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Minireview

Yeast ABC transporters – A tale of sex, stress, drugs and aging

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Abstract Yeast ATP-binding cassette (ABC) proteins are implicated in many biological phenomena, often acting at crossroads of vital cellular processes. Their functions encompass peptide pheromone secretion, regulation of mitochondrial function, vacuolar detoxification, as well as pleiotropic drug resistance and stress adaptation. Because yeast harbors several homologues of mammalian ABC proteins with medical importance, understanding their molecular mechanisms, substrate interaction and three-dimensional structure of yeast ABC proteins might help identifying new approaches aimed at combating drug resistance or other ABC-mediated diseases. This review provides a comprehensive discussion on the functions of the ABC protein family in the yeast *Saccharomyces cerevisiae*.

Keywords: ABC transporters; Drug resistance; Detoxification; Aging; Transcription factor

1. Introduction

ATP-binding cassette (ABC) proteins constitute one of the largest protein superfamilies, with more than 3000 members operating from bacteria to man [1]. Quite remarkably, most members of the ABC protein family share a similar molecular architecture and domain organization. Nevertheless, ABC proteins fulfill a stunning variety of functions, ranging from ATP-driven transmembrane transport of great many different molecules, to the regulation of important cellular processes (for recent comprehensive reviews see [1,2]). The genome of the baker's yeast *Saccharomyces cerevisiae* harbors 30 distinct genes encoding ABC proteins, several of which carry out membrane translocation of hundreds of structurally and functionally unrelated xenobiotics, mediating cellular detoxification or conferring pleiotropic drug resistance (PDR). These are generally referred to as ABC drug efflux pumps [1].

PDR in yeast is similar to multidrug resistance (MDR) phenomena in tumor cells [2], parasites, fungal pathogens or even in bacteria [1]. However, ABC proteins not only function as simple membrane transporters, they are also implicated in maintenance of mitochondrial function, maturation of cytosolic Fe/S proteins, pheromone secretion, peroxisome biogenesis, stress response, as well as lipid bilayer homeostasis and lipid uptake [1].

The domain organization of ABC proteins is defined by the presence of at least one nucleotide-binding domain (NBD). NBDs carry a highly conserved ATP-binding motif, as well as signature motifs that are diagnostic hallmarks for ABC proteins. The NBDs are required to fuel membrane transport or other functions by hydrolysis of ATP. In addition, membrane-bound ABC proteins usually contain variable numbers of predicted transmembrane-spanning segments (TMS) that somehow determine distinct substrate specificities of individual ABC proteins. TMSs are certainly instrumental for the architecture of substrate translocation pores, but their structure remains elusive. This review shall discuss yeast ABC proteins that have been studied beyond simple sequence identification, emphasizing the molecular structure–function properties of those operating in *S. cerevisiae*.

2. The molecular architecture of yeast ABC proteins

ABC proteins in *S. cerevisiae* were classified into five distinct classes, represented by the PDR, MRP/cystic fibrosis transmembrane conductance regulator (CFTR), MDR, ALDp, and the YEF3/RLI subfamilies [3]. The NBD domains cover approximately 250 residues, containing five conserved amino acid motifs. The most conserved features found in any given NBD, are the C-loop or ABC signature motif (LSGGQ), as well as the Walker A and B motifs, which are also present in all ATP-binding proteins. Moreover, two less conserved regions contain diagnostic hallmark features. The so-called center motif is located between Walker A and B, and a second sequence lies downstream of Walker B [1].

