was also estimated based on the position of the emission maximum at equilibrium of the diS-C_3(3), a dye sensitive to membrane potential. Since this diS-C_3(3) assay was used to compare two isogenic strains (parental cells and cells deleted for PDR18), the activity of other MDR-pumps is considered identical and the differences in λ_{max}^{eq} believed to reflect differences in membrane potential (Maresova et al., 2006).

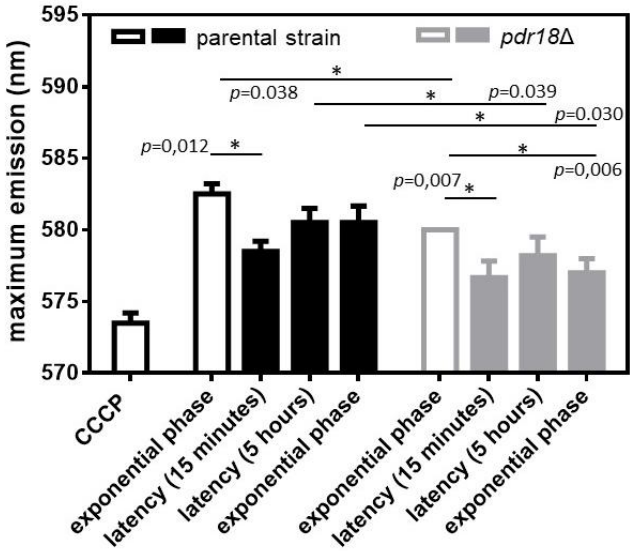


Figure 2-8. Plasma membrane electrochemical potential of the parental and*pdr18*Δ strains during cultivation in presence or absence of acetic acid.
Comparison of plasma membrane electrochemical potential based on the maximum emission wavelength of the fluorescent probe $DiSC_3$(3) in citrate-phosphate buffer at pH 6.0. Cells of parental (□,■) and *pdr18*Δ (□,■) strains were harvested at the exponential phase of growth in the absence of acetic acid (□,□) and at the indicated timepoints of the corresponding growth curves in the presence of 60 mM acetic acid (■,■). Error bars represent standard deviation resultant from at least two biological replicates with at least three technical replicates each. Asterisks represent statistical significance in t-student test. CCCP: Carbonyl cyanide *p*-chlorophenylhydrazone.

Results indicate that even in the absence of stress, parental strain cells exhibit a significantly higher (p=0.038) plasma membrane potential than *pdr18*Δ cells (figure 2-8), as previously observed using other methods (Cabrito et al., 2011). Acetic acid induced

a short-term decrease of plasma membrane potential for both the parental strain and *pdr18*Δ cells after 15 minutes of cultivation in the presence of acetic acid (figure 2-8). However, after five hours of cultivation in the presence of acetic acid, parental strain cells recovered to values that were not significantly different from unstressed cells, and these levels were maintained during exponential growth of adapted cells (figure 2-8). For *pdr18*Δ acetic acid-adapted cells such recovery was not observed (figure 2-8). The incubation of exponentially-growing parental strain cells for 15 minutes with carbonyl cyanide *p*-chlorophenylhydrazone (CCCP), used in this assay as a positive control, confirmed the disruption of membrane potential under these conditions (figure 2-8).

2.5 DISCUSSION

The yeast *Saccharomyces cerevisiae* has evolved numerous and diverse complex mechanisms that allow cells to grow and thrive in stressful environments. Among them are those related with the remodeling of lipid and protein composition of plasma membrane, an active interface between the cell interior and its environment that shields yeast cells against a potentially harmful environment (Caspeta et al., 2015; Thibault et al., 2012). The composition of phospholipids, sphingolipids, and sterols in the plasma membrane play a critical role in determining its physical properties, such as permeability and fluidity, and has a strong influence on the activity of the proteins associated to or embedded in the lipid bilayer (Bagnat et al., 2001; Opekarová and Tanner, 2003; Rattray et al., 1975; Van Meer et al., 2008). In this work we demonstrate the importance of the plasma membrane ABC transporter Pdr18 as a determinant of resistance to acetic acid. Pdr18 was previously involved in resistance to multiple stresses presumably by increasing plasma membrane ergosterol content (Cabrito et al., 2011; Teixeira et al., 2012). Ergosterol, the major sterol present in fungal plasma membrane, modulates its thickness, fluidity and permeability and regulates the activity of membrane associated transporters (Abe and Hiraki, 2009; Aguilera et al., 2006; Caspeta et al., 2014b; del Castillo Agudo, 1992; Eisenkolb et al., 2002; Kodedová and Sychrová, 2015; Parks and Casey, 1995). In the present work, *PDR18* expression was found to be essential to maintain maximum ergosterol content in yeast plasma membrane and to counteract acetic acid-induced decrease of ergosterol content and plasma membrane order and its non-specific permeability and electrochemical potential levels. This role for Pdr18 in the maintenance of adequate plasma membrane physical properties under acetic acid stress is essential for adequate physiological function of this important cell membrane as a selective barrier thus allowing the efficient import of nutrients and excretion of toxic metabolites, such as the counter-anion acetate, as well as the restriction of the diffusional entry of acetic acid.

The transcriptional activation under acetic acid stress of a number of genes of the ergosterol biosynthetic pathway has been revealed by transcriptomic analysis (Mira et al., 2010a). The *ERG* genes found to be activated under acetic acid stress are also required for tolerance to this weak acid (Mira et al., 2010c, 2010d, 2010a), reinforcing the importance of the ergosterol pathway in yeast adaptation and tolerance to acetic acid. The interconnection between the ergosterol pathway and the function of the multistress resistance determinant Pdr18 under acetic acid stress is also suggested by the data obtained in this study, for the transcription analysis of *PDR18* and several genes of the ergosterol biosynthetic pathway. Yeast growth under acetic acid stress was found

to involve the activation of all these genes with a peak of gene transcripts during advanced acetic acid-induced lag phase although the time-course profiles do not coincide. Remarkably, the peak of the mRNA level from *PDR18* was observed later than the peak registered for the first genes of the pathway tested (*ERG24*, *ERG6* and *ERG2*), consistent with the proposed role for Pdr18 at the end of the ergosterol transport process of the newly synthesized sterols from the endoplasmic reticulum (ER) to the plasma membrane. Moreover, the activation level of the late genes of the ergosterol pathway tested (*ERG3* and *ERG4*) was more moderate and the activation profile less dependent of the growth phase. It would be important to know the sensors that perceive the fluctuations in the membrane environment of acetic acid stressed cells and transduce the signals to *PDR18* gene resulting into the compensatory changes in the ergosterol profile and how these processes are controlled.

Yeast cells synthesize ergosterol in the membrane of the endoplasmic reticulum via a cascade of coupled enzymatic reactions (Klug and Daum, 2014). Ergosterol has then to be transported from the site of its synthesis to the plasma membrane and although this transport is known to be fast, the mechanism by which newly synthesized sterols are transported from the ER to the plasma membrane is not fully understood (Baumann et al., 2005; Schnabl et al., 2005)**.** An essential role for vesicular transport pathways in transporting ergosterol, from its site of synthesis to the plasma membrane has been ruled out and ergosterol transport was proposed to occur by equilibration ($t_{1/2} \sim 10-15$ min) of endoplasmic reticulum and plasma membrane ergosterol pools via a bidirectional, nonvesicular process (Baumann et al., 2005). To reconcile an equilibration process with the high ergosterol concentration present in plasma membrane, and based on the observation that a large fraction of ergosterol is found in the plasma membrane condensed with sphingolipids in membrane rafts that coexist with free sterol, Baumann *et a*l. (Baumann et al., 2005) hypothesized that the concentration of free sterol is similar in the plasma membrane and endoplasmic reticulum and that only free (non-raft) sterol molecules have access to a nonvesicular transport pathway that connects the two organelles. Based on the results of the present study, we propose the involvement of the plasma membrane associated ABC transporter Pdr18 in the active ergosterol transport process at the plasma membrane level, allowing the high physiological ergosterol concentration present in this membrane.

Like other organisms, the yeast *S. cerevisiae* has the ability to acquire resistance to multiple drugs (MDR) or to multiple xenobiotic compounds (MXR), *i.e.* to become less sensitive to a broad range of chemically and functionally unrelated cytotoxic compounds

(Balzi and Goffeau, 1995; dos Santos et al., 2012; dos Santos and Sá-Correia, 2015; Gulshan and Moye-Rowley, 2007; Paul and Moye-Rowley, 2014). The acquisition of resistance to multiple stressing agents is due to a number of mechanisms, among them the mechanism mediated by efflux pump proteins localized at the plasma membrane and belonging to either the ABC (ATP-binding cassette) superfamily or the MFS (major facilitator superfamily) and proposed to enhance the kinetics of extrusion of these compounds (dos Santos et al., 2014; Glavinas et al., 2004; Jungwirth and Kuchler, 2006; Sá-Correia et al., 2009). The presence of a large number of ABC and MFS transporters homologous to MDR/MXR characterized transporters in the genomes of yeasts and other organism strongly suggests that they may play important physiological roles even in the absence of the cytotoxic compounds. Accumulating evidence has shown that ABC proteins perform endogenous activities extending beyond their accepted role as drug exporters, among them the transport of lipids (Borst et al., 2000; Pomorski et al., 2004; Prasad et al., 2016; Van Meer et al., 2008) as schematized in figure 2-1. Although *PDR18* has a paralogous gene in *S. cerevisiae*, *SNQ2* (Seret et al., 2009), no demonstration of the eventual involvement of Snq2 in lipid transport and homeostasis was so far reported. However, the affinity of Snq2 transporter for sterols was hypothesized due to its role in alleviating estradiol toxicity in *S. cerevisiae* (Mahé et al., 1996b). Moreover, we were not able to identify any phenotype of resistance to acetic acid associated to *SNQ2* expression. The coordinate control of plasma membrane ergosterol composition by Pdr18 and the so far well accepted role of Pleiotropic drug resistance (PDR) transporters in drug/xenobiotic efflux activity (in this particular case, the putative role of Pdr18 in the active acetate efflux) is a possible model. However, based on the data gathered in this study, the involvement of Pdr18 in multistress resistance may merely result from its physiological role in ergosterol transport and proper distribution within plasma membrane proved to have a significant impact in plasma membrane properties, especially relevant under acetic acid stress. Apart from serving as substrates of certain ABC transporters, lipid molecules are also crucial molecular determinants that affect the trafficking and functioning of certain ABC transporter proteins (Aye et al., 2009; Gulati et al., 2015; Krishnamurthy and Prasad, 1999; Rice et al., 2014). Therefore, the role of Pdr18 in ergosterol homeostasis at the plasma membrane level is likely to affect the proper functioning of other efflux pumps of the ABC superfamily or the MFS, already proposed to be required for acetate efflux (Mira et al., 2010d), or the essential plasma membrane H^+-ATPase activity (Alexandre et al., 1996; Bagnat et al., 2001). Collectively, results support the notion that multistress resistance mediated by Pdr18 is closely linked to the status of plasma membrane lipid environment, specifically related with ergosterol content and the associated plasma membrane properties.

3 The paralogous genes *PDR18* and *SNQ2*, encoding multidrug resistance ABC transporters, derive from a recent duplication event, *PDR18* being specific of the *Saccharomyces* genus.

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Godinho CP, Dias PJ, Ponçot E, Sá-Correia I. The paralogous genes *PDR18* and *SNQ2*, encoding multidrug resistance ABC transporters, derive from a recent duplication event, *PDR18* being specific of the *Saccharomyces* genus.

3.1 ABSTRACT

Pleiotropic drug resistance (PDR) family of ATP-binding cassette (ABC) transporters play a key role in the simultaneous acquisition of resistance to a wide range of structurally and functionally unrelated cytotoxic compounds in yeasts. *Saccharomyces cerevisiae* Pdr18 was proposed to transport ergosterol at the plasma membrane, contributing to the maintenance of adequate ergosterol content and decreased levels of stress-induced membrane disorganization and permeabilization under multistress challenge leading to resistance to ethanol, acetic acid and the herbicide 2,4-D, among other compounds. *PDR*18 is a paralog of *SNQ2,* first described as a determinant of resistance to the chemical mutagen 4-NQO. The phylogenetic and neighborhood analysis performed in this work to reconstruct the evolutionary history of Sc*PDR18* gene in Saccharomycetaceae yeasts was focused on the 214 Pdr18/Snq2 homologs from the genomes of 117 strains belonging to 29 yeast species across that family. Results support the idea that a single duplication event occurring in the common ancestral of the *Saccharomyces* genus yeasts was at the origin of *PDR18* and *SNQ2*, and that chromosome translocation ocurred, conferring *PDR18* with a subtelomeric localization on chromosome XIV. The multidrug/multixenobiotic phenotypic profiles of *S. cerevisiae pdr18*Δ and *snq2*Δ deletion mutants were compared, as well as the susceptibility profile for *Candida glabrata snq2*Δ deletion mutant, given that this yeast species has diverged previously to the duplication event on the origin of *PDR18* and *SNQ2* genes and encode only one Pdr18/Snq2 homologue. Results show a significant overlap between ScSnq2 and CgSnq2 roles in multidrug/multixenobiotic resistance (MDR/ MXR) as well as some overlap in azole resistance between ScPdr18 and CgSnq2. The fact that ScSnq2 and ScPdr18 confer resistance to different sets of chemical compounds with little overlapping is consistent with the subfunctionalization and neofunctionalization of these gene copies. The elucidation of the real biological role of Sc*SNQ2* will enlighten this issue. Remarkably*, PDR18 i*s only found in *Saccharomyces* genus genomes and is present in almost all the recently available 1,000 deep coverage genomes of natural *S. cerevisiae* isolates, consistent with the relevant encoded physiological function.

3.2 INTRODUCTION

Several ABC (ATP-binding cassette) transporters that catalyze the ATP-dependent active solute transport across cell membranes in yeasts are associated with multidrug/multixenobiotic resistance (MDR/MXR) (Jungwirth and Kuchler, 2006; Monk and Goffeau, 2008; Piecuch and Obłak, 2014). Although these transporters are usually considered drug/xenobiotic pumps, evidence is arising supporting the idea that their involvement in MDR/MXR may result from their specific and, in general, not yet determined biological role in the active transport of physiological substrates (Cabrito et al., 2011; Godinho et al., 2018; Prasad et al., 2016; Prasad and Panwar, 2004). Moreover, the presence of a large number of ABC transporters involved in MDR/MXR in the genomes of yeasts and other organisms, from bacteria to man, also strongly suggests that these transporters may play important physiological roles even in the absence of the cytotoxic compounds to which they confer resistance. For example, the yeast ABC-MDR/MXR family of transporters that includes the Pleiotropic Drug Resistance (PDR) transporters, perform endogenous activities beyond their proposed role as drug exporters, in particular as lipid transporters (Balzi and Goffeau, 1995; Borst et al., 2000; Panwar et al., 2008; Pomorski et al., 2004; Prasad et al., 2016; Van Meer et al., 2008). *Saccharomyces cerevisiae* genome encodes 10 PDR proteins: Adp1, Aus1, Pdr5, Pdr10, Pdr11, Pdr12, Pdr15, Pdr18, Snq2 and YOL075c (Decottignies and Goffeau, 1997; Paulsen et al., 1998). A combined phylogeny and neighborhood analysis of the evolution of these ABC transporters in nine yeast species belonging to the *Saccharomycotina* subphylum has shown that Pdr18 is a paralog of Snq2 and that *SNQ2* and *PDR18* genes reside in unshared chromosomal environments (Seret et al., 2009). However, the small number of yeast species genomes available when this study was performed did not allow a firm conclusion concerning the hypothesized gene duplication event at the origin of these two PDR gene sub-lineages. In fact, it was doubtful whether the duplication event remounted to the whole genome duplication (WGD) event or if it was an independent event that occurred post-WGD (Seret et al., 2009).

The *S. cerevisiae* plasma membrane transporter Pdr18 was described as a MDR/MXR determinant required for ergosterol transport at the plasma membrane level (Cabrito et al., 2011; Godinho et al., 2018; Teixeira et al., 2012). Pdr18 expression was found to lead to increased yeast tolerance to the herbicides 2,4-dichlorophenoxyacetic acid (2,4-D), 2-methyl-4-chlorophenoxyaceticacid (MCPA), and barban, the agricultural fungicide mancozeb, the metal cations Zn^{2+}, Mn^{2+}, Cu^{2+} and Cd^{2+} (Cabrito et al., 2011) and to ethanol (Teixeira et al., 2012) and acetic acid (Godinho et al., 2018). The involvement of Pdr18 in the maintenance of yeast plasma membrane ergosterol content under 2,4-D or

acetic acid stresses was related with its role as a determinant of resistance to multiple stresses in yeast (Cabrito et al., 2011; Godinho et al., 2018; Teixeira et al., 2012). A coordinated response involving the transcriptional activation of *PDR18* and several ergosterol biosynthetic genes was found to occur in response to acetic acid stress, strongly suggesting the involvement of Pdr18 in ergosterol homeostasis in stressed yeast cells (Godinho et al., 2018). Moreover, the proposed role for Pdr18 in ergosterol homeostasis was demonstrated to be important to counteract acetic acid-induced decrease of plasma membrane lipid order, increase of plasma membrane non-specific permeability and decrease of transmembrane electrochemical potential (Godinho et al., 2018).

The *PDR18* paralog gene *SNQ2* was first described based on its involvement in yeast resistance to the chemical mutagens 4-nitroquinoline 1-oxide (4-NQO) and triaziquone (Servos et al., 1993). Later, several other publications extended the range of compounds to which *SNQ2* expression confers increased tolerance in yeast (Hirata et al., 1994; Kolaczkowski et al., 1998; Ling et al., 2013; Mahé et al., 1996a, 1996b; Miyahara et al., 1996; Nishida et al., 2013; Piper et al., 2003; Servos et al., 1993; Snider et al., 2013; Tsujimoto et al., 2015; van Leeuwen et al., 2012; Ververidis et al., 2001; Wehrschütz-Sigl et al., 2004). Although no role in lipid homeostasis was demonstrated for Snq2 transporter, it was shown that Snq2 is involved in the alleviation of estradiol toxicity in *S. cerevisiae* (Mahé et al., 1996a). For this reason, it was hypothesized that Snq2 could also have affinity for lipid transport, especially for the estradiol structurally-related molecule ergosterol (Kuchler et al., 1997; Mahé et al., 1996a).

Gene duplication is considered to be one of the most important forces driving the evolution of genetic functional innovation and genes encoding membrane transporters are one of the functional gene categories that exhibit high number of duplication events (Ohno, 1970; Taylor and Raes, 2004; Zhang, 2003). In yeast, for example, this is the case of genes encoding proteins of the Major Facilitator Superfamily (MFS) of transporters (Dias et al., 2010; Dias and Sá-Correia, 2013, 2014). Also, transporters from the PDR family were involved in multiple gene duplications and gene losses occurring during their evolutionary history (Kovalchuk and Driessen, 2010; Seret et al., 2009). After gene duplication, it is possible the occurrence of the inactivation of one of the copies (pseudogenization), the maintenance of the two copies (dosage effect), the adoption of part of the function or of the expression pattern of their parental gene (subfunctionalization), or the acquisition of a related or new function (neofunctionalization) (Conant and Wolfe, 2008; Zhang, 2003). The duplicate genes are

maintained in the genome depending upon their function, mode of duplication, expression rate and the organism taxonomic lineage (Taylor and Raes, 2004).

To better understand the duplication event that gave rise to the *PDR18* and *SNQ2* paralog genes, the evolutionary history of *PDR18* was reconstructed in this work by combining phylogenetic tree building methodology with gene neighborhood analysis in 117 strains genomes belonging to 29 species across the Saccharomycetaceae family. A systematic multidrug/multixenobiotic phenotypic profiling of *S. cerevisiae* deletion mutants for *PDR18* or *SNQ2* genes was also performed. Given that the genomes of the post-WGD *Candida glabrata* species encode only one Pdr18/Snq2 homolog (*CgSNQ2*) the susceptibility profiling for the *Cgsnq2*Δ deletion mutant was also examined to get additional insights into the functional divergence of *S. cerevisiae* Pdr18 and Snq2 and the common ancestral gene on the origin of the post-WGD single duplication event.

3.3 MATERIALS AND METHODS

3.3.1 Identification of the homologs of *S. cerevisiae* Pdr18 and Snq2 proteins in Hemiascomycete yeast genomes

A total of 1,110,525 Open Reading Frames (ORFs) encoded in the genomes of 171 strains belonging to 68 different yeast species of the *Saccharomycotina* subphylum were retrieved and compiled in a local Genome DB (Dias and Sá-Correia, 2013, 2014). A second in-house database built in this work, BLASTp DB, comprises the output values of the blastp algorithm (Altschul et al., 1990) for each pairwise entry of all possible combinations between the translated ORFs compiled in the Genome DB, including length of the alignment, e-value, percentage of identity and similarity, and alignment score. The blastp algorithm used a gapped alignment with the following parameters: open gap (-1), extend gap (-1), threshold for extending hits (11) and word size (3). This approach generated a total of 328 million pairwise alignments. The ORFs encoding PDR proteins were identified through the adoption of a network traversal strategy considering the whole set of blastp pairwise relationships as a network that was subsequently traversed at a range of different e-value thresholds (Dias and Sá-Correia, 2013, 2014; Palma et al., 2017). The *S. cerevisiae* Pdr18 was selected to represent the PDR sensu stricto (Seret et al., 2009) transporters and was used as a starting node for network traversal. The *S. cerevisiae* Adp1 and Yol075c were selected to represent the PDR sensu lato transporters (Seret et al., 2009) and also used as starting nodes in independent network traversals. The disjoint sets of translated ORFs obtained from these three traversals were merged. The amino acid sequences of these ORFs were analyzed for potential false positive members of the PDR protein family, protein fragments and/or frameshifts. The remaining amino acid sequences were aligned using MUSCLE software. Subsequently, the protdist and neighbor algorithms made available by the PHYLIP software suite were used to construct a preliminary phylogenetic tree. Using as reference the cluster of residence of the Snq2 and Pdr18 proteins, the branch comprising the homologs of these two *S. cerevisiae* proteins in this tree was identified. For species abbreviation a four letters code is used, composed by the first two letter of the genus and species. The number displayed after the first four letters is used to abbreviate the strain name when the genome of more than one strain from a given species was examined. To standardize the annotation used, translated ORFs are represented by small letters.