In contrast to the high conservation of NBDs, there is considerable variation in the appearance and arrangements of TMSs between different ABC subfamilies. While the PDR, MRP/CFTR, MDR and ALDp family members contain at least six predicted TMSs, YEF3/RLI proteins lack any obvious TMS (Fig. 1). Full-size ABC proteins have a tandemly duplicated organization, with usually six predicted membrane helices in each half, arranged either in a forward $(TMS_6\text{-}NBD)_2$

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Abbreviations: ABC, ATP-binding cassette; PDR, pleiotropic drug resistance; MDR, multidrug resistance; NBD, nucleotide-binding domain; TMS, transmembrane-spanning segment; ER, endoplasmatic reticulum; MRP, multidrug resistance-related protein; CFTR, cystic fibrosis transmembrane conductance regulator; NTE, N-terminal extension; LCFA, long chain fatty acid; PDREs, pleiotropic drug resistance elements

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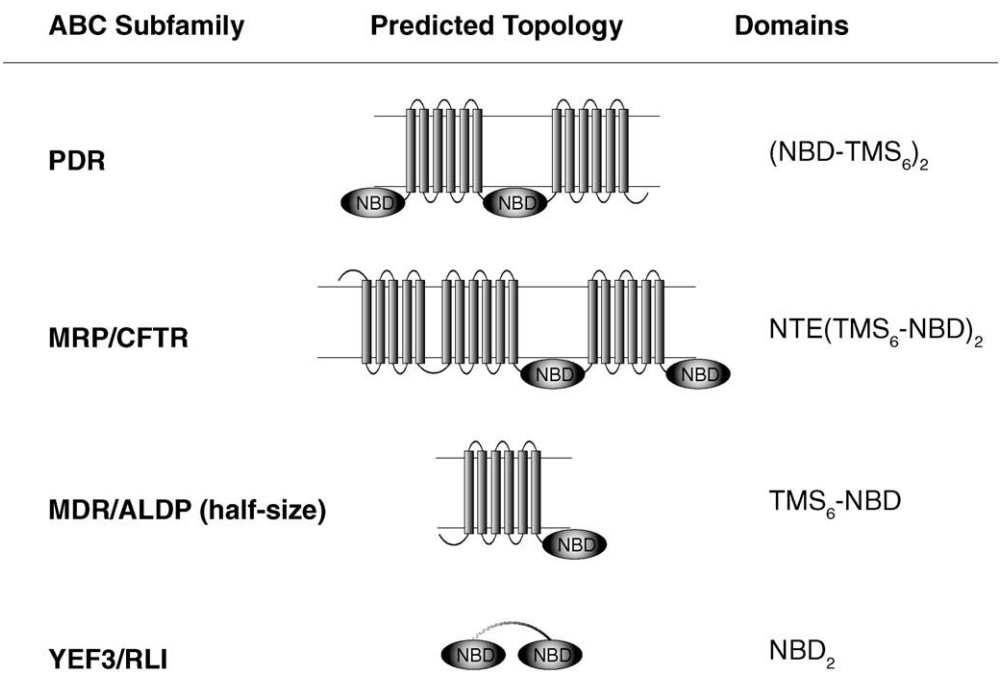

Fig. 1. Predicted topology and domain organization of ABC protein subfamilies. The cartoon depicts the predicted membrane topology and domain organization of all subfamilies encoding yeast ABC proteins (see text for details). NBD, nucleotide-binding domain; NTE, N-terminal extension; TMS, transmembrane segment.