3.3.2 Phylogenetic analysis and tree construction

The MUSCLE software suite (Edgar, 2004) was used to build a multiple alignment of the amino acid sequences of the Snq2 and Pdr18 proteins encoded in Saccharomycetaceae yeasts that was analyzed using the Jalview 2.9 software suite (Clamp et al., 2004; Waterhouse et al., 2009). The “read.fasta” and “write.nexus.data” functions made available by the seqinr 3.4-5 (Charif et al., 2007) and by the ape 4.1 R packages (Paradis et al., 2004; Popescu et al., 2012), respectively, were used to convert the multiple alignment in fasta format into a nexus file that was subsequently fed into MrBayes 3.2.6 (Huelsenbeck and Ronquist, 2001; Ronquist and Huelsenbeck, 2003), a Bayesian Markov chain Monte Carlo (MCMC) package for phylogenetic analysis. The Message Passing Interface (MPI) version of MrBayes (Altekar et al., 2004) was used to speed up phylogeny computation using Metropolis coupling of MCMC sampling. The MCMC simulations used 100000 generations, coupling one “cold” chain together with 9 heated chains. Two independent runs of MCMC sampling (each started from two distinct random trees) confirmed parameter convergence of the posterior probability distribution. The option of estimating the fixed-rate amino acid prior model made available by MrBayes was used, allowing the MCMC sampler to explore all of the nine available models by regularly proposing new ones (upon parameter convergence, each model contributes to the results in proportion to its posterior probability) and rate variation over sites was assumed to follow a gamma distribution. The remaining MrBayes MPI parameters were set to default values. The PhyML 3.0 software suite (Guindon et al., 2010) was used to construct a Maximum Likelihood (ML) derived phylogenetic tree to confirm the gathered results. The PhyML parameters and models used as input were the default ones, with exception of the method for searching the optimal phylogenetic tree, where the Subtree Pruning and Regrafting (SPR) were used as algorithm instead of Nearest Neighbor Interchange (NNI). The phylogenetic trees obtained were analyzed using the visualization software Dendroscope 3.5.7 (Huson et al., 2007; Huson and Scornavacca, 2012). The MrBayes clade credibility score and PhyML bootstrapping values calculated for each internal node of the Bayesian and ML trees, respectively, were inspected using either the FigTree 1.4.3 software suite (http://tree.bio.ed.ac.uk/software/figtree/) or PhyloTree 0.1 package (http://apps.cytoscape.org/apps/phylotree/) installed in Cytoscape 2.8.3 (Shannon et al., 2003).

3.3.3 Gene neighbourhood analysis

A chromosome block of 30 neighboring genes, 15 on each side of the pair of homologous genes under analysis, was selected to assess the conservation of the chromosome

region where the members of the Snq2/Pdr18 subfamily reside (Dias et al., 2010; Seret et al., 2009). Scripting in the R language was used to retrieve 15 neighbor genes on each side of the query genes as well as the corresponding sequence clustering classification from Genome DB. The classification of each of the thirty genes neighboring each query gene was obtained using a conservative blastp e-value of E-50 to limit the number of false positive sequences gathered together with true cluster members. When dubious synteny connections between genes needed corroboration, the amino acid sequence clustering was performed at a less restrictive e-value threshold of E-40. The existence of synteny between query genes was verified through the analysis of network topology (number of shared neighbor pairs) and the biological information associated with the corresponding edges. Three sources of biological information were used as independent evidence confirming the strength of the synteny between members of the Snq2 and Pdr18 protein subfamily (Dias et al., 2010): 1) distance of the neighbors in relation to the query genes, 2) similarity of the amino acid sequences of the shared neighbors and 3) total number of members comprised in the cluster of amino acid sequence to which the homologous neighbors belong to; sequence clusters comprising a small number of members are more reliable as synteny evidence since the probability that two homologous neighbors being in the vicinity of two query genes by chance is small.

3.3.4 Susceptibility phenotypes of *S. cerevisiae pdr18*Δ and *snq2*Δ and *C. glabrata snq2*Δ deletion mutants

3.3.4.1 Strains, media and growth conditions

S. cerevisiae BY4741 (*MATa*, *his3*Δ*1*, *leu2*Δ*0*, *met15*Δ*0*, *ura3*Δ*0*) and the derived deletion mutants *pdr18*Δ and *snq2*Δ were obtained from EUROSCARF collection. *C. glabrata* BPY55 (clinical isolate) and the derived deletion mutant *snq2*Δ built using the SAT1 flipper system were kindly provided by Professor Dominique Sanglard, Institut de Microbiologie, Centre Hospitalier Universitaire Vaudois (CHUV), Lausanne, Switzerland.

Cultivation of *S. cerevisiae* strains was performed in MM4 medium, containing 1.7 g/L yeast nitrogen base without amino acids and ammonium sulfate (Difco, Michigan, USA), 20 g/L glucose (Merck, Darmstadt, Germany), 2.65 g/L $(NH_4)_2SO_4$ (Panreac AppliChem, Connecticut, USA), 20 mg/L L-methionine, 20 mg/L L-histidine (both from Merck, Darmstadt, Germany), 60 mg/L L-leucine and 20 mg/L L-uracil (both from Sigma, Missouri, USA). *C. glabrata* strains were cultivated in MM medium, with the composition of MM4 medium, without supplementation with amino acids and uracil. YPD medium contained 20 g/L glucose, 20 g/L Bacto™ Peptone and yeast extract (both from BD Biosciences, New Jersey, USA). Solid media were prepared by the addition of 20 g/L

agar (Iberagar, Barreiro, Portugal) to the different liquid media. Media pH were adjusted to 4.5 with HCl. Growth in liquid media was performed at 30°C with orbital agitation (250 rpm).

3.3.4.2 Susceptibility tests

Growth susceptibility tests of *S. cerevisiae* parental strain BY4741, the corresponding *pdr18*Δ and *snq2*Δ deletion mutants and of *C. glabrata* BPY55 and derived deletion mutant *snq2*Δ to a wide range of growth inhibitory compounds was evaluated by spot assays. Yeast cell suspensions used for the spot assays were prepared from mid-exponential cell cultures grown in liquid media MM4 (*S. cerevisiae*), MM (*C. glabrata*), or YPD (both strains) by harvesting (5000 rpm, 5 minutes) and resuspending the cells in sterile ddH_2O to an OD_{600nm} of 0.25 followed by four serial dilutions of 1:5 each. These cell suspensions were plated as 4 µL spots onto the surface of MM4 (*S. cerevisiae*) or MM (*C. glabrata*) or YPD (both strains) at pH 4.5 solid media, supplemented or not with the toxic compound to be tested. The plates were incubated at 30°C for 72 hours and pictures were taken every 24 hours. The toxic compounds tested and the selected concentrations for the screening of *S. cerevisiae* and *C. glabrata* strains in MM4 and MM are listed in table 3-1, and those selected for experiments in YPD are indicated close to the respective pictures. The susceptibility of the deletion mutants was assessed by comparison of their growth performance with the growth performance of the corresponding parental strain in the presence of the different cytotoxic compounds tested. Moderately reduced growth of the deletion mutants compared with the parental strain was classified as minus (-), marked reduced growth as a double minus (- -), improved growth as a plus (+) and identical growth as zero (0). Results are representative from three independent experiments.

Table 3-1. Susceptibility of the deletion mutants *pdr18*Δ and *snq2*Δ of *S. cerevisiae* BY4741 and *snq2*Δ of *C. glabrata* BPY55 to a wide range of chemical compounds supplemented in minimal media. The phenotypes registered that were also published before are indicated together with the corresponding reference. 0: no growth phenotype was identified, - or --: susceptibility phenotype, moderate or marked, respectively, +: resistance phenotype. Results arise from three independent experiments.

Growth inhibitory compounds				*S. cerevisiae*		*C. glabrata*
Class	**Compound**	**Selected concentrations (*S. cerevisiae* / *C. glabrata*)**	**Manufacturer**	***snq*2Δ**	***pdr18*Δ**	***snq*2Δ**
Weak acids	Acetic acid	60 mM / 60 mM	Fluka, USA	0	- (Godinho et al., 2018)	0
	Benzoic acid	1.2 mM / 1 mM	Sigma, USA	0	--	0
	Propionic acid	15 mM	Sigma, USA	0	--	
	Formic acid	1 mM	Sigma, USA	0	--	
	Sorbic acid	30 mM	Fluka, USA	0	-	
Herbicides	2,4-D	2.5 mM / 2 mM	Sigma, USA	0	- (Cabrito et al., 2011)	0
	MCPA	1.5 mM / 1 mM	Sigma, USA	0	- (Cabrito et al., 2011)	0
	Barban	0.02 mM	Sigma, USA	-	- (Cabrito et al., 2011)	
	Alachlor	1.5 mM	Fluka, USA	-	-	
	Metolachlor	1.1 mM	Fluka, USA	--	-	
Alcohols	Ethanol	1,37 M / 1,37 M	Merck, USA	0	- (Teixeira et al., 2012)	0
	Propanol	1 M	Acros, USA	0	-	
	1-Butanol	0.21 M	Acros, USA	0	-	
	2-Butanol	0.5 M	Acros, USA	0	--	
	1,3 - Propanediol	0.98 M	Acros, USA	0	+	
	1,4-Butanediol	0.9 M / 0.9 M	Acros, USA	0	+	0
Clinical and agricultural fungicides	Itraconazole	14.2 µM / 71 µM	Sigma, USA	0	--	+
	Ketoconazole	28.2 µM / 226 µM	Sigma, USA	0	--	0
	Fluconazole	98 µM / 1.96 mM	Sigma, USA	0	--	0
	Clotrimazole	8.7 µM / 116 µM	Sigma, USA	0	--	0
	Miconazole	1.2 µM / 48 µM	Sigma, USA	0	--	+
	Tioconazole	1.3 µM	Sigma, USA	0	--	0
	Amphotericin B	0.33 µM / 0.33 µM	Sigma, USA	0	--	0
	Mancozeb	3.7 µM	Sigma, USA	0	- (Cabrito et al., 2011)	
Cations	Cd^{2+}	16 µM	Merck, USA	0	- (Cabrito et al., 2011)	
	Li^{+}	9 mM / 80 mM	Sigma, USA	- (Miyahara et al., 1996)	0	0
	Mn^{2+}	1 mM	Merck, USA	- (Miyahara et al., 1996)	-	
	Mg^{2+}	90 mM	Sigma, USA	0	0	
	Cu^{2+}	0.13 mM / 0.25 mM	Merck, USA	0	-	-
Mutagens	4-NQO	5.26 µM / 5.26 µM	Sigma, USA	-- (Servos et al., 1993)	0	-
Anti -arrythmic and -malarial compounds	Quinine	4 mM	Sigma, USA	-	--	
	Quinidine	5 mM	Sigma, USA	0	-	
Polyamines	Spermine	2.5 mM	Sigma, USA	-	-	
	Spermidine	7 mM	Sigma, USA	0	0	
	Putrescine	43 µM / 55 µM	Sigma, USA	0	-	0

3.4 RESULTS

3.4.1 Identification of Snq2 and Pdr18 proteins encoded in the Saccharomycetaceae yeast genomes

To identify the PDR proteins encoded in the examined yeast genomes, the network representing the amino acid sequence similarity of the translated ORFs comprised in the Genome DB was traversed at different blastp e-value thresholds using as starting nodes *Saccharomyces cerevisiae* proteins Pdr18 [representing the PDR sensu stricto proteins (Seret et al., 2009)] and Yol075c and Adp1 [representing the PDR sensu lato proteins (Seret et al., 2009)]. When Pdr18 was used as starting node the analysis of the protein sets obtained at different blastp e-values indicated that a threshold of E-142 was adequate, allowing the gathering of 1263 translated ORFs (figure 3-1). Using a similar approach, a threshold of E-104 and E-84 was found to be adequate for network traversal using Yol075c and Adp1 as starting nodes, allowing the gathering of 189 and 196 translated ORFs, respectively (figure 3-1). The three disjoint protein sets were merged, giving a total of 1648 translated ORFs homologous to the yeast PDR proteins in yeast strains belonging to the *Saccharomycotina* subphylum. This set included members of another family of transporters (hexose transporters) that were manually removed.

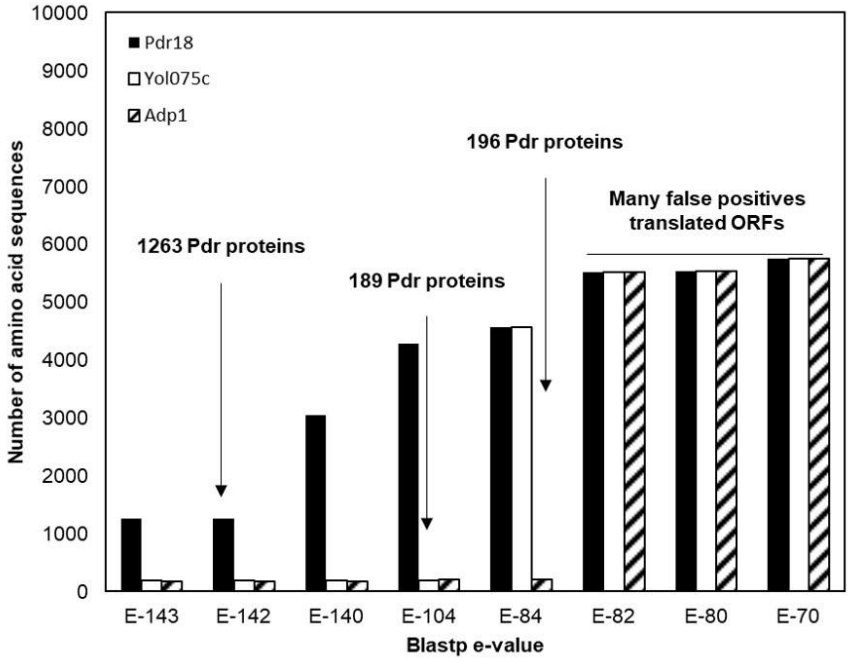

Figure 3-1. Identification of the PDR proteins encoded in the genomes of 117 strains, belonging to 29 different yeast species belonging to the Saccharomycetaceae family. Plot representing the number of amino acid sequences retrieved after constraining and traversing the pairwise similarity network at different e-values taking Pdr18, Yol075c and Adp1 proteins as starting nodes. The blastp e-value used as threshold for network constraining is marked by an arrow.

The corresponding amino acid sequences were aligned using MUSCLE software and the protdist and neighbor algorithms made available by the PHYLIP software suite were used to construct a preliminary phylogenetic tree. Using as reference the cluster of residence of the Snq2 and Pdr18 proteins, the phylogenetic branch comprising the homologs of these two *S. cerevisiae* proteins was identified. The comparison of the PDR protein set gathered in this study with those reported before encoded in the genomes of 10 yeast species (Seret et al., 2009) confirmed the co-clustering of the homologs of *S. cerevisiae* Snq2/Pdr18 proteins in a single branch of the phylogenetic tree. However, since it is well known that translated ORFs residing in long phylogenetic branches may correspond to protein fragments or sequence frameshifts, the blastp algorithm was used to test ORFs with dubious sequence similarity. After determining the homology of each of these proteins selected for blastp testing, a total of 214 translated ORFs, encoded in the Saccharomycetaceae yeast genomes examined, showing strong amino acid sequence similarity to the *S. cerevisiae* Snq2/Pdr18 transporters, were retained for further analysis (table 3-2 and supplementary table S3-1).

Table 3-2. Saccharomycetaceae yeast strains examined in this work, and the number of Snq2/Pdr18 homologs, Snq2 orthologs and Pdr18 orthologs identified. Pre-whole genome duplication (WGD) species are highlighted in grey. A detailed list of the strains analyzed, number of Pdr18 and Snq2 proteins and sources of genome information and annotation tools is provided as supplementary table S3-1.

Species	Nr of strains	Nr of Snq2 and Pdr18 homologs	Nr of Snq2 orthologs	Nr of Pdr18 orthologs
Saccharomyces cerevisiae	60	115	59	56
Saccharomyces paradoxus	25	50	25	25
Saccharomyces mikatae	1	3	1	1
Saccharomyces kudriavzevii	1	2	1	1
Saccharomyces arboricola	1	2	1	1
Saccharomyces eubayanus	2	4	2	2
Saccharomyces bayanus (HYBRID)	2	1	1	0
Saccharomyces uvarum	1	1	0	1
Kazachstania africana	1	2	2	0
Kazachstania naganishii	1	1	1	0
Naumovozyma castellii	2	4	4	0
Naumovozyma dairenensis	1	2	2	0
Candida glabrata	2	2	2	0
Tetrapisispora blattae	1	2	2	0
Tetrapisispora phaffii	1	2	*2*	0
Vanderwaltozyma polyspora	1	2	2	0
Zygosaccharomyces bailii	2	6	6	0
Zygosaccharomyces rouxii	1	2	2	0
Torulaspora delbrueckii	1	1	1	0
Lachancea kluyvery	1	2	2	0
Lachancea thermotolerans	1	1	1	0
Lachancea waltii	1	1	1	0
Kluyveromyces aestuarii	1	1	1	0
Kluyveromyces lactis	1	1	1	0
Kluyveromyces marxianus var. marxianus	1	1	1	0
Kluyveromyces wickerhamii	1	1	1	0
Ashbya aceri	1	1	1	0
Eremothecium cymbalariae	1	1	1	0
Eremothecium gossypii	1	1	1	0

3.4.2 Phylogenetic analysis of Snq2 and Pdr18 proteins.

For the phylogenetic analysis of Snq2 and Pdr18 proteins, more than one strain from a number of yeast species were used (50 strains for *S. cerevisiae*, 25 for *Saccharomyces paradoxus*, 2 for *Saccharomyces eubayanus*, 2 for *Saccharomyces bayanus*, 2 for *Nauvozyma castellii*, 2 for *Candida glabrata* and 2 for *Zygosaccharomyces bailii*). All the repeated sequences were removed from the protein dataset, leaving just a representative member of each species. This resulted in 146 unique translated ORFs encoded in the Saccharomycetaceae yeast genomes that were used to construct a

phylogenetic tree for Pdr18 and Snq2 proteins. For this, the 146 unique translated ORFs were aligned and the corresponding phylogenetic tree constructed using the MrBayes software suite (figure 3-2). After assuring that all model parameters had converged, the corresponding consensus Bayesian phylogenetic tree was retained for further analysis. The translated ORF lakl_1_h21010g was selected as root of the phylogenetic tree because it comprised the most divergent amino acid sequence present in the protein dataset. Complementing the Bayesian approach adopted for the construction of the phylogenetic tree, the PhyML software suite was also used to obtain a Maximum Likelihood (ML) derived tree (not shown). The analysis of the bipartition probabilities and bootstrap values obtained for each internal node of the Bayesian and ML trees, respectively, indicates that the two distinct statistical approaches generated similar trees. Due to their similarity, the analysis presented in the manuscript is solely based on the Bayesian tree (figure 3-2).

The analysis of i) the tree topology, ii) the bipartition probability of the branches and iii) the phylogenetic distances separating the 146 unique translated ORFs led to the proposal of dividing the tree into thirteen clusters, labeled from 1 to 13 (figures 3-2A and 3-2B). The Snq2 and Pdr18 homologs encoded in Lachancea, Kluyveromyces and Eremothecium species occupy a basal position in this phylogenetic tree (figure 3-2B - clusters A1, A11, A12 and A13). On the other hand, the early divergence of the Snq2 and Pdr18 homologs encoded in the genomes of the pre-WGD Zygosaccharomyces and Torulaspora species is not observed (figure 3-2B - clusters A8 and A9).

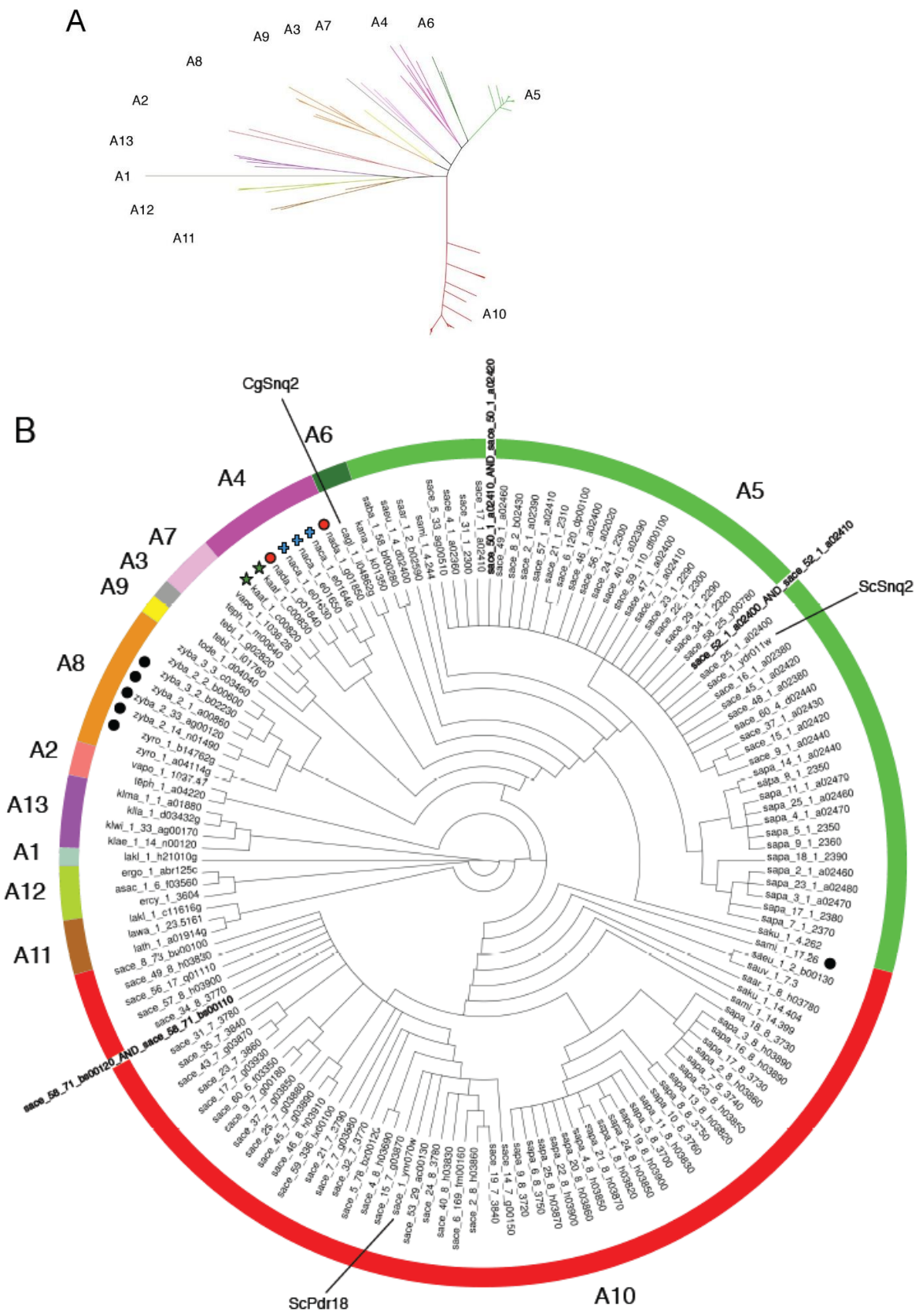

Figure 3-2. Phylogenetic tree of the Pdr18 and Snq2 homologs encoded in the genome sequences of 117 strains of 29 yeast species belonging to the Saccharomycetaceae family. (A) Radial phylogram showing the amino acid sequence similarity distances between the protein homologs of Pdr18 and Snq2. **(B)** Circular cladogram showing the tree topology. Singleton genes were identified with graphical labeling of the translated ORFs using black circles, and tandemly duplicated genes were identified with graphical labeling of the translated ORFs using green stars, red circles and blue crosses. The translated ORF lakl_1_h21010g was chosen as outgroup due to the strong dissimilarity of its amino acid sequence.

All translated ORFs showing strong amino acid sequence similarity to the *S. cerevisiae* Snq2 or Pdr18 proteins are only encoded in the genomes of yeast species classified in the *Saccharomyces* genus and are divided in two distinct ranches of the phylogenetic tree, A5 and A10. The analysis of the phylogenetic tree also indicates that the Snq2 homologs cagl_1_i04862g and kana_1_k01350 also share strong sequence similarity (cluster 6, 74.5% and 85.9% of identity and similarity, respectively). The percentage of identity and similarity shown between these Snq2/Pdr18 homologs is unexpectedly high suggesting that a lateral gene transfer event to an ancestral strain of *K. naganishii* species might have mediated the acquisition of the Snq2/Pdr18 homolog encoded in this species. The homologs of the Snq2 and Pdr18 proteins encoded in yeast species of the remaining post-WGD taxonomic genera (*Kazachstania*, *Naumovozyma*, *Tetrapisispora* and *Vanderwaltozyma*) reside in the phylogenetic clusters A2, A3, A4 and A7 (figure 3-2B).

3.4.3 Gene neighbourhood analysis of Pdr18 and Snq2 orthologs in Saccharomycetaceae yeasts

Gene neighborhood analysis of *S. cerevisiae SNQ2* and *PDR18* homolog genes encoded in the examined Saccharomycetaceae yeast strains genomes was performed. This study, together with the phylogenetic analysis, is useful to contribute to the elucidation of their ortholog/paralog status.

Results show that for the pre-WGD yeasts species of *Torulaspora*, *Lachancea*, *Kluyveromyces* and *Eremothecium* genera there is a single lineage, with genes sharing strong synteny (figures 3-3 and 3-4). The main exception to this rule is the *Lachancea kluyvery* CBS 3082 strain because, in addition to the gene lakl_1_c11616g residing in the above mentioned conserved chromosome environment, this yeast strain also encodes one singleton gene, lakl_1_h21010g, sharing very weak synteny with *Kluyveromyces lactis* and *Kluyveromyces marxianus* var. marxianus genes (figure 3-3). The pre-WGD yeast strains of the *Zygosaccharomyces* genus, *Z. rouxii* CBS 732 and *Z. bailii* CLIB213, both encode two *SNQ2*/*PDR18* homolog genes (figure 3-3). However, *Z. bailii* IST302 encodes two additional *SNQ2*/*PDR18* homolog genes, zyba_2_14_n01490 and zyba_2_33_ag00120, lacking common neighbors with the remaining *SNQ2*/*PDR18* homolog genes in the Saccharomycetaceae strains genomes examined (figure 3-3; supplementary figure S3-1). For this reason, these two genes were considered singletons. The high amino acid sequence identity shared by these two genes suggests that they are a paralog pair originated in a duplication event that occurred recently in the evolution of *Zygosaccharomyces* species.

After the whole genome duplication (WGD) event, the above described single gene lineage gave rise to two gene sub-lineages and the chromosome regions where these genes reside in pre- and early-divergent post-WGD yeast species are conserved (figure 3-3). For instance, the analysis of the neighborhood of the pre-WGD *Zygosaccharomyces rouxii* gene zyro_1_a04114g and of the post-WGD species *Vanderwaltozyma polyspora* gene vapo_1_1037.47 shows the existence of 9 common neighboring genes, some of them absent from figure 3-3 but that can be seen in supplementary figure S3-1. *Z. rouxii* gene zyro_1_a04114g also shares 9 common neighboring genes with the second gene encoded in *V. polyspora* (vapo_1_1036.28), belonging to the second sub-lineage of ohnolog genes originating from whole genome duplication (figure 3-3 and supplementary figure S3-1).

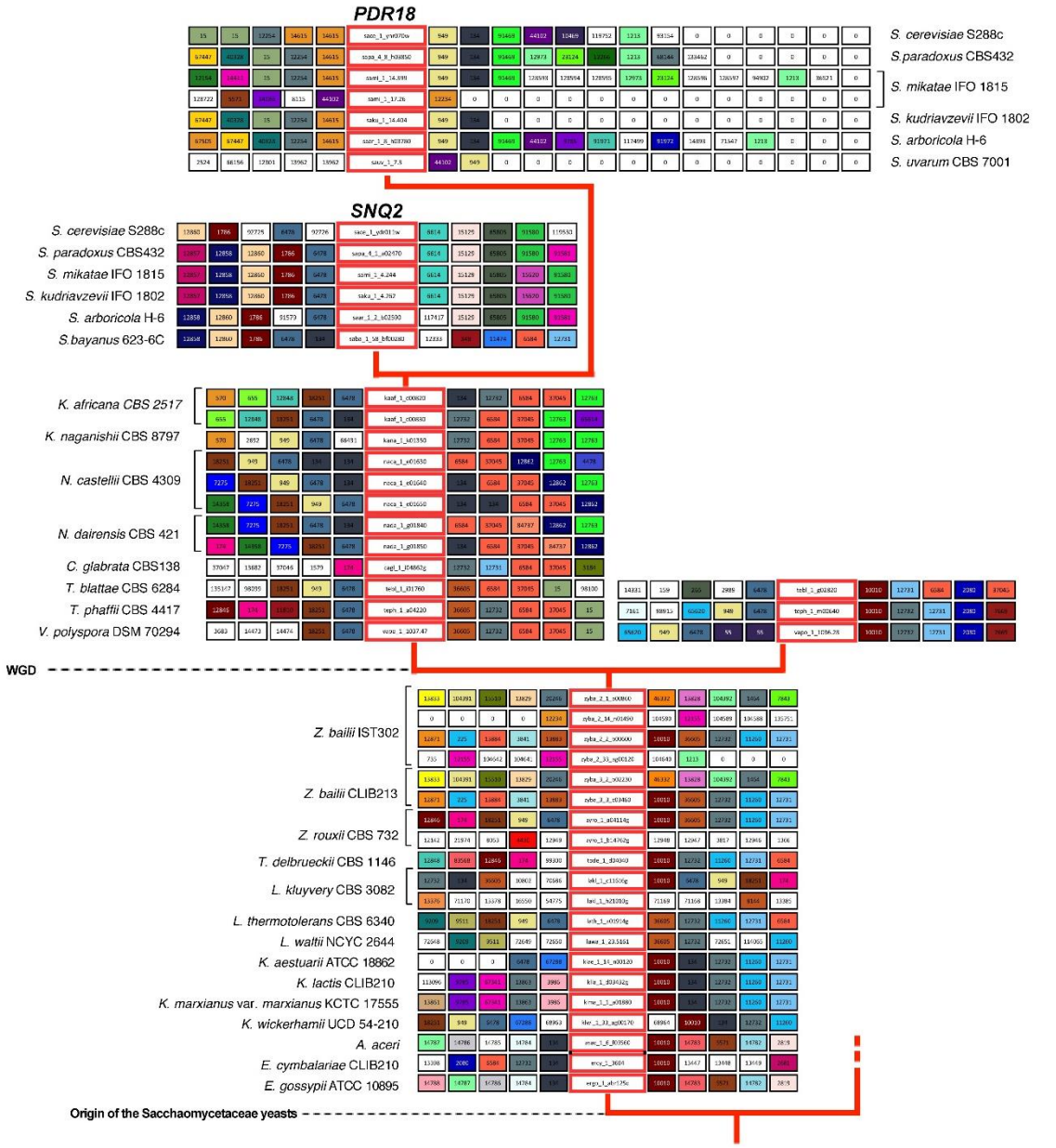

Figure 3-3. Gene neighborhood of *SNQ2* and *PDR18* genes and corresponding homologs encoded in the genomes examined. Genes in the central boxes represent *SNQ2*, *PDR18* and their orthologs. Adjacent boxes represent gene neighbors and homologous neighbors are highlighted in the same colour. A white box represents genes with no homologous neighbors in the represented chromosome region and white boxes with a zero represent the end of the contig/chromosome. The synteny was assessed with 15 neighbors on each side but this representation was truncated to 5 neighbors. In the case of *PDR18* orthologs, the gene neighborhood data was not truncated in one of the sides to highlight the sub-telomeric position occupied by these genes.

The *Candida glabrata* strains' genomes analyzed only encode one Pdr18/Snq2 homolog gene sharing strong synteny with all the homologs encoded in post-WGD species, from *Naumovozyma dairensis* to *Kazachstania africana* (figure 3-3 and supplementary figure S3-1). This sub-lineage also shows strong synteny with one of the gene sub-lineages encoded in *Saccharomyces* genus species, corresponding to the Sc*SNQ2* orthologs in

Saccharomyces yeasts, supporting the Sc*SNQ2*-ortholog status for the *SNQ2*/*PDR18* homologs encoded in the post-WGD species, from *C. glabrata* to *S. cerevisiae* (figure 3- 3 and 3-4). We also hypothesize the loss of the ohnolog gene from the second sub-lineage in the common ancestor of Saccharomyces, Nakaseomyces, Kazachstania and Naumovozyma genera, given that all the encoded *PDR18*/*SNQ2* homologs from *C. glabrata* to *K. africana* appear to be Sc*SNQ2* orthologs.

The gene neighborhood analysis of the *S. cerevisiae SNQ2*/*PDR18* homologs encoded in the genomes of the *Saccharomyces* genus yeast species *S. paradoxus*, *S. mikatae*, *S. kudriavzevii* and *S. arboricola*, shows the existence of two gene sub-lineages, one comprising the above described *SNQ2* orthologs and the other comprising *PDR18* orthologs. *PDR18* orthologs occur exclusively in the *Saccharomyces* genus yeast species and the sub-lineage constituted by these orthologs shows a very strong synteny (figure 3-3). However, the analysis of the gene neighborhood of the *SNQ2* and *PDR18* orthologs in *Saccharomyces* species residing in a basal phylogenetic position could not be performed due to lack of data: the translated ORF encoding the *S. uvarum SNQ2* ortholog, and the *PDR18* ortholog encoded in the genome of *S. bayanus* 623-6C were fragmented into two different contigs of small dimension. Therefore the closely related species *S. eubayanus* (Baker et al., 2015), whose genome had not been at first included in Genome DB, was used instead. The chromosome environment where the *SNQ2* and *PDR18* orthologs reside in the *S. eubayanus* genome was inspected by performing manual blastp pairwise comparisons of the amino acid sequence of the neighboring genes against the full protein set of *S. cerevisiae*. This analysis also showed that *S. eubayanus* CBS12357 and FM1318 strains encode a single *SNQ2* ortholog and a single *PDR18* ortholog sharing strong synteny with the remaining genes within each sub-lineage. These results support the notion that the chromosome environment where *SNQ2* and *PDR18* paralogs reside in the genome of *Saccharomyces* species has been conserved since their appearance in the last common ancestor of the species comprised in this taxonomic genus.

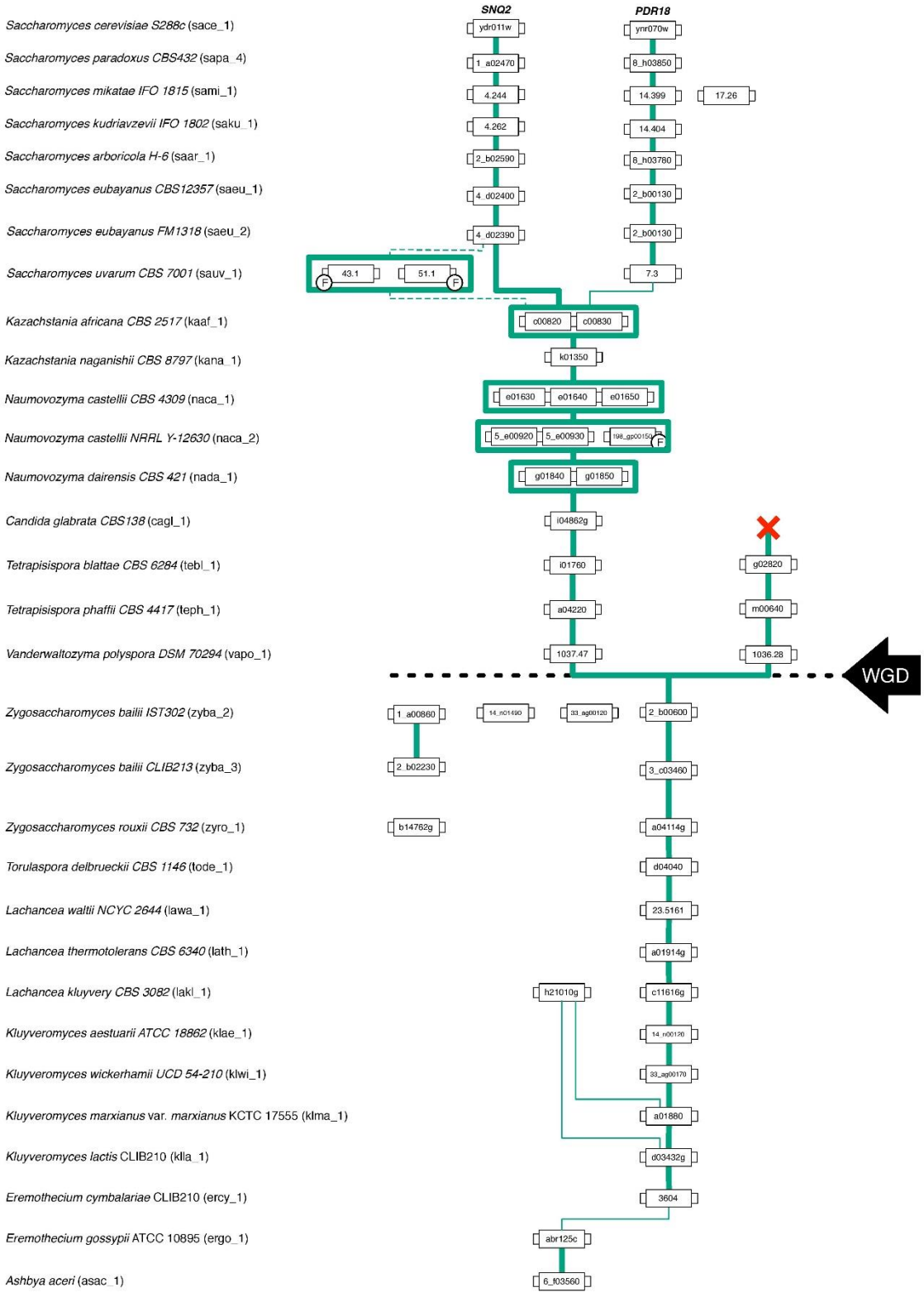

Figure 3-4. Gene lineage comprising the homologs of *S. cerevisiae PDR18* and *SNQ2* genes encoded in the Saccharomycetaceae species examined. Each box represents a gene and the lines connect genes sharing common neighbors. F indicates that the corresponding gene was classified as a fragment. Line thickness represents the strength of synteny between genes. The black dashed line marks the point in time where the Whole Genome Duplication (WGD) event occurred.

The *S. cerevisiae PDR18* gene resides in the subtelomeric region of chromosome XIV (figure 3), a region poorly conserved throughout Saccharomycetaceae yeasts´ evolution, sharing little synteny with the homologs of the *SNQ2/PDR18* genes encoded in the genomes of yeast species belonging to the other post-WGD taxonomic genera. In fact, the only neighbor in the vicinity of the *PDR18* gene that did not belong to large gene families and is also shared with the *K. naganishii*, *N. castelli* and *N. dairensis* species is ORF YNR071C, a non-biochemically characterized member of a gene family of aldose 1-epimerases (Li et al., 2013). This ORF and the other *S. cerevisiae* members of this gene family, *GAL10* gene and ORF YHR210C, are comprised in the cluster of amino acid similarity 949 (figure 3-3). This cluster of amino acid sequence similarity comprises a total of 366 members in the 170 hemiascomycetous strains of the Genome DB (gathered at an e-value threshold of E-50). The gene neighborhood analysis showed that the members of this cluster are present in the chromosome environment where Snq2 orthologs from pre- and post-WGD species and Pdr18 orthologs from *Saccharomyces* genus species reside (figure 3-3).

The analysis of the gene neighbourhood of the Snq2 and Pdr18 homologs encoded in the protoploid yeast species classified in *Torulaspora*, *Lachancea*, *Kluyveromyces* and *Eremothecium* genera showed the existence of a single lineage, with the comprised genes sharing strong synteny among them (figures 3-3 and 3-4). The main exception to this rule was the *Lachancea kluyvery* CBS 3082 strain. In addition of comprising ORF lakl_1_c11616g residing in the above mentioned conserved chromosome environment, this yeast strain also encodes one singleton gene, lakl_1_h21010g, sharing very weak synteny with *K. lactis* and *K. marxianus* var. marxianus genes. The Snq2 homologs encoded in the genomes of the two yeasts species classified in the *Zygosaccharomyces* genus analyzed in this study, *Z. baillii* and *Z. rouxii*, divide into two distinct sub-lineages. Similarly to the *Z. rouxii* CBS 732, the type strain *Z. bailii* CLIB213 encodes two *SNQ2*/*PDR18* homolog genes. *Z. bailii* IST302 strain encodes two additional Snq2 and Pdr18 homologs, zyba_2_14_n01490 and zyba_2_33_ag00120. The gene neighbourhood analysis concluded that these two translated ORFs lack common neighbours with the remaining Snq2/Pdr18 homologs encoded in the protoploid Saccharomycetaceae strains, and therefore these two genes were considered singletons. The high amino acid sequence identity shared by these two genes suggests that these are a paralog pair originated in a duplication event that occurred recently in the evolution of the *Zygosaccharomyces* yeast species.

3.4.4 Susceptibility profiling of *S. cerevisiae snq2*Δ and *pdr18*Δ *and C. glabrata snq2*Δ deletion mutants.

The post-WGD *C. glabrata* species genome encodes a sole ScSnq2 ortholog, the CgSnq2 (Sanglard et al., 2001). The encoded gene has diverged before the hypothesized duplication event that originated *S cerevisiae PDR18*. For this reason, *C. glabrata* was the selected species to get insights into the functional divergence of the ancestral gene and *S. cerevisiae PDR18* and *SNQ2* genes. Since ScPdr18, ScSnq2 and CgSnq2 were reportedly involved in MDR/MXR, the functional divergence of these proteins was examined by profiling the growth susceptibility of the corresponding deletion mutants against a wide range of cytotoxic compounds, some of them already described as potential substrates for these drug/xenobiotic pumps. The susceptibility of *S. cerevisiae* BY4741-derived *snq2*Δ and *pdr18*Δ deletion mutant strains to a wide range of chemical compounds was screened under identical conditions in minimal medium MM4 (*S. cerevisiae*), ranging from weak acids, alcohols, polyamines, metal cations to herbicides, fungicides and anti -arrhythmic and -malarial compounds (table 3-1). The deletion of the *SNQ2* gene in *S. cerevisiae* was found to lead to increased susceptibility to 4-NQO, Li^{+} and Mn^{2+}, in agreement with previous studies (Miyahara et al., 1996b; Servos et al., 1993). Moreover, this systematic screening contributed to extend to the herbicides barban, alachlor and metolachlor, the anti-malarial anti-arrythmic quinine and the polyamine spermine the list of toxic compounds to which Snq2 confers protection in *S. cerevisiae* (table 3-1).

The deletion of the *PDR18* gene was found to render *S. cerevisiae* cells more susceptible towards almost all the compounds tested, contrasting with *SNQ2* whose MDR/MXR spectrum is apparently more limited, the phenotypes only coinciding for 8 of the 35 compounds tested (table 3-1). In particular, the higher toxic effect of weak acids and of fungicides that target either ergosterol biosynthesis (azoles) or the ergosterol molecule itself (amphotericin B) towards the *pdr18*Δ strain is evident (table 3-1), consistent with the role described for this ABC transporter in acetic acid resistance and in ergosterol transport at plasma membrane (Cabrito et al., 2011; Godinho et al., 2018; Teixeira et al., 2012).

The expression of *CgSNQ2* gene in *C. glabrata* was found not to confer protection or increased susceptibility to stress induced by the weak acids acetic and benzoic acid, the herbicides 2,4-D and MCPA, the alcohols ethanol and 1,4-butanediol, and the polyamine putrescine in minimal medium, similarly to the observed in the case of the expression of *ScSNQ2* in *S. cerevisiae*.

This profile is considerably different from the exhibited by *S. cerevisiae* cells devoid of *PDR18,* which show increased susceptibility all these compounds, except for 1,4-butanediol, towards which this strain proved to be more tolerant (table 3-1). Moreover, susceptibility phenotypes for *Cgsnq2*Δ deletion mutant were detected with toxic concentrations of the mutagen 4-NQO [which is a described putative substrate for *S. cerevisiae* efflux pump Snq2 (Servos et al., 1993)]. However, the susceptibility phenotype for the lithium cation exhibited by *Scsnq2*Δ mutant, was not detected for the *Cgsnq2*Δ mutant (table 3-1). Moreover, CgSnq2 is apparently a determinant of resistance to Cu^{2+}, while no phenotype was found for *Scsnq2*Δ (table 3- 1). The toxic effect of the azole drugs clotrimazole, ketoconazole, and fluconazole, and of amphotericin B was not alleviated by the expression of *CgSNQ2* when tested in minimal medium, consistent with what was observed for *S. cerevisiae* Sc*SNQ*2 expression. Surprisingly, in the presence of itraconazole and miconazole, the deletion of *CgSNQ2* was apparently advantageous (table 3-1).

Although the results in table 3-1 suggest that CgSnq2 has no positive effect in *C. glabrata* BPY55 resistance to azoles, a susceptibility phenotype for the BPY55_*snq2*Δ mutant in the presence of the azole drugs fluconazole and ketoconazole was previously reported (Torelli et al., 2008). However, the spot assays performed in the referred study were performed in YPD medium while all the phenotypes described in table 3-1 for *C. glabrata* were performed in minimal medium MM. Therefore, we investigated the susceptibility to azole drugs in rich medium YPD for *C. glabrata* strains, as well as for *S. cerevisiae* strains as a confirmation of the results obtained in minimal media MM4 (figure 3-5). We also included the well-characterized phenotypes for *Scpdr18*Δ and *Scsnq2*Δ in the presence of acetic acid and 4-NQO, respectively, and confirmed there is no interference of the medium used in these phenotypes (figure 3-5). The higher susceptibility of *Scpdr18*Δ deletion mutant, when compared to the corresponding parental strain, to the azole drugs ketoconazole, clotrimazole, miconazole, fluconazole, and to a lower extent, to itraconazole was confirmed in YPD (figure 3-5). Also, as found in minimal medium MM4 (table 3-1), *Scsnq2*Δ shows no susceptibility phenotype in the presence of the azole drugs in YPD (figure 5). In summary, even in YPD media, CgSnq2 is not a determinant of *C. glabrata* tolerance to itraconazole, clotrimazole or miconazole (figure 3-5), but the phenotypes previously reported for fluconazole and ketoconazole (Torelli et al., 2008) were confirmed.