Table 1
ABC proteins in the yeast *S. cerevisiae*

ABC proteins	Family	Length	Topology	Function	Substrates	Reference
Plasma membrane						
Pdr5p	PDR	1511	$(NBD\text{-}TMS_6)_2$	PDR, lipid transport	Cycloheximide, azoles, mycotoxins	[9,57]
Snq2p	PDR	1501	$(NBD\text{-}TMS_6)_2$	PDR	Drugs, mutagens	[10,60]
Pdr12p	PDR	1511	$(NBD\text{-}TMS_6)_2$	Weak acid stress response	Weak organic acids	[17]
Pdr15p	PDR	1529	$(NBD\text{-}TMS_6)_2$	Gerneral stress response	Drugs, herbicides, 2,4-DCP	[18,61]
Aus1p	PDR	1394	$(NBD\text{-}TMS_6)_2$	Sterol uptake	Sterols	[19]
Pdr11p	PDR	1411	$(NBD\text{-}TMS_6)_2$	Sterol uptake	Sterols	[19]
Yor1p	MRP/CFTR	1477	$NTE(TMS_6\text{-}NBD)_2$	PDR, lipid transport	Oligomycin, phospholipids	[21]
Ste6p	MDR	1290	$(TMS_6\text{-}NBD)_2$	Mating factor transport	**a**-factor pheromone	[23,24]
Vacuole						
Ycf1p	MRP/CFTR	1515	$NTE(TMS_6\text{-}R\text{-}NBD)_2$	Cellular detoxification	GS-conjugates, heavy metals	[29]
Bpt1p	MRP/CFTR	1559	$NTE(TMS_6\text{-}NBD)_2$	Cellular detoxification	Unconj. bilirubin, cadmium, arsenate	[37–39]
Ybt1p	MRP/CFTR	1661	$NTE(TMS_6\text{-}NBD)_2$	Unknown	Bile acids, taurocholate	[40]
Mitochondria						
Atm1p	MDR	694	$TMS_6\text{-}NBD$	Fe(S)-translocation	Fe(S)-proteins	[41,42]
Mdl1p	MDR	696	$TMS_6\text{-}NBD$	Oxidative stress response	Peptides	[46,47]
Mdl2p	MDR	820	$TMS_6\text{-}NBD$	Unknown	Unknown	[1]
Peroxisomes						
Pxa1p	ALDp	870	$TMS_6\text{-}NBD$	Fatty acid transport	LCFA	[50,54]
Pxa2p	ALDp	853	$TMS_6\text{-}NBD$	Fatty acid transport	LCFA	[50,54]

Abbreviations. NBD, nucleotide binding domain; TMS, transmembrane spanning segment; NTE, N-terminal extension; PDR, pleiotropic drug resistance; PL, phospholipids; GS, glutathione S; LCFA, long chain fatty acid.

or reverse $(NBD\text{-}TMS_6)_2$ configuration (Table 1). Yeast MRP/CFTR proteins carry an additional TMS at the N-terminus, known as N-terminal extension (NTE).

A catalytic cycle of yeast ABC proteins as originally proposed for mammalian P-glycoprotein [4] has not been experimentally verified. Indeed, the transport mechanisms of yeast ABC transporters or their molecular mode of action has remained obscure.

3. Functions of ABC proteins in *S. cerevisiae*

Yeast cells can quickly counteract toxic environmental challenges through efficient detoxification systems such as the PDR machinery. PDR originates from overexpression of plasma membrane pumps as well as vacuolar transporters (Table 1). It is important to recognize that PDR not only causes hypertolerance to many unrelated exogenous drugs or xenobiotics,

it also protects cells from unwanted side effects of endogenous toxic metabolites. This typically involves members of the major facilitator superfamily (MFS) [5,6], as well as several ABC pumps homing in cellular membrane compartments (Fig. 2). Furthermore, many ABC transporters are tightly regulated by transcription factors within the so-called PDR network (Fig. 3) that modulates levels of numerous membrane transporters under physiological as well as adverse conditions [7].

4. Cellular distribution of ABC transporters

4.1. Plasma membrane ABC transporters – the first defense line

Except for Adp1p, all PDR members are full-size ABC proteins sharing a duplicated reverse $(NBD\text{-}TMS_6)_2$ configuration (Fig. 1 and Table 1). Because certain PDR-transporters mediate translocation of anticancer drugs, phospholipids, peptides, steroids and herbicides, they can be regarded as the first line of defense in *S. cerevisiae* [8].

The best characterized plasma membrane members of the PDR-subfamily are the related ABC transporters Pdr5p [9] and Snq2p [10]. Overexpression of Pdr5p and Snq2p leads to PDR, since they extrude hundreds of structurally and functionally unrelated xenobiotics across the plasma membrane [10,11]. Furthermore, Pdr5p levels are high in the logarithmic growth phase, while levels decrease significantly when cells exit exponential growth. Thus, Pdr5p may operate in detoxification mainly during exponential growth [12], although endogenous substrates remain undisclosed.