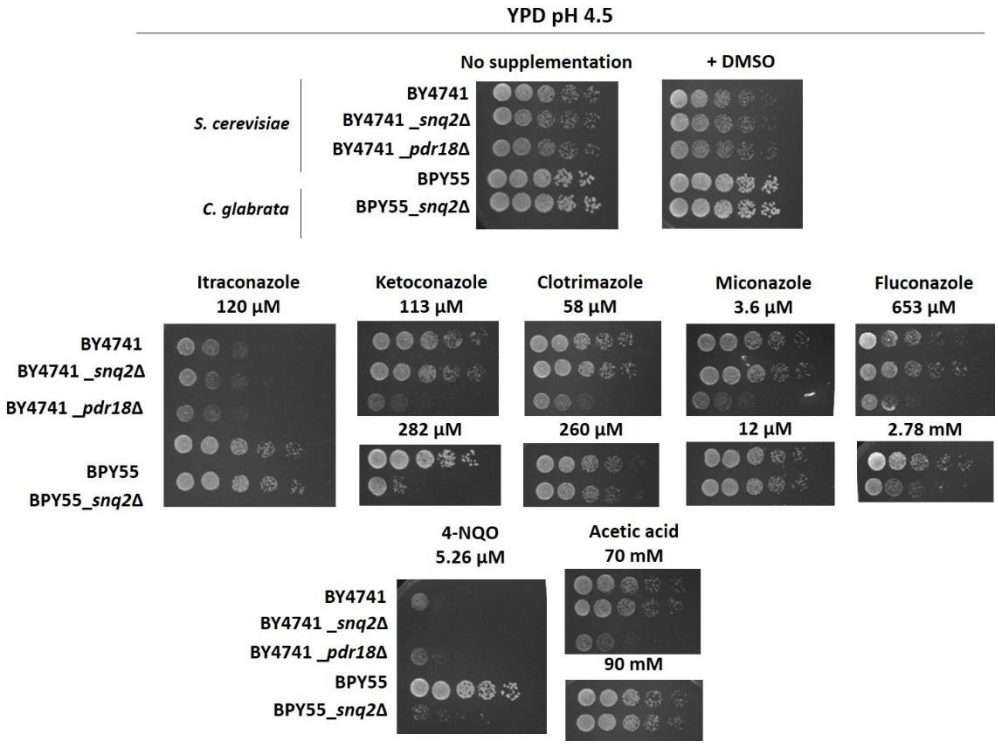

Figure 3-5. Susceptibility profiling of the *S. cerevisiae pdr18*Δ and *snq2*Δ and of *C. glabrata snq2*Δ deletion mutants to azole drugs, 4-NQO and acetic acid in rich media YPD. Comparison of growth by spot assays of the *S. cerevisiae* parental strain BY4741 and derived *pdr18*Δ and *snq2*Δ, and of *C. glabrata* parental strain BPY55 and derived *snq2*Δ mutant cell suspensions plated in solid YPD media supplemented or not with azole drugs, 4-NQO or acetic acid (at pH 4.5). All pictures were obtained after 24 hours of incubation. The results are representative from three independent experiments.

3.5 DISCUSSION

The evolutionary history of *Saccharomyces cerevisiae PDR18* gene in Saccharomycetaceae yeasts was reconstructed in this study. Compared with a former analysis based on the genome sequences of only nine yeast species belonging to the *Hemiascomycetes* phylum (Seret et al., 2009), our study took advantage of the increasing number of yeast genomes currently available, and has examined sixteen post-WGD yeast species instead of only two, spanning six different taxonomic genera, instead of only two. This fact allowed the clarification of the evolution of *S. cerevisiae PDR18* and *SNQ2* genes homologs after the WGD event and led us to propose that a single gene loss event has occurred in the last common ancestor of *Nakaseomyces*, *Naumovozyma*, *Kazachstania* and *Saccharomyces* yeasts. This event explains the "interruption" of one of the post-WGD sub-lineages given that yeast strains of the *Nakaseomyces*, *Naumovozyma* and *Kazachstania* genera encode only one *PDR18*/*SNQ2* homolog gene with high synteny between them. The probability that the duplication event has occurred during the WGD event is negligible, as it would imply the gene loss in the five yeast species belonging to the *Nakaseomyces*, *Naumovozyma* and *Kazachstani* genera.. Also, the gene neighborhood analysis does not provide support to the second evolutionary scenario, as only one common neighboring gene in these sub-lineages belongs to the similarity cluster 949, which is also present in the chromosome environment of many genes comprised in the other post-WGD sub-lineage proposed to give rise to the duplication event. Altogether, results do not support the ohnolog status for *S. cerevisiae SNQ2* and *PDR18* genes, but instead support the first proposed scenario.

Concerning the point in time where the duplication event originating the *SNQ2* and *PDR18* sub-lineages might have occurred in the evolution of the post-WGD yeast species, it is possible that the *PDR18* gene ancestor was nurtured in one of the tandem repeats encoding *PDR* genes that, with exception of the *K. naganishii* genome, are found in the genomes of all yeast species belonging to the *Kazachstania* and *Naumovozyma* genera.. Under this scenario, the amino acid sequence of the ancient gene that gave rise to *PDR18* did not diverge until the last common ancestral in the origin of the *Saccharomyces* genus split apart from the ancestral yeast population giving rise to the *Kazachstania* genus. In the second scenario, the *PDR18* gene ancestral was originated in an independent duplication event not related with the events on the origin of the tandem repeats observed in the genomes of the *Kazachstania* and *Naumovozyma* species. Independently of which of these evolutionary scenarios is true, subsequent genome shuffling and/or other mechanisms of genome evolution should have been

responsible for the transposition of the two ancestral genes encoding Pdr18 and the neighboring gene, encoding the aldose 1-epimerase, into a new chromosome environment.

Beside the gene duplication events in the origin of the *SNQ2* and *PDR18* paralog genes, other gene duplication events were identified in the genomes of the protoploid Saccharomycetaceae (pre-WGD). The yeast species belonging the *Zygosaccharomyces* genus and the *Lachancea kluyvery* species encoded more than one Snq2/Pdr18 homolog in their genomes, escaping the typical pattern observed in the majority of the pre-WGD species analyzed in this study, where a sole member of the Snq2/Pdr18 protein subfamily were found encoded in the corresponding genome sequences. Interestingly, the amino acid sequences of the two singletons encoded in the genome of the *Z. bailii* IST302 strain showed the existence of a strong divergence in respect to the amino acid sequences of the Snq2/Pdr18 homologs encoded in the other *Zygosaccharomyces* strains analyzed in this study. This fact and the high amino acid sequence identity shared by these two singletons suggests that these are a paralog pair originated in a duplication event that occurred recently in the evolution of the *Zygosaccharomyces* yeast species. The consultation of the MIPS website comprising the aligning of the presently available genome sequences of yeast strains belonging to the *Zygosaccharomyces* genus showed that these two singleton genes are also absent in the interspecies hybrid Z. bailii ISA1307 (http://mips.helmholtz-muechen.de). This result furthers strengths the hypothesis of a recent origin of these two singletons occurring at the intraspecific level.

Since it was found that *PDR18* is specific for the *Saccharomyces* genus, with very high conservation among the 93 *Saccharomyces* yeast genomes examined, the MDR/MXR profiling of this transporter in *S. cerevisiae* was examined for a wide number of relevant toxic compounds and the possible overlapping of the susceptibility phenotypes exhibited by the *Scpdr18*Δ mutant and the *Scsnq2*Δ mutant with its paralog gene *ScSNQ2* deleted was systematically examined. ScSnq2 was found to confer resistance to a more restricted range of the toxic compounds tested, compared with ScPdr18 with a demonstrated physiological function as plasma membrane transporter in the maintenance of plasma membrane ergosterol content specially under chemical stress. This biological role was related with the decreased levels of stress-induced membrane disorganization and permeabilization and counteracting transmembrane electrochemical potential dissipation (Cabrito et al., 2011; Godinho et al., 2018) and thus with the maintenance under stress of a functional plasma membrane as a selective barrier and a suitable lipid environment for the physiological activity of the embedded proteins (Abe

and Hiraki, 2009; Aguilera et al., 2006; Caspeta et al., 2014b; del Castillo Agudo, 1992; Eisenkolb et al., 2002; Kodedová and Sychrová, 2015; Parks and Casey, 1995).

Since the anticipated functional divergence between ScPdr18, ScSnq2 and the ancestral gene on the origin of the duplication event is of relevance to understand the evolutionary process acting on the two duplicate genes, the post-WGD pathogenic species *Candida glabrata* was selected for a systematic analysis of the susceptibility phenotype of the corresponding deletion mutant *Cgsnq2*Δ. Based on the susceptibility assays performed, the sole *SNQ2*/*PDR18* homolog in *C. glabrata* encoding CgSnq2 appeared to be functionally closer to ScSnq2 then to ScPdr18, playing a role in 4-NQO resistance in both species and having no impact in *C. glabrata* tolerance to the weak acids acetic and benzoic, the herbicides 2,4-D and MCPA, the alcohols ethanol and 1,4-butanediol, and the polyamine putrescine, to which *ScPDR18* expression confers tolerance. It is noteworthy that CgSnq2 was previously found to play a role in *C. glabrata* BPY55 tolerance to fluconazole and ketoconazole by susceptibility assays in solid YPD medium (Torelli et al., 2008). This phenotype was confirmed in this study under similar conditions, a fact that could indicate some overlapping of the function associated to CgSnq2 and ScPdr18. However, these phenotypes were not reproduced in minimal media MM, the growth conditions used for the systematic analysis of the phenotypic profiling performed.

The apparent overlapping between ScPdr18 and CgSnq2 role observed in azole resistance in rich media can be related with species-specific adaptation of *C. glabrata* to these fungicides, given that this yeast species is one of the most common in nosocomial fungal infections and that azoles are one of the main families of drugs that are currently being used to treat or prevent fungal infections (Jandric and Schüller, 2011; Perlroth et al., 2007; Roetzer et al., 2011) and that the *C. glabrata* strain used in this work is a highly azole resistant clinical isolate (Torelli et al., 2008).. The sensitivity phenotype exhibited by *Cgsnq2*Δ towards Cu^{2+} toxicity might also be related to the fact that BPY55 is a clinical isolate and that adaptation to high Cu^{2+} environmental concentrations is a described determinant for survival of human pathogens (Chaturvedi and Henderson, 2014; Festa and Thiele, 2012; Fu et al., 2014; García-Santamarina and Thiele, 2015; Samanovic et al., 2012).

The fact that *S. cerevisiae* Snq2 and Pdr18 confer resistance to a very different set of chemical compounds with little overlapping, appears to exclude the evolutionary scenario where these highly similar MDR/MXR transporters have been retained in the *S. cerevisiae* genome due to functional redundancy or dosage effect, suggesting as the most consistent scenarios the subfuncionalization and the neofunctionalization of the

gene copies. Although subfunctionalization is commonly associated with the mere division of functions of the ancestral protein by the two duplicates, another possible model is that one of the duplicate proteins becomes more efficient at performing one of the original functions of the progenitor gene (Zhang, 2003). Considering the neofunctionalization process, in most of the cases the function adopted by one of the duplicate proteins is a related function rather than an entirely new function (Conant and Wolfe, 2008; Zhang, 2003). Although there are no detailed studies focusing on *SNQ2* expression impact on yeast plasma membrane lipid content and properties, Snq2 was previously found to contribute to alleviate estradiol toxicity in *S. cerevisiae*, a molecule highly similar to ergosterol (Mahé et al., 1996a) and Pdr18 is described to be an ergosterol transporter at the plasma membrane (Godinho et al., 2018). The clarification of whether ScSnq2 and/or CgSnq2 may play some role on lipid homeostasis and of whether ScPdr18 is the result from subfunctionalization by function specialization or whether the biological function acquired by ScPdr18 is totally different from ScSnq2 and CgSnq2, require further work and the elucidation of *SNQ2* biological function.

Pdr18 is encoded in at least 87 out of the 93 genomes from the *Saccharomyces* genus yeasts examined in this study: in 56 out of 60 *S. cerevisiae* genomes, in the 25 *S. paradoxus* genomes, in the genomes of *S. mikatae*, *S. kudriavzevii*, *S. arboricola* and *S. uvarum*, and in the two *S. eubayanus* genomes. No Pdr18 ortholog was found in the two *S. bayanus* genomes examined, although it should be taken into consideration that the genome sequence of *S. bayanus* 623-6C was fragmented in the chromosomal region of residence of Pdr18. Also, the *PDR18* gene was not found in the genome sequence of *S. cerevisiae* cen.pk113-7d strain but the genome sequencing shows no coverage for the right arm of chromosome XIV, where *PDR18* gene resides (Nijkamp et al., 2012). Moreover, *PDR18* gene was found to be present in the genomes of 964 isolates of more than 1,000 natural *S. cerevisiae* isolates that were a recently examined using deep coverage genome sequencing (Peter et al., 2018). This fact is consistent with the relevant physiological function encoded by *PDR18* in this yeast species (Godinho et al., 2018).

4 Yeast response and tolerance to benzoic acid involves the Gcn4- and Stp1- regulated multidrug/multixenobiotic resistance transporter Tpo1

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4.1 ABSTRACT

The action of benzoic acid in the food and beverage industries is compromised by the ability of spoilage yeasts to cope with this food preservative. Benzoic acid occurs naturally in many plants and is an intermediate compound in the biosynthesis of many secondary metabolites. The understanding of the mechanisms underlying the response and resistance to benzoic acid stress in the eukaryotic model yeast is thus crucial to design more suitable strategies to deal with this toxic lipophilic weak acid.

In this study, the *Saccharomyces cerevisiae* multidrug transporter Tpo1 was demonstrated to confer resistance to benzoic acid. *TPO1* transcript levels were shown to be up-regulated in yeast cells suddenly exposed to this stress agent. This up-regulation is under the control of the Gcn4 and Stp1 transcription factors, involved in the response to amino acid availability, but not under the regulation of the multidrug resistance transcription factors Pdr1 and Pdr3 that have binding sites in *TPO1* promoter region. Benzoic acid stress was further shown to affect the intracellular pool of amino acids and polyamines. The observed decrease in the concentration of these nitrogenous compounds, registered upon benzoic acid stress exposure was not found to be dependent on Tpo1, although the limitation of yeast cells on nitrogenous compounds was found to activate Tpo1 expression. Altogether, the results described in this study suggest that Tpo1 is one of the key players standing in the crossroad between benzoic acid stress response and tolerance and the control of the intracellular concentration of nitrogenous compounds. Also, results can be useful to guide the design of more efficient preservation strategies and the biotechnological synthesis of benzoic acid or benzoic acid-derived compounds.

4.2 INTRODUCTION

Benzoic acid is a lipophilic weak acid that occurs naturally in many plants, is largely used in the preservation of foods and beverages and is an intermediate compound in the biosynthesis of many secondary metabolites. Uncovering the complexity of cellular responses to stress induced by benzoic acid in the experimental eukaryotic model *Saccharomyces cerevisiae*, might be instrumental to improve food preservation action and microbial performance in biotechnological processes. In particular, the identification of candidate genes and signalling pathways involved in the response and resistance to this stress is essential to find targets for genetic engineering to increase stress robustness for biotechnological processes or to guide preservation strategies (dos Santos and Sá-Correia, 2015; Mira et al., 2010d; Teixeira et al., 2011b). Multidrug/multixenobiotic resistance (MDR/MXR) is many times the result of the action of MDR/MXR transporters found at the membranes of all living cells (Sá-Correia et al. 2009; Teixeira et al. 2011a). Therefore, our laboratory has dedicated research efforts to the study of the biological role and regulation of drug/xenobiotic pumps of the Major Facilitator Superfamily (MFS) and the ATP-Binding Cassette superfamily (ABC), and the link between their physiological role and the MDR/MXR phenomenon in yeast (Sá-Correia et al. 2009). The *S. cerevisiae* plasma membrane Drug:H^+ Antiporter (DHA) Tpo1, a MDR/MXR transporter of the MFS, has been found to mediate tolerance of this yeast species to a high number of cytotoxic compounds including the metal ions cadmium and aluminium (Cabrito et al. 2009), the antimalarial drugs quinidine and artesunate (Alenquer et al. 2006; do Valle Matta et al. 2001), the immunosuppressant mycophenolic acid (Desmoucelles et al. 2002), the herbicides 2,4-dichlorophenoxyacetic acid (2,4-D) and barban (Cabrito et al. 2009; Teixeira and Sá-Correia 2002), the anticancer agent bleomycine (Berra et al. 2014; Hillenmeyer et al. 2008), the antifungals nodoconazole and mancozeb (Dias et al. 2010; Hillenmeyer et al. 2008), the nonsteroidal anti-inflammatory drug diclofenac (Mima et al. 2007) and the weak acids acetic, propionic, decanoic and octanoic acids (Borrull et al. 2015; Legras et al. 2010; Mira et al. 2009). The apparent promiscuity of Tpo1 and other yeast MFS-MDR transporters in conferring protection to a wide range of structurally unrelated xenobiotic compounds has been questioning the idea that these transporters contribute to MDR by directly mediating the extrusion of the drugs (Dos Santos et al. 2014; Mira et al. 2010a; Sá-Correia et al. 2009). Within this line of thought, evidences have been obtained showing that the beneficial effect of some yeast MDR pumps in conferring drug resistance is indirect and results from their effect in the transport of a given physiological substrate whose partition ends up contributing to reduce the internal accumulation of drugs or to counteract their

deleterious effects (Cabrito et al. 2011; Sá-Correia et al. 2009; Teixeira et al. 2011a; Vargas et al. 2007). A paradigmatic example has been Qdr2, whose protective effect against quinidine action was correlated with its role in K^+ uptake (Vargas et al. 2007). Tpo1 has been found to be involved in the export of polyamines, in particular of spermidine and spermine (Albertsen et al. 2003; Krüger et al. 2013; Uemura et al. 2005), however, this physiological role has, so far, not been linked to its role in MDR.

The extensive amount of information that has been gathered regarding the transcriptional regulatory networks underlying the control of the expression of yeast MFS-MDR encoding genes, largely compiled in the YEASTRACT database (Teixeira et al. 2006), has been contributing to elucidate the function of these transporters in the MDR context and also outside of it, in a more physiological perspective. Key transcriptional regulators of MDR in yeast, such as Pdr1, Pdr3 and Yap1, were found to control drug-induced transcriptional activation of MFS-MDR-encoding genes (as recently reviewed in Dos Santos et al. 2014), including *TPO1* (Alenquer et al. 2006; do Valle Matta et al. 2001; Teixeira and Sá-Correia 2002). Nevertheless, a closer inspection of the data available in the YEASTRACT database shows that most yeast MFS-MDR-encoding genes have more documented regulatory associations with transcription factors not specifically linked to the MDR phenomenon than with those that are known to control MDR (Dos Santos et al. 2014). Gcn4, a transcription factor involved in signaling amino acid internal homeostasis, and Bas2, an activator of the histidine and purine biosynthetic pathways, stand out as they are associated to the transcriptional regulation of around 70% of all genes encoding MFS-MDR transporters in yeast (Dos Santos et al. 2014). In some cases, the transcriptional regulation by non-MDR transcription factors of MFS-MDR transporters is consistent with their proposed physiological function. This is the case of the Gcn4-regulated transporters Aqr1, implicated in the vesicle-mediated extrusion of homoserine, threonine and other amino acids (Velasco et al. 2004); Vba1-5, proposed to catalyse transport of amino acids into the vacuole (Shimazu et al. 2005), and Qdr2, which was also demonstrated to affect amino acid homeostasis (Vargas et al. 2007).

In this work, Tpo1 was identified as a determinant of yeast resistance to benzoic acid. A strong up-regulation of the *TPO1* gene was registered in response to benzoic acid stress; however, this was found to be independent of Pdr1 and other transcription factors specifically related with drug stress response and tolerance. Instead, the benzoic acid-induced transcriptional activation of *TPO1* was found to be dependent of Gcn4 and Stp1, which were also found to play an essential role in tolerance of *S. cerevisiae* to benzoic acid. Taking into consideration the crucial role played by the Gcn4- and Stp1-dependent pathways in yeast sensing and signalling of internal amino acid homeostasis (Ljungdahl

and Daignan-Fornier 2012), the hypothesized involvement of Tpo1 in polyamines and in amino acid internal homeostasis under benzoic acid stress was dissected. The results obtained provide useful insights into the link between the intracellular homeostasis of nitrogenous compounds and Tpo1 regulation and protective role in response to benzoic acid.

4.3 MATERIALS AND METHODS

4.3.1 Strains and plasmids

The parental strain *S. cerevisiae* BY4741 (*MATa*, *ura3*Δ0, *leu2*Δ0, *his3*Δ1, *met15*Δ0) and the derived deletion mutants (BY4741_*tpo1*Δ, BY4741_*pdr1*Δ, BY4741_*pdr3*Δ, BY4741_*pdr8*Δ, BY4741_*yap1*Δ, BY4741_*yap2*Δ, BY4741_*yap3*Δ, BY4741_*yap4*Δ, BY4741_*yap5*Δ, BY4741_*war1*Δ, BY4741_*gcn4*Δ, BY4741_*stp1*Δ and BY4741_*stp2*Δ) were obtained from the Euroscarf collection. The amino acid prototrophic strain 23344c (*MATa*, *ura3*) was kindly provided by B. André (Université Livre de Bruxelles, Belgium). *S. cerevisiae* BY4741 strains in which genome the vector pRS303*GPD* or the construction pRS303*GPD_TPO1* were integrated (herein referred to as BY4741.*GPD* and BY4741.*GPD_TPO1*, respectively) were kindly provided by M. Ralser (University of Cambridge, United Kingdom). The plasmids prepared and/or used in this study are listed in Table 4-1.

Tabela 4-1. Plasmids used in this study

Plasmid name	Description	Reference
pYEP351	Yeast/*E. coli* shuttle vector with a LEU2 marker.	(Hill et al. 1986)
pYEP351_*TPO1*	Yeast/*E. coli* shuttle vector with a LEU2 marker, in which *TPO1* gene was cloned.	(Tomitori et al. 2001)
p*TPO1*::*lacZ*	Expression fusion plasmid in which 1000 bp of *TPO1* promoter region were fused with *lacZ* coding sequence at pAJ152 basal vector	(Alenquer et al. 2006)
pYEP354	Yeast episomal vector with URA3 marker for construction of *lacZ* fusions	(Myers et al., 1986)
pYEP354_*TPO1*::*lacZ*	Expression fusion plasmid in which 1000 bp of *TPO1* promoter region were fused with *lacZ* coding sequence at pYEP354 basal vector	This study
p*YEP354_TPO1(GRE1***)*::*lacZ*	Plasmid derived from pYEP354_*TPO1*::*lacZ* in which the Gcn4 binding site TGACTC located at position -771 of *TPO1* promoter region was replaced by TGAGGC	This study
p*YEP354_TPO1(GRE2***)*::*lacZ*	Plasmid derived from pYEP354_*TPO1*::*lacZ* in which the Gcn4 binding site TGACTC located at position -175 of *TPO1* promoter region was replaced by TTTCTC	This study
p*YEP354_TPO1(SRE1***)*::*lacZ*	Plasmid derived from pYEP354_*TPO1*::*lacZ* in which the St1p binding site CGGCTC located at position -630 of *TPO1* promoter region was replaced by CGGATC	This study

4.3.2 Growth media

Cells were batch-cultured at 30 ºC, with orbital agitation (250 rpm), in MM4 growth media which contains, per liter: 20 g glucose (Merck, Darmstadt, Germany), 1.7 g yeast nitrogen base without amino acids or NH_4^+ (Difco, Detroit, Michigan, USA) and 2.65 g $(NH_4)_2SO_4$ (Merck, Darmstadt, Germany). To cultivate BY4741 and the deletion mutant strains derived from BY4741, the MM4 growth medium was further supplemented with 20 mg/L methionine, 20 mg/L histidine, 60 mg/L leucine and 20 mg/L uracil (all from Sigma,

Missouri, USA). The amino acid prototrophic strain 23344c was cultivated in MM4 growth medium supplemented with 20 mg/L uracil. Solid MM4 growth medium was obtained by supplementing the liquid medium with 2% agar (Iberagar, Barreiro, Portugal). The pH of liquid MM4 growth medium was adjusted to 4.0 using HCl as the acidulant. The stock solution of benzoic acid (potassium salt; Sigma, St. Louis, Missouri, USA) used to supplement the media was prepared in water and the pH of this solution was adjusted to 4.0 using HCl.

4.3.3 Benzoic acid susceptibility assays

The susceptibility of the *S. cerevisiae* strains tested to benzoic acid was examined by comparing the growth of these two strains in liquid medium. Mid-exponential cells (OD_{600nm} 0.5 ± 0.05) cultivated in liquid MM4 medium (at pH 4.0) were used to re-inoculate (at initial OD_{600nm} of 0.05) this same basal medium either or not supplemented with 0.9 mM benzoic acid. Growth in the presence or absence of benzoic acid was monitored by accompanying the increase in the OD_{600nm} of the cultures. Susceptibility of the BY4741.*GPD* and BY4741.*GPD_TPO1* was assessed in a range of 0.7-1.1 mM benzoic acid, in the same minimal media. To assess the effect of pH established without the addition of any weak acid, the MM4 growth medium pH was adjusted in the range of 2-5 with HCl and NaOH.

4.3.4 Subcultivation of benzoic acid-adapted yeast cells

S. cerevisiae BY4741 and the derived deletion mutant *tpo1*Δ cells were cultivated until mid-exponential phase (OD_{600nm}= 0.6 ± 0.05) in liquid MM4 medium (at pH 4.0) and reinoculated in this same basal medium supplemented with 0.9 mM benzoic acid. When cultures resumed growth, they were subcultivated in fresh MM4 medium supplemented with the same concentration of benzoic acid. Growth was followed by measuring OD_{600nm} and colony forming units.

4.3.5 Measurement of *TPO1* expression based on *lacZ* fusions

S. cerevisiae BY4741 and the derived deletion mutants *pdr1*Δ, *pdr3*Δ, *pdr8*Δ, *yrr1*Δ, *yap1*Δ, *yap2*Δ, *yap3*Δ, *yap4*Δ, *yap5*Δ, *gcn4*Δ, *stp1*Δ, *stp2*Δ, and *war1*Δ transformed with p*TPO1*::*lacZ* plasmid (Alenquer et al., 2006) were cultivated until mid-exponential phase (OD_{600nm}=0.6±0.01) in MM4 growth medium lacking uracil (at pH 4.0) and then re-inoculated (at an OD_{600nm}=0.2±0.01) into this same basal growth medium supplemented or not with 0.9 mM benzoic acid. The expression of the *TPO1* gene in the wild-type and in the mutant strains was compared in mid-exponential phase cells incubated for 12h in the presence of benzoic acid. This time-point was found to lead to maximum expression

of the *TPO1* gene measured from the p*TPO1*::*lacZ* plasmid in wild-type benzoic acid-challenged cells. In control cultures, cells were harvested in the mid-exponential phase of growth (6h of incubation in MM4 growth medium). The determination of β-galactosidase activity was carried out as described before (Alenquer et al., 2006), the enzyme specific activity units (U; Miller units) being defined as the increase in A_{420} min^{-1} $(OD_{600nm})^{-1} \times 1000$.

To examine the effect that the Gcn4- response elements (GRE) or of the Stp1-responsive elements (SRE) motifs present in the *TPO1* promoter have in the expression of the *TPO1* gene under benzoic acid stress, these DNA motifs were inactivated by site-directed mutagenesis using as a template pYEP354w_*TPO1*::*lacZ* fusion plasmid. This plasmid was constructed by cloning the promoter region of *TPO1* (considered as the 1000 bp ustream of the start codon) into the *Bam*HI and *Pst*I sites of the pYEP354w vector. The p*TPO1*::*lacZ* plasmid could not be used as template in the mutagenic PCR reactions due to its high molecular weight (~14 kb) and thus a shorter *lacZ* fusion, based on the YEP354w vector (~6kb), was constructed for this purpose. *lacZ* expression from pYEP354_*TPO1*::*lacZ*, pYEP354_*TPO1*(GRE1)::*lacZ*, pYEP354_*TPO1*(GRE2)::*lacZ* and pYEP354_*TPO1*(SRE2)*::lacZ* plasmids was assessed by real-time RT-PCR. For that, cell samples were obtained by centrifugation [5000 rpm in a Beckman (Brea, California, USA) JA20 rotor, 4ºC, 5 min] and immediately frozen at -80ºC until total RNA extraction. One µg of total RNA was used for cDNA synthesis. The reverse transcription step was performed using the multiscribe reverse transcriptase kit (Applied Biosystems, Foster City, California, USA) in a 7500 RT-PCR thermal cycler block (Applied Biosystems, Foster City, California, USA). Approximately 10 ng of the synthesized cDNA were used for the subsequent PCR step. In all experiments the transcript level of *ACT1* mRNA was used as an internal control. The primers used for amplification of *ACT1* cDNA (3'-CTCCACCACTGCTGAAAGAGAA-5', 5'- CCAAGGCGACGTAACATAGTTTT-3') and of *lacZ* cDNA (3'-AAAGCTGCAAGTCTGCATCACAC-5' and 5'-GCACGATAGAGATTCGGGATTT-3') were designed using Primer Express Software (Applied Biosystems, Foster City, California, USA). The relative values obtained for the expression from the native promoter in control conditions were set as 1 and the remaining values presented are relative to that control.

4.3.6 Measurement of *TPO1* transcription based on real time RT-PCR

Real time RT-PCR was used to compare *TPO1* mRNA levels during cultivation of *S. cerevisiae* BY4741 cells or of the deletion mutants *pdr1*Δ*, pdr3*Δ*, stp1*Δ, *stp2*Δ and

*gcn4*Δ in MM4 growth medium (at pH 4.0) either or not supplemented with benzoic acid (0.9 mM), as described in the previous section. The primers used for the amplification of the probes selected to monitor *TPO1* (3'- TCTGACAATTCACTACCGAACAATC-5', 5'-GGCGTGCCGCTGCTT-3') and *ACT1* expression (the same as indicated in the previous section), were designed using Primer Express Software (Applied Biosystems, Foster City, California, USA). The specificity of the probe selected for monitoring *TPO1* transcription was confirmed by the absence of an amplification product in the *tpo1*Δ mutant. The relative values obtained for the wild-type strain in control conditions were set as 1 and the remaining values presented are relative to that control. A similar experimental setup was used to monitor *TPO1* transcription in conditions of leucine exhaustion during growth of wild-type and *gcn4*Δ cells in MM4 growth medium or during growth of the 2344c strain in MM4 growth medium having a limiting (0.00265 g/L) or a saturating (2.65 g/L) concentration of ammonium.

4.3.7 Quantification of intracellular concentration of polyamines and amino acids

The intracellular concentration of amino acids and polyamines in wild-type or Δ*tpo1* cells was compared after one hour of incubation in MM4 growth medium (at pH 4.0) either or not supplemented with 0.9 mM benzoic acid. Yeast cells were harvested by centrifugation (5000 rpm in a Beckman JA20 rotor, 4ºC, 5 min), washed two times with ice-cold distilled water and frozen at -80ºC until further use. Polyamine extraction was performed by resuspending the harvested cells in 600 µL 10% trichloroacetic acid (TCA) supplemented with 1 mM 1,6-diaminohexane (Fluka, Buchs, Switzerland) which was used as the internal standard. The cell suspension obtained was incubated at 70ºC for one hour, centrifuged for 5 minutes (at 13000 *g*) and the supernatant was recovered into a new tube. Quantification of the content of spermine, spermidine and putrescine present in the 600 µL supernatant recovered was determined, by HPLC, as a service at the Instituto Biologia Experimental e Tecnológica (Oeiras, Portugal). The method used had a detection limit of 5 µM.

Intracellular amino acid pools were obtained using the method described before (Klasson et al., 1999). Briefly, yeast cells were harvested in the same conditions as those used for polyamine quantification and were washed twice with 1.5 mL of water and resuspended in 1.5 mL of AA buffer (2.5 mM K_2HPO_4-KH_2PO_4 at pH 6.0; 0.6 M sorbitol; 10 mM glucose). The washed filters were boiled in 3 mL of water for 15 min. 1-mL aliquots of this suspension were taken and centrifuged to remove particles of filter. The concentrations of amino acids present in this 1 mL sample was determined, by HPLC,

as a service at the Laboratório Nacional Dr Ricardo Jorge (Lisbon, Portugal). The method used had a detection limit ranging between 0.1 and 0.5 µg/mL for the different amino acids.

4.4 RESULTS

4.4.1 *TPO1* is a determinant of yeast resistance to benzoic acid

The comparison of the growth curves of unadapted cell populations of the parental strain *S. cerevisiae* BY4741 and of the *tpo1*Δ derived mutant in MM4 medium supplemented with 0.9 mM benzoic acid (at pH 4.0) shows that the elimination of *TPO1* significantly increases the duration of the adaptation period to the acid (from 18h to approximately 30h) and reduces the maximum specific growth rate of the adapted cell population (from 0.097 to 0.057 h^{-1}) (figure 4-1A). However, *TPO1* deletion had no detectable effect in yeast growth during cultivation in MM4 growth medium acidified to pH 2.0 using a strong acid (HCl) as the acidulant (figure 4-1B). This result shows that Tpo1 is specifically required for protection against benzoic acid and not against low pH by itself.

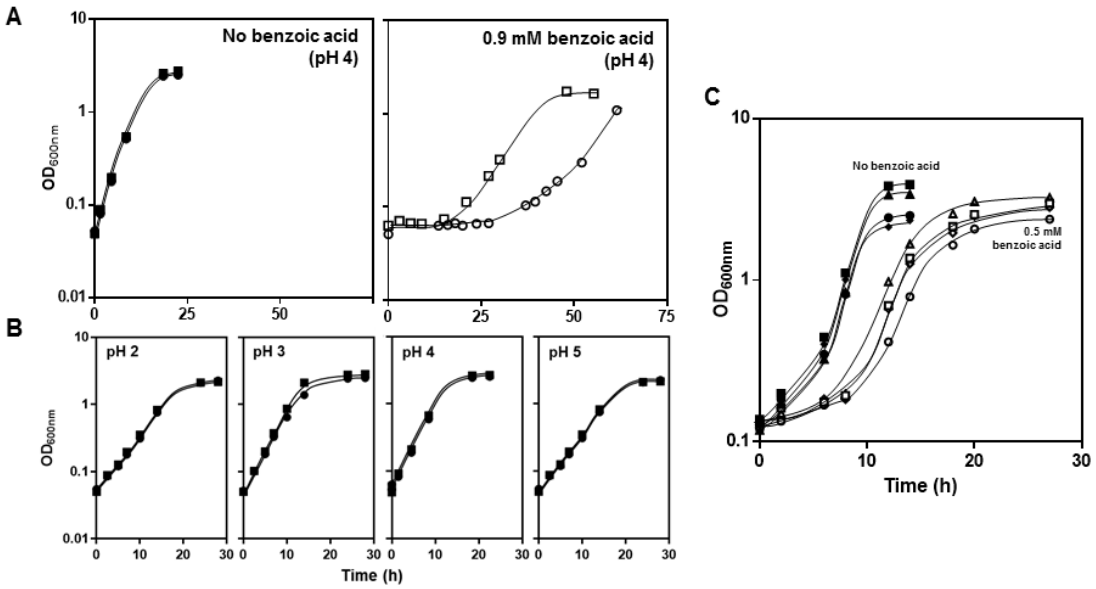

Figure 4-1. *TPO1* expression is required for a more rapid yeast adaptation to benzoic acid stress but not to low pH when a strong acid is used as the acidulant.
(A) Growth curves of *S. cerevisiae* BY4741 (■,□) or of the derived deletion mutant *tpo1*Δ (●,○) in MM4 growth medium (at pH 4.0) either (open symbols) or not (closed symbols) supplemented with benzoic acid (0.9 mM) or **(B)** in this same basal medium acidified at pH 2, 3.5 or 5 using HCl as the acidulant (lower panel). **(C)** Growth curves of *S. cerevisiae* BY4741 and *tpo1*Δ harbouring an empty vector (□,○ respectively) or the same pYEP351 vector with the *TPO1* gene cloned (Δ,◊, respectively) supplemented (open symbols) or not (closed symbols) with 0.5 mM benzoic acid. The growth curves shown are representative of, at least, three independent experiments that gave rise to the same growth patterns.

Expression of *TPO1* from a centromeric plasmid (pYEP351_*TPO1*) was further found to rescue the benzoic acid susceptibility exhibited by the *tpo1*Δ deletion mutant, enabling this mutant strain to display a susceptibility profile similar to the one exhibited by the BY4741 wild-type strain harbouring the empty vector pYEP351 (figure 4-1C). Insertion of the pYEP351_*TPO1* in the wild-type strain resulted in improved resistance to exposure to benzoic acid (figure 4-1C).

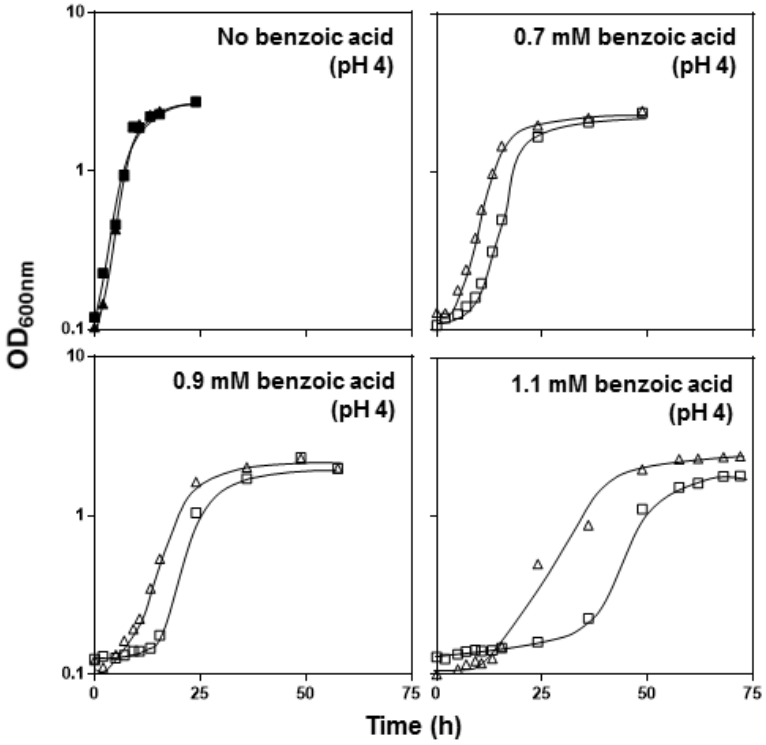

Figure 4-2. *TPO1* overexpression leads to an enhanced tolerance to benzoic acid induced stress. **(A)** Growth curves of *S. cerevisiae* BY4741.GPD (■,□) and BY4741.GPD_*TPO1* (▲,△) in MM4 growth medium (pH 4.0) either or not supplemented with benzoic acid (0.7 – 1.1 mM). The growth curves shown are representative of, at least, three independent experiments that gave rise to the same results.

The BY4741.*GPD_TPO1* strain (Krüger et al., 2013), overexpressing the *TPO1* gene by insertion of an extra copy in the genome controlled by the strongest constitutive yeast promoter *GPD*, proved to be less susceptible to benzoic acid stress in all the concentrations tested when compared to BY4741.*GPD*, the respective wild-type parental strain (figure 4-2). In fact, overexpression of *TPO1* proved to reduce the adaptation phase of yeast cells to half when exposed to a very high benzoic acid concentration (1.1 mM), also rendering a higher final biomass when compared to the BY4741.*GPD* cell culture (figure 4-22).

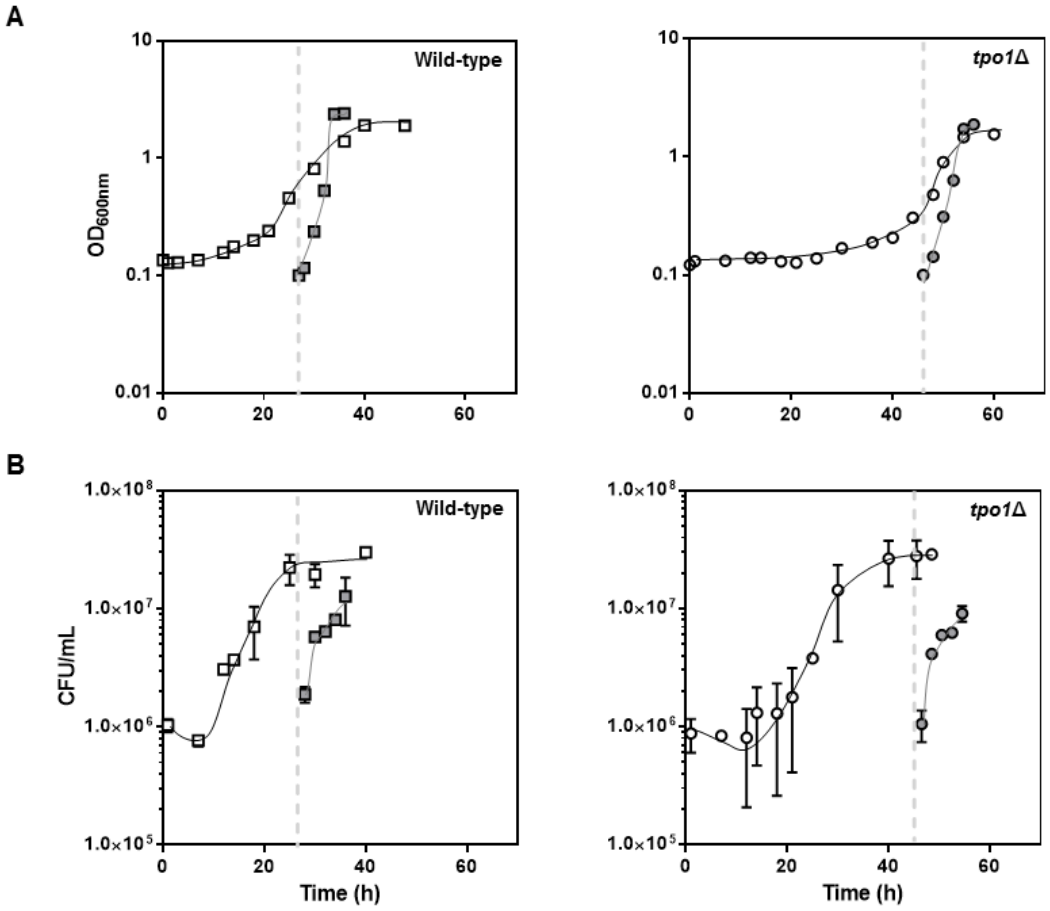

Figure 4-3. Subcultivation of benzoic acid-adapted cells shows yeast cell adaptation independently of Tpo1 expression.
Growth curves of wild-type (□,■) and derived deletion mutant *tpo1*Δ (○,●) in the presence of 0.9 mM benzoic acid (open symbols) in MM4 (pH 4.0). Grey symbols represent the subcultivation in fresh medium MM4 supplemented with 0.9 mM benzoic acid of cells harvested in the time point marked with the dashed line. Cells cultures were followed by measuring OD_{600nm} **(A)** and colony forming units per mL **(B)** The results are representative of, at least, three independent experiments, and error bars represent standard deviation.

The ability of wild-type and *tpo1*Δ mutant cells to adapt to benzoic acid-induced stress was tested by harvesting yeast cultures that resumed growth after a latency phase in MM4 media supplemented with 0.9 mM benzoic acid and re-inoculating them in fresh media with the same benzoic acid concentration. Pre-adapted cell populations did not exhibit a lag-phase period when exposed for the second time to the same stress (figure 4-3A). Although benzoic acid stress seems to induce a more severe and longer period of loss of viable cells in *tpo1*Δ cultures, the sub-cultivation of the adapted population rendered a similar growth in both wild-type and *tpo1*Δ cell cultures (figure 4-3B), consistently with the observed in optical density measurements. This phenomenon suggests that the role of Tpo1 is predominantly sensed in the period of adaptation to sudden benzoic acid, enabling the population to adapt faster to the presence of this food preservative.

4.4.2 *TPO1* transcription is activated under benzoic acid stress in the dependence of Gcn4 and Stp1 transcription factors

A dramatic increase (up to 30-fold) in *TPO1* transcript levels was registered during cultivation of an unadapted *S. cerevisiae* BY4741 population in the presence of a growth-inhibiting concentration of benzoic acid (0.9 mM at pH 4.0) compared with cells grown in unsupplemented medium (control cells) (figure 4-4A). This strong, but transient, stimulation of *TPO1* transcript levels reached a maximum value during the period of latency induced by benzoic acid, after 3h of incubation to the acid, after which mRNA levels decreased steeply to basal levels once adapted cells resumed exponential growth (figure 4-4A).

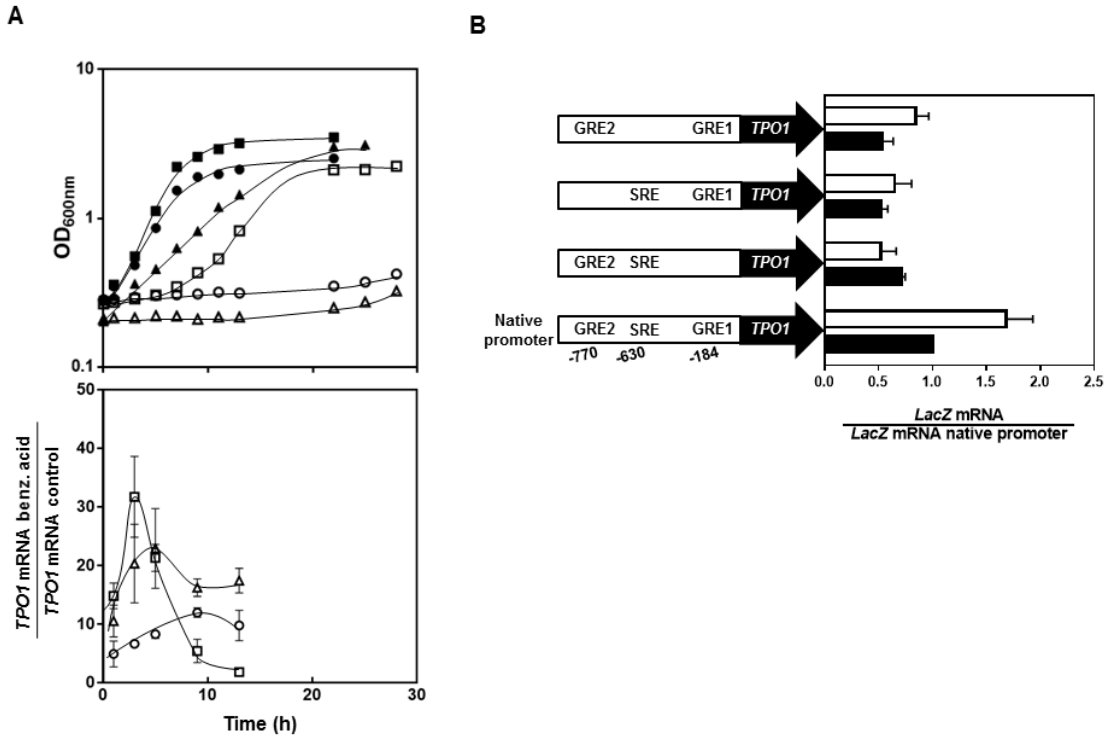

Figure 4-4. *TPO1* is up-regulated under benzoic acid stress in a Gcn4- and Stp1- dependent manner. (A) Growth curve of *S. cerevisae* BY4741 (■,□) and of the deletion mutants BY4741_*gcn4*Δ (▲,△) and BY4741_*stp1*Δ (●,○) in MM4 growth medium (at pH 4.0) (closed symbols) or in this same basal medium supplemented with 0.9 mM benzoic acid (open symbols). Quantification of *TPO1* mRNA levels during the growth curve of the three strains in the presence or absence of benzoic acid was based on quantitative real time RT-PCR. For each strain the transcript levels of *TPO1* gene shown are relative to the transcript levels registered in exponential-phase cells (at an OD_{600nm} of 0.4) cultivated in unsupplemented MM4 growth medium (at pH 4.0). In all samples *TPO1* mRNA levels were normalized using *ACT1* transcript levels; **(B)** Effect of the Gcn4-responsive elements (GRE) and Stp1-responsive elements (SRE) located in *TPO1* promoter in the benzoic acid-induced up-regulation of *TPO1,* measured, through RT-PCR, as the mRNA level of the *LacZ* gene, expressed under the control of the *TPO1* promoter. Wild-type cells harbouring the pYEP354w_*TPO1*::*lacZ* plasmid (which contains natural *TPO1* promoter) or the derived mutant constructs having the GRE and SRE motifs individually inactivated, were cultivated in MM4 growth medium (at pH 4) (black bars) or in this same growth medium supplemented with 0.9 mM benzoic acid (white bars) and harvested after 3 hours of growth.

The *TPO1* promoter region harbours at least each one binding site for twelve transcription factors known to be involved in yeast response to stress and other environmental challenges: Pdr1, Pdr3, Pdr8, Yrr1, Yap1, Yap2, Yap3, Yap4, Yap5, Msn2, Msn4, War1, Gcn4, Stp1 and Stp2) (figure S4-1 in the Supplementary Material). To examine the role of these transcription factors in benzoic acid-induced up-regulation of *TPO1* expression, the parental strain and several deletion mutants individually lacking these regulators, were transformed with the p*TPO1*::*lacZ* fusion plasmid (Alenquer et al., 2006). The levels of β-galactosidase produced in benzoic acid-stressed cells harbouring the p*TPO1*::*lacZ* plasmid were only found to be reduced, but not fully abrogated, in mutants devoid of Gcn4 or Stp1 transcription factors (figure S4-1 in the Supplementary Material). Consistent with this observation, *TPO1* mRNA levels produced in benzoic acid-challenged *gcn4*Δ and *stp1*Δ populations were significantly below the levels registered in the parental strain (figure 4-4A). Indeed, *TPO1* transcription in the *gcn4*Δ and *stp1*Δ mutant strains was found to reach only a 20-fold transient activation under benzoic acid stress, which represents a 30% reduction in the maximum level of *TPO1* up-regulation registered in the wild-type strain. Also, the period of time during which *TPO1* up-regulation appears to be extended in the mutant strains, possibly due to the fact that the lag-phase induced by benzoic acid in the mutant cell populations is also much longer than the one registered for the wild-type strain. The undetectable effect of Pdr1, Pdr3 and Stp2 in the activation of *TPO1* transcription under benzoic acid stress was also confirmed by real time RT-PCR (results not shown). To know if the effect of Gcn4 and Stp1 on *TPO1* transcription is direct, the three predicted Gcn4/Stp1 DNA binding sites found in the *TPO1* promoter (two for Gcn4 and one for Stp1, designated GRE and SRE motifs) were individually removed in the pYEP354:*TPO1 lacZ* fusion by mutagenesis, after which the responsiveness of the mutagenized constructs to benzoic acid stress was assessed by measurement of *lacZ* mRNA by RT-PCR (figure 4-4B). Inactivation of either of the two Gcn4-binding sites reduced the benzoic acid-induced up-regulation of the *TPO1* gene (figure 4-4B). Inactivation of the Stp1-binding site also abrogated the benzoic acid-induced up-regulation of *TPO1* (figure 4-4B). Significantly, even in the absence of benzoic acid the inactivation of the Gcn4- and Stp1- binding sites had a moderate effect in the *TPO1* transcription level (figure 4-4B). Altogether the results obtained are consistent with the concept that under benzoic acid stress, *TPO1* overexpression is under the coordinated action of Gcn4 and Stp1. Also, elimination of Gcn4 or Stp1 led to a dramatic increase in yeast susceptibility to benzoic acid, even higher than the one obtained upon deletion of *TPO1* (figure 4-4A), suggesting that there are other determinants of yeast tolerance to benzoic acid among the Gcn4 and Stp1 target genes.

4.4.3 Effect of Tpo1 expression and benzoic acid stress in internal homeostasis of polyamines and amino acids

The effect of *TPO1* expression and of benzoic acid stress in the internal concentration of polyamines was also examined considering the described involvement of Tpo1 in the export of these nitrogenous compounds (Albertsen et al., 2003; Krüger et al., 2013; Tomitori et al., 2001) (figure 4-5). Since benzoic acid-induced up-regulation of *TPO1* transcription was found to be controlled by Gcn4 and Stp1 and these transcription factors are key regulators of amino acid sensing and signalling in yeast (Ljungdahl and Daignan-Fornier, 2012), we also examined the effect of Tpo1 expression in internal amino acid homeostasis, either in the presence or absence of benzoic acid stress (figure 4-5). For this, the internal pools of amino acids and polyamines recovered from wild-type and *tpo1*Δ cells cultivated for 1 hour in the presence or absence of benzoic acid (0.9 mM, at pH 4) were compared by HPLC, these being the same experimental conditions that were found to lead to the strong transcriptional activation of *TPO1* mediated by Gcn4 and Stp1 (figure 4-5).

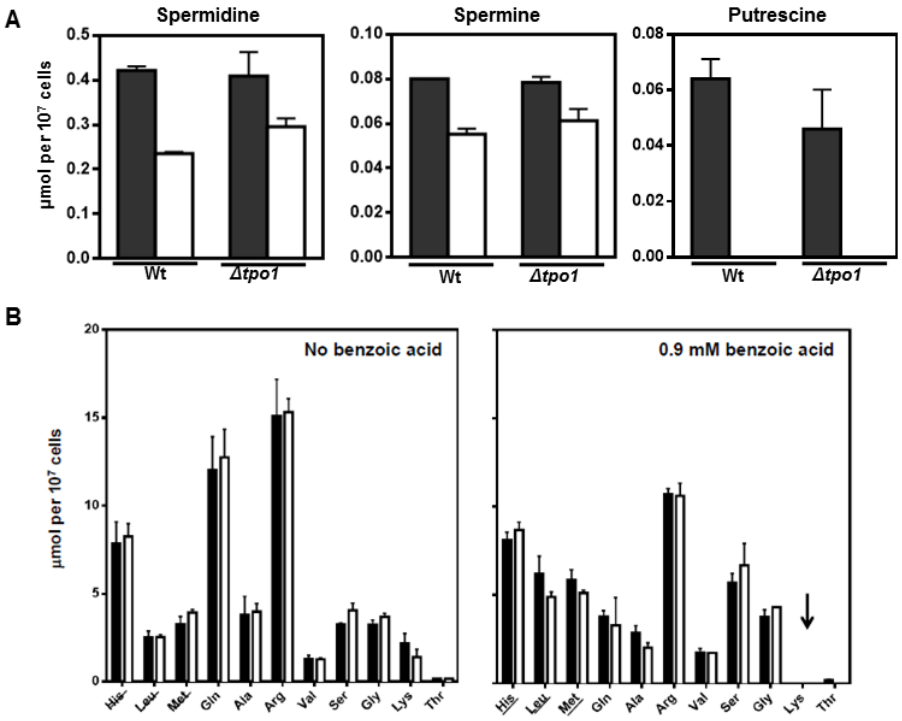

Figure 4-5. Effect of benzoic acid stress and *TPO1* expression in the internal pool of polyamines and amino acids.
(A) Spermidine, spermine and putrescine intracellular levels after one hour of incubation in the absence (black bars) or presence (white bars) of benzoic acid (0.9 mM); **(B)** Intracellular amino acid pools of BY4741 (black bars) and BY4741_*tpo1*Δ (white bars) in the presence or absence of 0.9 mM benzoic acid. The amino acids added to the MM4 growth medium to supress the auxotrophies of the BY4741 strain are underlined. The arrows indicate amino acids whose concentration was below the detection limit in the acid-challenged cells. Wt: wild-type.

The internal concentration of putrescine, spermine and spermidine was found to be similar in wild-type and *tpo1*Δ cells in the exponential phase of growth in MM4 growth medium (figure 4-5A). Exposure, for one hour, of unadapted BY4741 cell population to benzoic acid stress (0.9 mM, at pH 4.0), corresponding to the early period of adaptation to the acid, led to a reduction in the internal pool of the three polyamines, when compared to the values registered for control cell cultures (figure 4-5A). In fact, the putrescine levels fell under the detection limit. Surprisingly, no significant differences were observed between the wild-type and *tpo1*Δ yeast cell cultures regarding any of the polyamine intracellular levels determined (figure 4-5A).

The parental and *tpo1*Δ strains were found to exhibit, in general, similar amino acid pools (figure 4-5B). After one hour of exposure to 0.9 mM benzoic acid (at pH 4.0) the intracellular pool of several amino acids decreased in the parental strain and in the *tpo1*Δ mutant, in particular glutamine, arginine and lysine, the internal concentration of lysine having decreased to levels below the detection limit (figure 4-5B). The exception to this pattern of reduction of intracellular amino acid concentration were the amino acids corresponding to the yeast strain auxotrophies (histidine, leucine and methionine) and that for this reason, were supplemented to the growth medium (figure 4-5B). The intracellular levels of asparagine, isoleucine, proline, cysteine, phenylalanine and tyrosine could not be determined because they were below the detection limit of the technique. This could be the result of the cultivation of yeast cells in minimal growth medium (Kitamoto et al., 1988).

4.4.4 *TPO1* transcription is up-regulated in response to amino acid and nitrogen limitation

The transcription level of yeast MFS-MDR transporter-encoding genes *QDR2*, *AQR1* and *QDR3,* also required for resistance to polyamines, has been found to increase in response to amino acid and nitrogen limitation in a Gcn4-dependent manner (dos Santos et al., 2014; Sá-Correia et al., 2009; Teixeira et al., 2011a). This indication prompted us to examine whether *TPO1* transcription was also responsive to this type of physiological perturbation (figure 4-6). The results obtained show that cells of the prototrophic strain 2344c cultivated for 3h in minimal medium, supplemented with a limiting concentration of ammonium sulphate as the sole nitrogen source (0.0265 g L^{-1}), exhibit a 3-fold higher expression of *TPO1*, comparing with cells cultivated under the same conditions in a saturating concentration of ammonium (2.65 gL^{-1}) (figure 4-6). *TPO1* transcript levels were also found to increase (by around 3-fold) when *S. cerevisae* BY4741 cells entered stationary-phase of growth in MM4 medium due to leucine exhaustion (figure 4-6).

Leucine is one of the amino acids that has to be added to the growth medium to complement the auxotrophies of BY4741 strain and the concentration used in the MM4 medium, 60 mg L^{-1}, was demonstrated to be growth-limiting (Vargas et al., 2007). Indeed, supplementation of the exhausted growth medium with fresh leucine restored growth of BY4741 cells and resulted in a decrease in the level of *TPO1* mRNA to values similar to those registered in the leucine-replete growth medium (figure 4-6). No significant increase in *TPO1* expression induced by leucine exhaustion during growth in the MM4 growth medium was observed in the *gcn4*Δ mutant (figure 4-6).

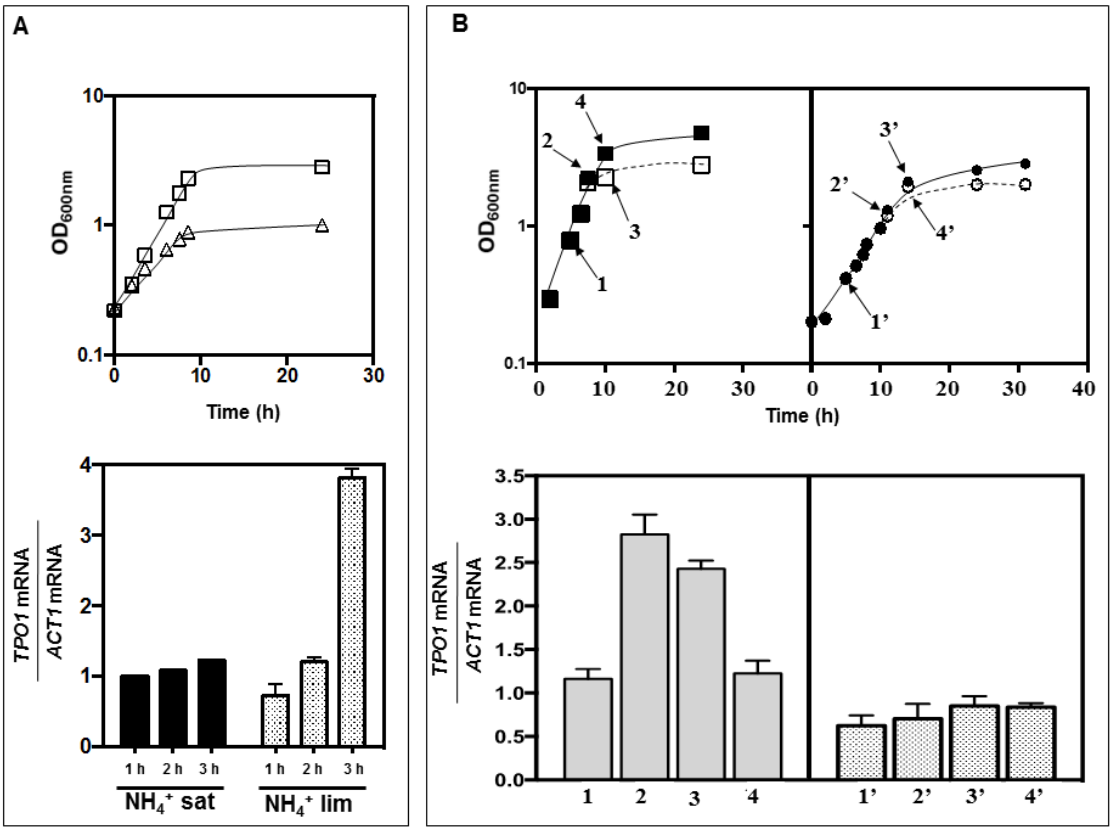

Figure 4-6. *TPO1* is up-regulated under ammonium or leucine limitation.
(A) Expression of *TPO1* gene during the first 3 hour of growth of the prototrophic strain 2344c in minimal growth medium supplemented with a limiting (lim; △ and grey bars)(0.0265 g L^{-1}) or a saturating (sat; □ and black bars) (2.65 g L^{-1}) concentration of ammonium sulphate as the sole nitrogen source. Growth curve of the 2344c strain in the two conditions of ammonium availability is also shown. **(B)** Expression of *TPO1* during growth of *S. cerevisiae* BY4741 (■,□) or of *gcn4*Δ (●,○) in MM4 growth medium. Cell samples were harvested during exponential growth (sample 1) and when cells approached stationary phase at an OD_{600nm} of 1.0 (sample 2). At this point the cultures were split in two and fresh leucine was added to one of the cultures (closed symbols) while the other remained in the leucine-exhausted growth medium (open symbols, dashed curve). After one hour of incubation in the leucine-exhausted (sample 3) or in the leucine-replete growth medium (sample 4) cells were harvested and *TPO1* transcription levels were assessed. The results presented are means of at least three independent experiments and error bars represent standard deviation.

4.5 DISCUSSION

In this work the *S. cerevisiae* plasma membrane drug:H^+ antiporter Tpo1 was implicated for the first time in yeast tolerance to benzoic acid. Tpo1 was shown to be predominantly required in the period of adaptation to sudden benzoic acid, enabling the population to adapt faster to the presence of this food preservative. These results are actually consistent with previous observations, showing that Tpo1 (Alenquer et al., 2006; Teixeira and Sá-Correia, 2002) and many other drug transporters are key players in the early response to sudden stress (Sá-Correia et al., 2009). Upon exposure to weak acids, such as acetic acid or 2-4-dichlorophenoxyacetic acid, it was demonstrated that together with the transient overexpression of drug transporters during the stress-induced lag-phase, cells appear to activate additional mechanisms involved in cell wall and plasma membrane remodelling that decrease the permeability of the cell envelope (Mira et al., 2010d, 2010b; Simões et al., 2003; Teixeira et al., 2006, 2007; Viegas et al., 2005) avoiding a futile and energetically expensive cycle, in which the acid diffuses back into the cells, counteracting the active expulsion of its counterion.

During the adaptive response to benzoic acid stress, *TPO1* transcription was found to be strongly up-regulated (up to 30-fold), this activation being partially dependent on the Gcn4 and Stp1 transcription factors. Elimination of Gcn4 and/or Stp1 led to a dramatic increase in yeast susceptibility to benzoic acid, even higher than the one obtained upon deletion of *TPO1*, suggesting that there are other determinants of yeast tolerance to benzoic acid among Gcn4 and Stp1 target genes. The demonstration that none of the stress-responsive transcription factors that have a binding site in the *TPO1* promoter (Pdr1, Pdr3, Yrr1, Msn2, Msn4, Yap1, Yap2, Yap3, Yap4 and Yap5) are involved in the regulation of *TPO1* transcriptional activation under benzoic acid stress was surprising. In particular, it was unexpected to observe the lack of effect of Pdr1 in this activation, since this transcription factor is known to mediate all the previously described drug/xenobiotic-induced up-regulations of *TPO1* (Alenquer et al., 2006; Do Valle Matta et al., 2001; Lucau-Danila et al., 2005; Teixeira and Sá-Correia, 2002). Since Pdr1 function appears to be activated upon direct binding of xenobiotics/drugs (Thakur et al., 2008), it is possible that this lack of Pdr1 in the control of the benzoic acid response might result from the inability of benzoic acid (or benzoate) to bind to this protein. Interestingly, while the transcriptional association between Stp1 and *TPO1* is described here for the first time, Gcn4 had already been seen to play a role in the up-regulation of *TPO1*, in cells exposed to stress induced by 3-aminotriazole, a drug that mimics the effect of histidine limitation (Moxley et al., 2009). It is also interesting to observe that both Gcn4 and Stp1 have been documented to regulate the expression of several other genes

involved in polyamine biosynthesis and transport (figure 4-7). The regulatory network schematized in figure 4-7 rejoins the information available in the YEASTRACT database (Teixeira, 2006; Teixeira et al., 2014) with the new data obtained in this study, and shows that Stp1 and/or Gcn4 are involved in the regulation of both uptake, biosynthesis and excretion of polyamines. Indeed, they are involved in the regulation of *TPO1* [this work and (Moxley et al., 2009)] and, according to the data gathered in the YEASTRACT database, of its orthologues *TPO2*, *TPO3* and *TPO4*, required for polyamine excretion (Albertsen et al., 2003; Tomitori et al., 2001; Uemura et al., 2005), as well as of *AGP2*, *DUR3* and *SAM1*, that mediate polyamine uptake (Aouida et al., 2013; Uemura et al., 2007). Despite the fact that benzoic acid-induced transcriptional activation of *TPO2* and *TPO3* is fully dependent of Haa1 (Fernandes et al., 2005), this transcription factor was found to have no effect in the regulation of *TPO1* transcription under the same conditions (our unpublished results).

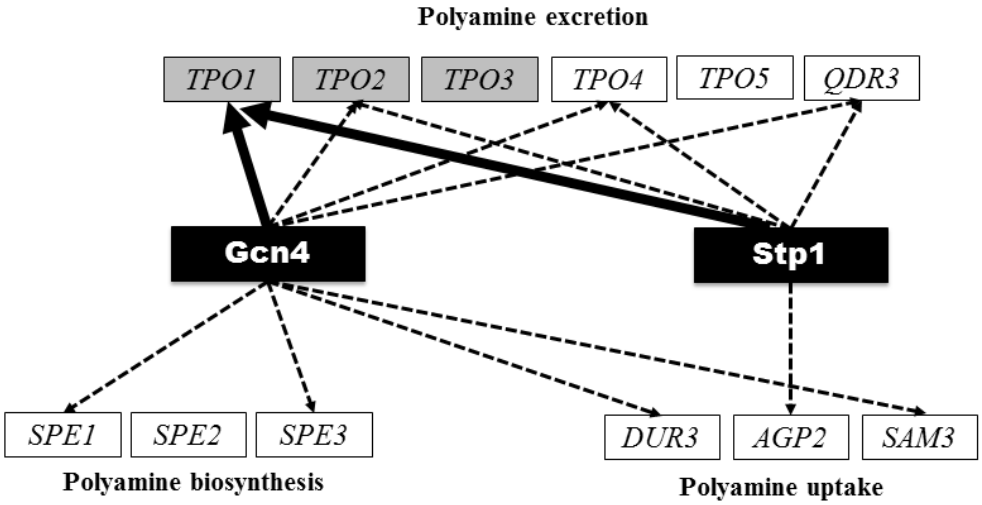

Figure 4-7. Effect of Gcn4 and Stp1 in the regulation of genes involved in synthesis, uptake and excretion of polyamines.
Documented regulatory associations between the genes involved in synthesis, uptake and excretion of polyamines and Gcn4 and Stp1 are shown according to the information available in the YEASTRACT database (dashed lines), plus the data described herein on the effect of Stp1 and Gcn4 over *TPO1* expression (full lines). Genes up-regulated by benzoic acid stress are highlighted in grey boxes while down-regulated genes are shown in white boxes (Abbott et al. 2007).

The regulatory association between *TPO1* and Stp1 and Gcn4 is consistent with the idea that the transcriptional control of *TPO1*, as well as other MFS-MDR transporters-encoding genes, under stress may involve more than just transcription factors directly related to the response to drugs/xenobiotics/stress conditions (dos Santos et al., 2014). This notion is further reinforced by previous observations showing that the regulators of methionine and leucine biosynthesis, Leu3 and Met32, and the activator of oleate

catabolism, Pip2, are other non-MDR transcription factors described as regulators of *TPO1* expression (Carrillo et al., 2012; Smith et al., 2007; Tang et al., 2006). Interestingly, Gcn4 and Stp1 have no apparent role in the transcriptional activation of *TPO1* gene induced by the lipophilic weak acid herbicide 2,4-D (results not shown) rendering clear that the regulatory network controlling *TPO1* transcription is largely dependent on the environmental stressor under study. Further studies are required to understand what are the physiological cues that determine which players of the *TPO1* regulatory network are activated in each condition.

Given the essential role played by Gcn4 and Stp1 in sensing and signalling internal amino acid homeostasis in yeast (Hinnebusch, 2005; Ljungdahl and Daignan-Fornier, 2012), the indications gathered prompted us to examine the effect of benzoic acid and *TPO1* transcription in the internal amino acid pool during early response to sudden exposure to this stress. However, *TPO1* deletion was found to have no detectable effect in the intracellular concentration of any of the measured amino acids in benzoic acid supplemented media. These results suggest that, despite being a target of Gcn4/Stp1 regulatory control, Tpo1 is apparently not involved in the control of amino acid homeostasis in benzoic acid stressed cells, a biological role that was proposed for its close homologues Aqr1 and Qdr2, also transcriptionally regulated by Gcn4 (Vargas et al., 2007; Velasco et al., 2004). Nonetheless, benzoic acid challenge was indeed seen to alter the intracellular amino acid pool in *S. cerevisiae* cells. In particular, the significant reduction in the internal concentration of glutamine, arginine and lysine (the latter reached undetectable levels) registered in benzoic acid-challenged cells could trigger activation of Gcn4 as this transcription factor responds when the internal concentration of any amino acid becomes limiting (Hinnebusch, 2005). Intracellular acidification, a known deleterious effect of benzoic acid stress (Piper et al. 2001), was recently shown to reduce the activity of aminoacyl tRNA synthetases thereby leading to an accumulation of uncharged tRNAs (Hueso et al., 2012), a signal that is also known to activate Gcn4 (Hinnebusch, 2005). The pool of the amino acids leucine and methionine that corresponds to the yeast strain auxotrophies did not suffer a reduction on the intracellular pools in benzoic acid stressed cells, the levels of this amino acids were even found to increase. The increase in methionine and leucine intracellular levels in the same yeast cells challenged with propionic acid stress was previously registered by metabolomic analysis (Lourenço et al., 2010). The activation of the transcription factor Stp1 is dependent on the external sensing of amino acids by the receptor membrane protein Ssy1. It was proposed that when the concentration of an inducing amino acid in the exterior is higher than the concentration found in the cytosol, Ssy1 conformation is

altered and Stp1 becomes active (Ljungdahl and Daignan-Fornier, 2012). The activation of Stp1 registered under the experimental conditions used in our study was unexpected since a saturating concentration of ammonium was present in the growth medium, this being a condition that represses all pathways required for utilization of amino acids, including the Stp1-pathway (Ljungdahl and Daignan-Fornier, 2012) . It is possible that some of the amino acids whose internal concentration is reduced upon benzoic acid challenge could be accumulating in the exterior, either as the result of excretion and/or leakage, thereby resulting in the conformational change of Ssy1 and consequently in the activation of Stp1 as hypothesized before (Gaber et al., 2003; Ljungdahl and Daignan-Fornier, 2012). Sudden exposure to benzoic acid stress was also found to lead to a reduction in the internal concentrations of spermidine, spermine and putrescine, this response being independent of the expression of the *TPO1* gene. This result appears to suggest that Tpo1 does not mediate the export of polyamines in response to benzoic acid stress. Altogether the results of our study confirm that the transcriptional regulatory network that governs the expression of *TPO1* is complex and involves regulators that are not themselves directly implicated in MDR/MXR. Despite the strong Gcn4- and Stp1-dependent up-regulation of the *TPO1* gene registered under benzoic acid stress, the expression of Tpo1 did not have a significant effect in the internal amino acid pool in benzoic acid-stressed cells. Since the modulation of the internal concentration of polyamines was found to have a very pleiotropic effect in yeast cells (Chattopadhyay et al., 2008, 2009; Eisenberg et al., 2009), it is likely that the reduction in the internal concentration of polyamines could contribute in various manners to improve cell tolerance to benzoic acid, but this does not seem to be related with the role of the Tpo1 transporter in alleviating benzoic acid stress effects.

In conclusion, the results obtained in this study are expected to advance current understanding of the regulation and function of drug/xenobiotic efflux pumps in the MDR phenomenon in the yeast model and in less accessible organisms. Furthermore, since benzoic acid is largely used as a food preservative, it is expected that the identification of molecular mechanisms of tolerance to this weak acid in *S. cerevisiae* can be used to guide the design of more efficient preservation strategies, also at the level of medium composition in the food industry (Mira et al., 2010d). This is particularly expected considering that multiple robust homologues of Tpo1 are found in the genome sequence of several spoilage yeasts and fungi tolerant to benzoic acid, including strains of the food spoilage *Zygosaccharomyces bailii* species (Mira et al., 2014). Also, production of industrially relevant aromatic compounds using microorganisms is gathering increased research interest. The production of benzoic acid through the assimilation of many

carbon sources via a plant-like β-oxidation pathway was recently reported (Noda et al., 2012). Therefore, unveiling the mechanisms by which microbial strains are able to tolerate increasingly higher concentrations of this stress agent is also of extreme value to improve industrial robustness and reach enhanced production yield of benzoic acid-derived compounds.

5 General Discussion

The ability that a microorganism has to sense and respond to the challenging conditions in its ever-changing environment is essential for its survival. In the case of industrial microorganisms, their ability to cope with the multistresses occurring during the bioprocess, and their ability to thrive, can determine the success and profitability of an industrial process (Liu, 2012). Weak acids are intensively used as preservatives in the food industry as they are naturally-occurring compounds that can inhibit growth of spoilage microorganisms in acidic food products (Booth et al., 1989; Booth and Stratford, 2003). Also, some weak acids are important inhibitors in alcoholic fermentation-based processes, either because their production together with the increasing accumulation of ethanol are toxic to the microorganism, or because they can already be present in the substrates used for example for second-generation bioethanol production, thus inhibiting the fermentation of the sugars into the final product, ethanol (Palma et al., 2018). This thesis work contributed to deepen the knowledge regarding the mechanisms of yeast tolerance to stress induced by the weak acids acetic and benzoic acids by exploiting the functional role and regulation of the multidrug/multixenobiotic resistance (MDR/MXR) transporters Pdr18 (ATP-binding cassette superfamily) and Tpo1 (Major Facilitator Superfamily), along with the interplay with plasma membrane lipid composition and structure.

Yeast plasma membrane is not only the first line of cell defense towards any physical and chemical stress, but also provide the adequate environment for embedded proteins that aim at alleviating weak acid deleterious effects (Kodedová and Sychrová, 2015; Mukhopadhyay et al., 2002; Peetla et al., 2013; Rank et al., 1978; Shahi and Moye-Rowley, 2009). In yeast cell adaptation to acetic acid stress, the remodeling of plasma membrane lipids and proteins by increasing the content of complex sphingolipids has been reported (Lindberg et al., 2013). Although ergosterol biosynthesis pathway genes are known to be determinants of acetic acid resistance in yeast (Mira et al., 2010c), and that higher ergosterol incorporation is described to reduce plasma membrane permeability and contribute to membrane stabilization in response to ethanol (del Castillo Agudo, 1992; Vanegas et al., 2010, 2012; You et al., 2003), no role in weak acid response had been shown before the work herein presented. In fact, the same authors that reported the increase in sphingolipid content in yeast adaptation to weak acids, find a significant decrease in ergosterol content in total cell extracts and no changes in total sterols in acid-adapted cells of *S. cerevisiae* (Lindberg et al., 2013). This thesis work contributes to add a role for ergosterol biosynthesis and incorporation in yeast plasma membrane remodeling in response to acetic acid stress. This finding opens the door for relevant questions, since ergosterol is present in the plasma membrane of yeast in non-

raft and lipid-raft domains, the latter being intrinsically related to more condensed and less permeable domains (Alvarez et al., 2007). It remains to be clarified if increased ergosterol content itself or an increase in lipid raft formation is the key for yeast plasma membrane remodeling in the process of yeast adaptation and tolerance to weak acid-induced stress.

Previous works have showed that different weak acids can induce different levels of toxicity in the cell and different levels of different deleterious effects, therefore triggering different signaling pathways and responses in yeast cells. This is the case of the two weak acids studied in this work, acetic and benzoic acids, as they have different hydrophobicities, and consequently different passive uptake rates into the cell (Sikkema et al., 1995; Ullah et al., 2012). This is consistent with results reported before in model membranes showing that membrane remodeling by sphingolipid increase reduces membrane permeability to acetic acid, but not to benzoic acid (Lindahl et al., 2016). It would be interesting to understand if an increase in ergosterol content reduces the permeability of model membranes to benzoic acid. Also, it can be that the full plasma membrane remodeling process in response to benzoic acid stress requires not only sphingolipid but also ergosterol increased incorporation to become more benzoic acid-impermeable. The dependence of the permeability to benzoic acid on ergosterol content is a likely hypothesis, considering the results in this thesis showing that *S. cerevisiae* cells devoid of *PDR18* are highly susceptible to benzoic acid toxicity, when compared to the parental strain.

The contribution of this work is not only on the impact of plasma membrane composition in weak acid tolerance, but also provides useful information on the role of key elements acting in yeast plasma membrane: the MDR/MXR transporters. The mechanisms behind their action have been a matter of discussion since the disclosure of the first *S. cerevisiae* genome sequence (dos Santos et al., 2014; Prasad and Panwar, 2004; Sherlach and Roepe, 2014). The number, apparent redundancy and conservation among different yeast species, of these transporters has raised questions regarding their true function in the cell, both in the absence and in the presence of drugs/xenobiotics. This work further contributes to support the idea that MDR/MXR transporters can act in alleviation of stress by many mechanisms, rather than the traditional view of simple drug pumping out of the cell. In fact, most of MDR/MXR transporters seem to contribute to decrease intracellular concentration of drug/xenobiotics, but such as the case of the action of Pdr18, some transporters can alter the drug partition by physical or chemical changes in plasma membrane (Sherlach and Roepe, 2014).

Although the exact mechanism by which Tpo1 exerts a protective effect against benzoic acid stress was not clearly found, a role in benzoate extrusion was ruled out. Also, the physiological role of Tpo1 in polyamine export that was found previously to alleviate yeast stress in oxidative stress response (Krüger et al., 2013) was not found to be the mechanism by which this MFS transporter confers yeast with tolerance to benzoic acid. Tpo1 may therefore play in the cell other mechanisms than those previously proposed. Given the fact that benzoic acid is quite hydrophobic and was found to permeate yeast plasma membrane very effectively, and that its main toxicity is associated with the accumulation of the counterion that can lead to plasma membrane damage, a role in lipid homeostasis can not be excluded. In fact, the deletion of Tpo1 was previously found to induce an up-regulation in squalene syntehase (*ERG9*) transcription (Kennedy and Bard, 2001). Therefore, the clear role of Tpo1 requires further investigation.

Snq2 profile in MDR/MXR was also investigated in this thesis work, as it has a described paralogous relation with Pdr18 (Seret et al., 2009).This contributed to extend the knowledge on Snq2 relevance in resistance to chemical stress, but no physiological role in *S. cerevisiae* was yet disclosed. Given its paralogue relation with the ergosterol transporter Pdr18, and the fact that it reduces intracellular accumulation of the steroid estradiol (Mahé et al., 1996a), a role in lipid homeostasis can also be hypothesized (Kuchler et al., 1997; Mahé et al., 1996a). More detailed studies are required to better understand the role of this MDR/MXR transporter in yeast cells and would contribute to get more insights into the understanding of the evolutionary processes acting on the two copies originated in the duplication event described herein that gave rise to Snq2 and Pdr18.

The regulatory network of the MDR/MXR transporters is complex and the fact that these transporters are not only regulated by transcription factors associated to stress response, provides another clue of their relevance even in the absence of any stressful condition. This is the case of the regulatory network found for Tpo1 in benzoic acid stress response, in which benzoic acid deleterious effect in the reduction of polyamine and amino acid intracellular levels triggered the Gcn4 and Stp1 transcription factors, typically associated with nutrient limitation response, leading to the up-regulation of Tpo1. Also, the up-regulation of Pdr18 seems to be coordinated with the up-regulation of genes of the ergosterol biosynthetic pathway, in response to the presence of acetic acid stress. This is not the first described case of co-regulation of metabolic processes and MDR/MXR transporter-encoding genes (Coste et al., 2004; DeRisi et al., 2000; Khakhina et al., 2015; Kihara and Igarashi, 2004) and opens the door to the future understanding of the signaling pathways and regulatory networks behind the control of both Pdr18 and the

ergosterol pathway. It would certainly be an interesting topic of research the study of the cell sensors that perceive the fluctuations in the membrane environment and lead to the activation of the regulatory network behind the *PDR18* and *ERG* genes upregulation, resulting in the compensatory changes observed in this work to alleviate acetic acid-induced stress.

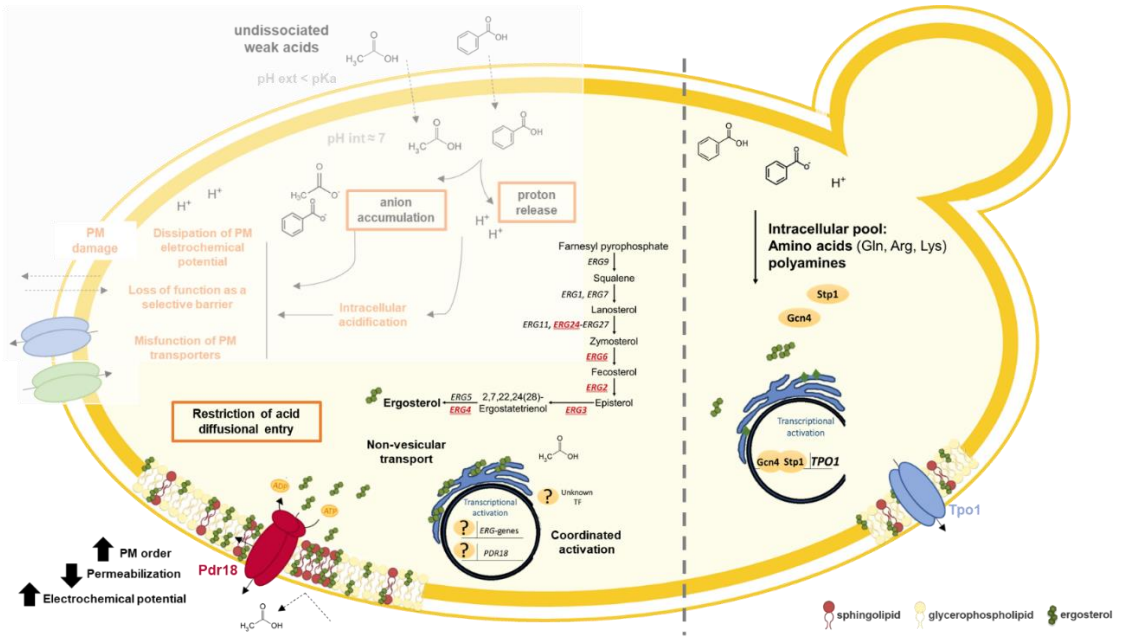

Figure 5-1. Proposed model for the knowledge gathered in this thesis work on the role and regulation of Pdr18 and Tpo1 in yeast tolerance to stress induced by the weak acids acetic and benzoic acid.
Abbreviations: PM plasma membrane, TF transcription factor, Gln glutamine, Arg arginine, Lys lysine.

6 Thesis publications

Peer-reviewed scientific publications directly related to this thesis

Godinho CP, Dias PJ, Ponçot E, Sá-Correia I. The paralogous genes *PDR18* and *SNQ2*, encoding ABC multidrug resistance transporters, derive from a recent duplication event, *PDR18* being specific of the *Saccharomyces* genus (in preparation)

Godinho CP, Prata CS, Pinto SN, Cardoso C, Bandarra NM, Fernandes F, Sá-Correia I. 2018. Pdr18 is involved in yeast response to acetic acid stress counteracting the decrease of plasma membrane ergosterol content and order. *Sci. Rep.* **8**.

Godinho CP, Mira NP, Cabrito TR, Teixeira MC, Alasoo K, Guerreiro JF, Sá-Correia I. 2017. Yeast response and tolerance to benzoic acid involves the Gcn4- and Stp1-regulated multidrug/multixenobiotic resistance transporter Tpo1. *Appl. Microbiol. Biotechnol.* **101**:5005–5018.

Poster presentation in international scientific meetings

Godinho CP, Prata CS, Ponçot E, Mira NP, Pinto SN, Fernandes F, Sá-Correia I. The ABC transporter Pdr18 and ergosterol homeostasis are involved in yeast response and tolerance to acetic acid by counteracting stress-induced decrease of plasma membrane order. 6th Physiology of Yeast and Filamentous Fungi (PYFF), Lisbon, 11-14 July, 2016.

Dias PJ, Godinho CP, Ponçot E, Sá-Correia I. Evolutionary and physiological insights into *Saccharomyces cerevisiae* multidrug/multixenobiotic resistance ABC transporter genes *PDR18* and *SNQ2*. 6th Physiology of Yeast and Filamentous Fungi (PYFF), Lisbon, 11-14 July, 2016.

Oral presentation in national scientific meetings

Godinho CP, Prata CS, Pinto SN, Cardoso C, Bandarra NM, Fernandes F, Sá-Correia I. Expression of the ABC transporter Pdr18 is essential to counteract the negative impact of acetic acid stress on yeast plasma membrane. Microbiotec'17, Porto, 7-9 December, 2017.

Godinho CP, Prata CS, Mira NP, dos Santos S, Palma M, Sá-Correia I. Acetic acid resistance in Saccharomyces cerevisiae: role of the ABC transporter Pdr18, proposed to mediate plasma membrane sterol incorporation. XX Jornadas de Biologia de Leveduras Professor Nicolau van Uden, Lisboa, 11-12 July, 2014.

Poster presentation in national scientific meetings

Godinho CP, Mira NP, Cabrito TR, Teixeira MC, Alasoo K, Guerreiro JF, Sá-Correia I. Yeast response and tolerance to benzoic acid: focus on the expression and regulation of the multidrug/multixenobiotic resistance transporter Tpo1. Microbiotec'15. Évora, 10-12 December, 2015.

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Remy E, Niño-González M, Godinho CP, Cabrito TR, Teixeira MC, Sá-Correia I, Duque P. 2017. Heterologous expression of the yeast Tpo1p or Pdr5p membrane transporters in Arabidopsis confers plant xenobiotic tolerance. *Sci. Rep.* **7**.

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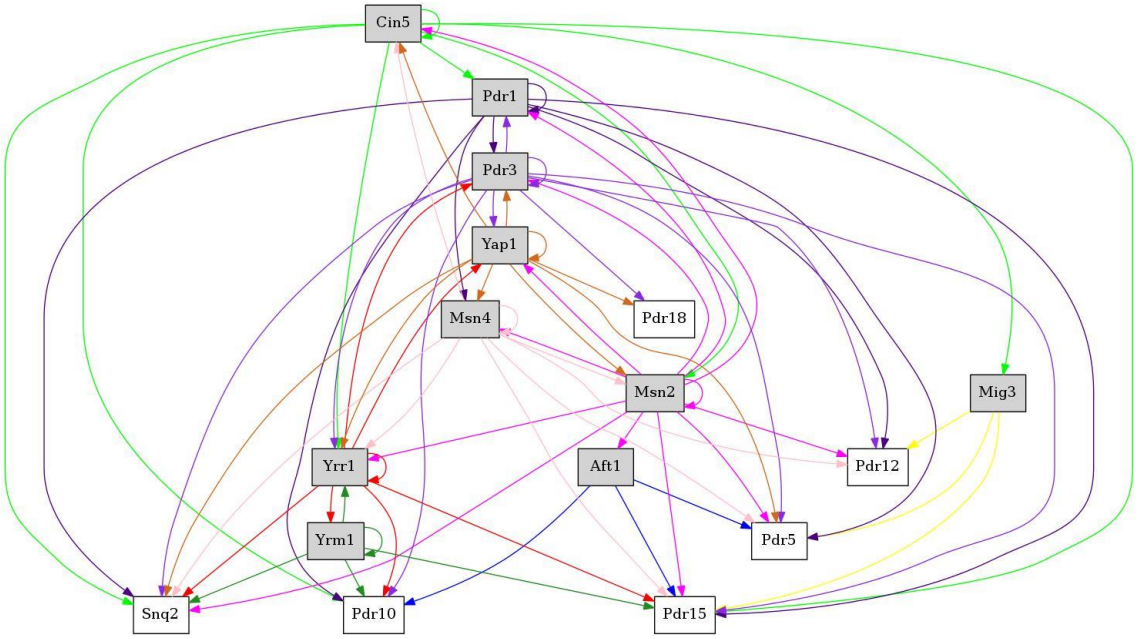
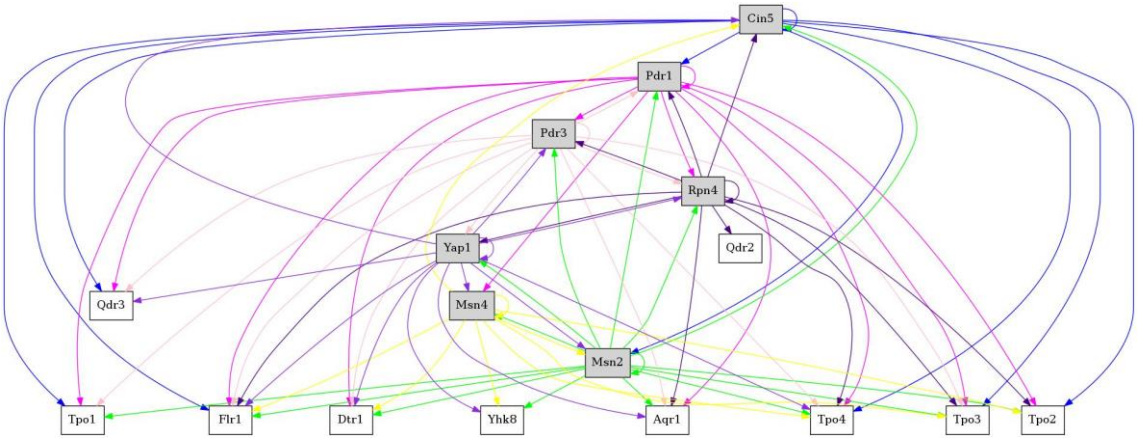
8 Suplementary Material

8.1 Supplementary Figures

Supplementary figure S1-1. Transcriptional regulatory network controlling the expression of *S. cerevisiae* PDR transporters genes under stress, as extracted from YEASTRACT database.

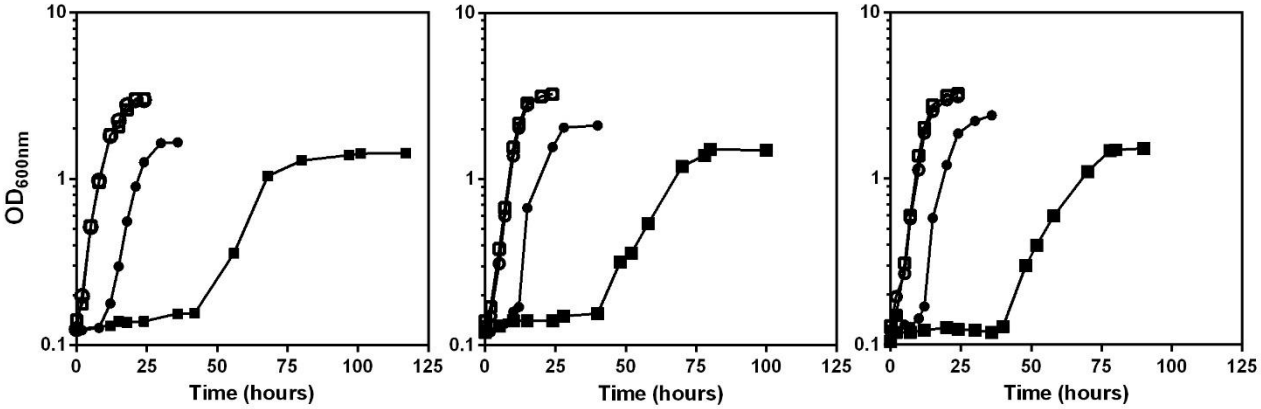


Supplementary figure S1-2. Transcriptional regulatory network controlling the expression of *S. cerevisiae* MFS-MDR/MXR transporters genes from the DHA1 family, under stress, as extracted from YEASTRACT database.

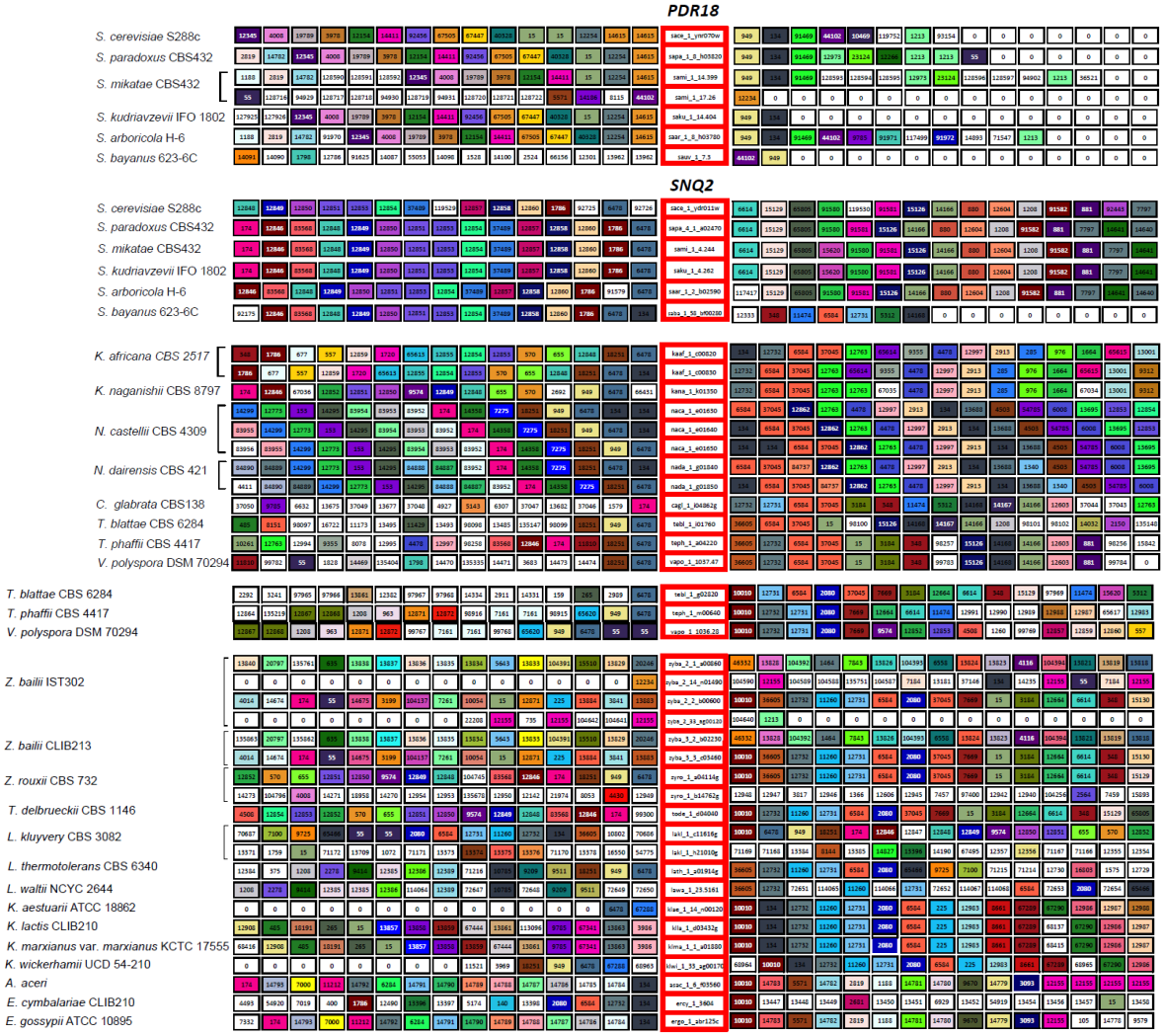


Supplementary figure S2-1. Growth curve of *S. cerevisiae* BY4741 and derived deletion mutant for *PDR18* gene, in the presence or absence of acetic acid.

Biological replicates of the growth curves of the parental (○, ●) and *pdr18*Δ (□, ■) strains in MM4 liquid medium supplemented (●, ■) or not (○, □) with 60 mM acetic acid at pH 4.0 based on culture OD_{600nm}.

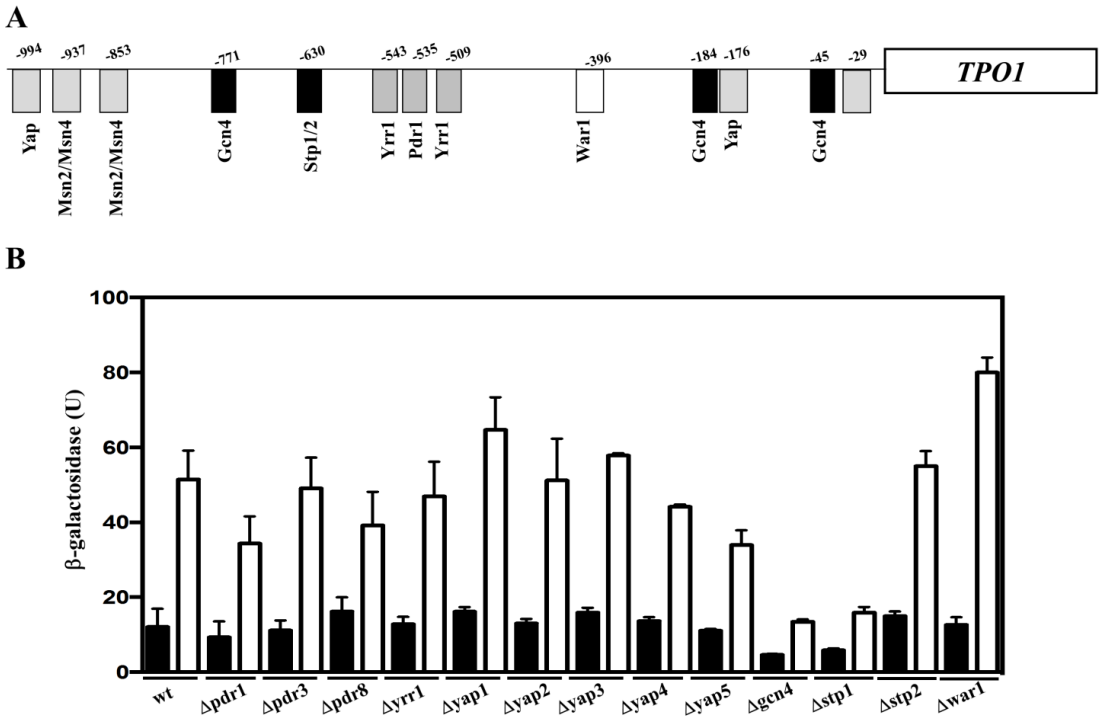


Supplementary figure S3-1. Gene neighborhood of the *ScPDR18* and *ScSNQ2* ortholgs encoded in the Saccharomycetaceae species genomes examined. Central boxes represent *ScPDR18*/ *ScSNQ2* orthologs and the adjacent boxes represent gene neighbors. Homologous neighbours are highlighted in the same colour and identified with the same number. A white box represents genes with no homologous neighbours in the represented chromosome region and white boxes with a zero represent the end of the contig/chromosome.



Supplementary figure S4-1. **Transcription factors involved in activation of Tpo1 under benzoic acid stress conditions.**

(A) Schematic representation of the DNA motifs existing in the *TPO1* promoter region that serve as binding sites for the stress-responsive transcription factors Pdr1, Pdr3, Yrr1, Stp1, Msn2, Msn4, Yap1, Yap2, Yap3, Yap4, Yap5 and Gcn4; **(B)** Expression of *TPO1* gene, assessed based on the levels of β-galactosidase activity produced from the plasmid p*TPO1*::*lacZ* (Alenquer et al. 2006), in wild-type and in mutants deleted for the above referred transcription factors after 12 h of growth in MM4 growth medium (at pH 4) (black bars) or in this same growth medium supplemented with 0.9 mM benzoic acid (white bars). The results presented are means of at least three independent experiments.



8.2 Supplementary Tables

Supplementary table S2-1. Primers used for qRT-PCR analysis.

Target gene	Sequence (5'-3')
ACT1	fw: CTCCACCACTGCTGAAAGAGAA rev: CCAAGGCGACGTAACATAGTTTT
PDR18	fw: TTGGCAAGCCGGATCTGT rev: CCACGCGGATTGGGAAT
ERG2	fw: TTCCTTTGCCCTTGAATTGG rev: AAACCCGAATGGCAACATACA
ERG3	fw: GCTCTGCACAAGCCTCATCA rev: GGAAAGAATGAGATGCGAAAGG
ERG4	fw: CAACTCGGTGTTCCCATGGT rev: AAGGCTCTGTGAATCAGGACAAC
ERG6	fw: GCTGGTATTCAAAGAGGCGATTT rev: GCTGGGCCCCCAACA
ERG24	fw: GAATTGGGATGGGTGAAAGTT ev: TGGAAGATGTGGAAACCCAAA

Supplementary Table S3-1. Saccharomycetaceae yeast strains examined in this work, belonging to (A) *Saccharomyces* genus or (B) non-*Saccharomyces* genera. List of Saccharomycetaceae yeast strains genomes analysed in this study, corresponding source and annotation tool, and number of Snq2/Pdr18 homologs, Snq2 orthologs and Pdr18 orthologs identified. Pre-whole genome duplication (WGD) species are highlighted in grey. SGD Saccharomyces Genome Database (http://www.yeastgenome.org/), Genbank (http://www.ncbi.nlm.nih.gov/genome/browse/), Sanger Center (http://www.sanger.ac.uk/research/projects/genomeinformatics/sgrp.html), YGOB Yeast Gene Order Browser (http://ygob.ucd.ie/), YGAP Yeast Genome Annotation Pipeline (http://wolfe.ucd.ie/annotation/), DOE Joint Genome Institute (https://genome.jgi.doe.gov/saccharomycotina/saccharomycotina.info.html)

Supplementary Table S3-1A

Species	Strain	Strain acronym	Snq2 and Pdr18 homologues	Snq2 orthologues	Pdr18 orthologues	Annotation software/ site	Genome source
Saccharomyces cerevisiae	S288c	sace_1	2	1	1	SGD	SGD
	AWRI796	sace_2	2	1	1	YGAP	SGD
	cen.pk113-7d	sace_3	1	1	nd	YGAP	SGD
	FostersB	sace_4	2	1	1	YGAP	SGD
	FostersO	sace_5	2	1	1	YGAP	Genbank
	JAY291	sace_6	2	1	1	YGAP	Genbank
	BC187	sace_7	2	1	1	YGAP	Sanger centre
	Lalvin QA23	sace_8	2	1	1	YGAP	Genbank
	M3707	sace_9	2	1	1	YGAP	DOE JGI
	M3836	sace_10	2	1	1	YGAP	DOE JGI
	M3837	sace_11	2	1	1	YGAP	DOE JGI
	M3838	sace_12	2	1	1	YGAP	DOE JGI
	M3839	sace_13	2	1	1	YGAP	DOE JGI
	RM11-1a	sace_14	1	0	1	YGAP	Broad Institute
	273614N	sace_15	2	1	1	YGAP	Sanger centre
	322134S	sace_16	2	1	1	YGAP	Sanger centre
	378604X	sace_17	2	1	1	YGAP	Sanger centre
	DBVPG1106	sace_18	2	1	1	YGAP	Sanger centre
	DBVPG1373	sace_19	2	1	1	YGAP	Sanger centre
	DBVPG1788	sace_20	2	1	1	YGAP	Sanger centre
	DBVPG1853	sace_21	2	1	1	YGAP	Sanger centre
	DBVPG6040	sace_22	2	1	1	YGAP	Sanger centre
	DBVPG6044	sace_23	2	1	1	YGAP	Sanger centre
	DBVPG6765	sace_24	2	1	1	YGAP	Sanger centre
	K11	sace_25	2	1	1	YGAP	Sanger centre
	L 1374	sace_26	2	1	1	YGAP	Sanger centre
	L 1528	sace_27	2	1	1	YGAP	Sanger centre
	NCYC110	sace_28	2	1	1	YGAP	Sanger centre
	NCYC361	sace_29	2	1	1	YGAP	Sanger centre
	SK1	sace_30	2	1	1	YGAP	Sanger centre
	UWOPS03 461 4	sace_31	2	1	1	YGAP	Sanger centre
	UWOPS05 217 3	sace_32	2	1	1	YGAP	Sanger centre
	UWOPS05 227 2	sace_33	2	1	1	YGAP	Sanger centre
	UWOPS83 787 3	sace_34	2	1	1	YGAP	Sanger centre
	UWOPS87 2421	sace_35	2	1	1	YGAP	Sanger centre
	Y9	sace_36	2	1	1	YGAP	Sanger centre
	Y12	sace_37	2	1	1	YGAP	Sanger centre
	Y55	sace_38	2	1	1	YGAP	Sanger centre
	YIIc17 E5	sace_39	2	1	1	YGAP	Sanger centre
	YJM975	sace_40	2	1	1	YGAP	Sanger centre

Supplementary Table 1A (continued)

Species	Strain	Strain acronym	Snq2 and Pdr18 homologues	Snq2 orthologues	Pdr18 orthologues	Annotation software/ site	Genome source
	YJM978	sace_41	2	1	1	YGAP	Sanger centre
	YJM981	sace_42	2	1	1	YGAP	Sanger centre
	YPS128	sace_43	2	1	1	YGAP	Sanger centre
	YPS606	sace_44	2	1	1	YGAP	Sanger centre
	YS2	sace_45	2	1	1	YGAP	Sanger centre
	YS4	sace_46	2	1	1	YGAP	Sanger centre
	YS9	sace_47	2	1	1	YGAP	Sanger centre
	Sigma1278b	sace_48	1	1	0	YGAP	SGD
	vin13	sace_49	2	1	1	YGAP	SGD
	VL3	sace_50	1	1	0	YGAP	SGD
Saccharomyces cerevisiae	w303	sace_51	1	1	0	YGAP	SGD
	YJM789	sace_52	2	1	1	YGAP	Genbank
	EDRL	sace_53	2	1	1	YGAP	DOE JGI
	BY4741	sace_54	2	1	1	YGAP	SGD
	BY4742	sace_55	2	1	1	YGAP	SGD
	EC1118	sace_56	2	1	1	YGAP	Genbank
	Kyokai7	sace_57	2	1	1	YGAP	SGD
	T7	sace_58	2	1	1	YGAP	SGD
	YB210	sace_59	2	1	1	YGAP	DOE JGI
	ZTW1	sace_60	2	1	1	YGAP	Genbank
	reference consensus	sapa_1	2	1	1	YGAP	Sanger centre
	A4	sapa_2	2	1	1	YGAP	Sanger centre
	A12	sapa_3	2	1	1	YGAP	Sanger centre
	CBS432	sapa_4	2	1	1	YGAP	Sanger centre
	CBS5829	sapa_5	2	1	1	YGAP	Sanger centre
	DBVPG4650	sapa_6	2	1	1	YGAP	Sanger centre
	DBVPG6304	sapa_7	2	1	1	YGAP	Sanger centre
	IFO1804	sapa_8	2	1	1	YGAP	Sanger centre
	KPN3828	sapa_9	2	1	1	YGAP	Sanger centre
	KPN3829	sapa_10	2	1	1	YGAP	Sanger centre
	N 17	sapa_11	2	1	1	YGAP	Sanger centre
	N 43	sapa_12	2	1	1	YGAP	Sanger centre
Saccharomyces paradoxus	N 44	sapa_13	2	1	1	YGAP	Sanger centre
	N 45	sapa_14	2	1	1	YGAP	Sanger centre
	Q31 4	sapa_15	2	1	1	YGAP	Sanger centre
	UFRJ50791	sapa_16	2	1	1	YGAP	Sanger centre
	UFRJ50816	sapa_17	2	1	1	YGAP	Sanger centre
	UWOPS91 917 1	sapa_18	2	1	1	YGAP	Sanger centre
	W7	sapa_19	2	1	1	YGAP	Sanger centre
	Y6 5	sapa_20	2	1	1	YGAP	Sanger centre
	Y7	sapa_21	2	1	1	YGAP	Sanger centre
	Y8 5	sapa_22	2	1	1	YGAP	Sanger centre
	YPS138	sapa_23	2	1	1	YGAP	Sanger centre
	Z1	sapa_24	2	1	1	YGAP	Sanger centre
	Z1 1	sapa_25	2	1	1	YGAP	Sanger centre

Supplementary Table 1A (continued)

Species	Strain	Strain acronym	Snq2 and Pdr18 homologues	Snq2 orthologues	Pdr18 orthologues	Annotation software/ site	Genome source
Saccharomyces mikatae	IFO 1815	sami_1	3	1	1	YGOB	YGOB
Saccharomyces kudriavzevii	IFO 1802	saku_1	2	1	1	YGOB	YGOB
Saccharomyces arboricola	H-6	saar_1	2	1	1	YGAP	Genbank
Saccharomyces eubayanus	FM1318	saeu_1	2	1	1	YGAP	Genbank
	CBS12357	saeu_2	2	1	1	YGAP	Genbank
Saccharomyces bayanus (HYBRID)	623-6C	saba_1	1	1	0	YGAP	SGD
	MCYC 623	saba_2	0	0	0	YGAP	SGD
Saccharomyces uvarum	CBS 7001	sauv_1	1	0	1	YGOB	YGOB

Supplementary Table 1B

Species	Strain	Strain acronym	Snq2 orthologues	Annotation software/site	Genome source
Kazachstania africana	CBS 2517	kaaf_1	2	YGOB	YGOB
Kazachstania naganishii	CBS 8797	kana_1	1	YGOB	YGOB
Naumovozyma castellii	CBS 4309	naca_1	3	YGOB	YGOB
	NRRL Y-12630	naca_2	1	YGAP	SGD
Naumovozyma dairenensis	CBS 421	nada_1	2	YGOB	YGOB
Candida glabrata	CBS138	cagl_1	1	YGOB	YGOB
	CCTCC M202019	cagl_2	1	YGAP	Genbank
Tetrapisispora blattae	CBS 6284	tebl_1	2	YGOB	YGOB
Tetrapisispora phaffii	CBS 4417	teph_1	2	YGOB	YGOB
Vanderwaltozyma polyspora	DSM 70294	vapo_1	2	YGOB	YGOB
Zygosaccharomyces bailii	IST302	zyba_2	4	MIPS	EBI
	CLIB 213	zyba_3	2	YGAP	Genbank
Zygosaccharomyces rouxii	CBS 732	zyro_1	2	YGOB	YGOB
Torulaspora delbrueckii	CBS 1146	tode_1	1	YGOB	YGOB
Lachancea kluyvery	CBS 3082	lakl_1	2	YGOB	YGOB
Lachancea thermotolerans	CBS 6340	lath_1	1	YGOB	YGOB
Lachancea waltii	NCYC 2644	lawa_1	1	YGOB	YGOB
Kluyveromyces aestuarii	ATCC 18862	klae_1	1	YGAP	Genbank
Kluyveromyces lactis	CLIB210	klla_1	1	YGOB	YGOB
Kluyveromyces marxianus var. marxianus	KCTC 17555	klma_1	1	YGAP	Genbank
Kluyveromyces wickerhamii	UCD 54-210	klwi_1	1	YGAP	Genbank
Ashbya aceri	-	asac_1	1	YGAP	Genbank
Eremothecium cymbalariae	DBVPG 7215	ercy_1	1	YGOB	YGOB
Eremothecium gossypii	ATCC 10895	ergo_1	1	YGOB	YGOB