A three-dimensional reconstruction of Pdr5p revealed a dimeric organisation, in which each monomer protruded from

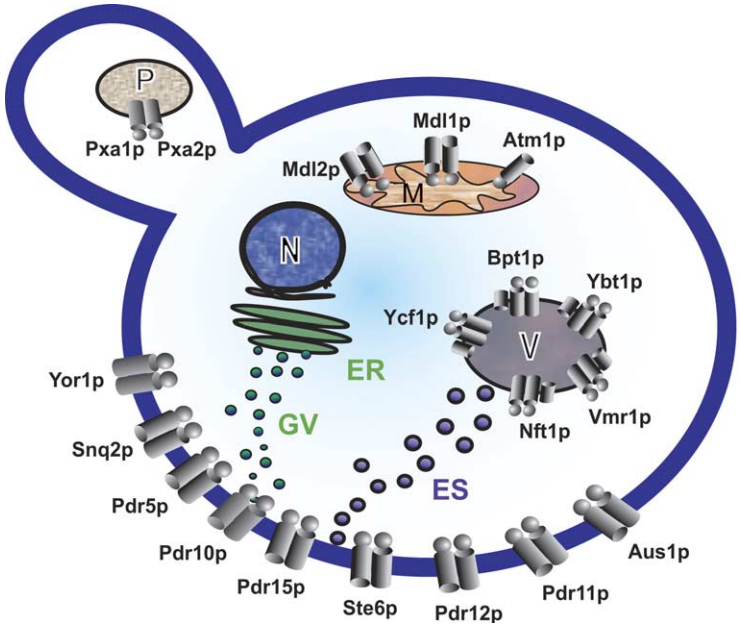

Fig. 2. Membrane localization of yeast ABC proteins. The cartoon depicts the subcellular localization of prominent membrane ABC transporters at the cell surface, in the vacuole, as well as in mitochondria and peroxisomes. Only ABC transporters whose functions have been studied beyond sequencing are depicted (see text for details). N, nucleus; V, vacuole; ER, endoplasmic reticulum, GV, Golgi vesicles; ES, endosomes. M, mitochondrion, P, peroxisome.

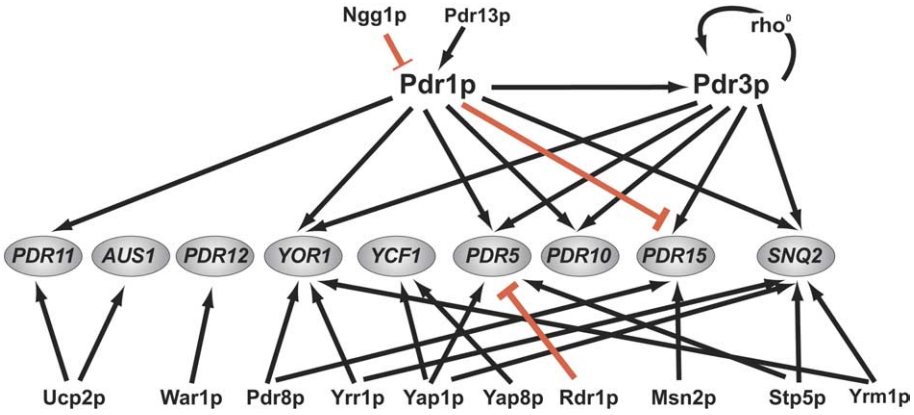

Fig. 3. The PDR network in yeast. Genes in the centerline represent target genes of transcriptional regulators depicted above and below. Note that the cartoon only lists genes of the ABC family. The yeast PDR network also contains non-ABC genes whose function has not established in many cases (see text for details). Red lines indicate a negative regulatory impact, while black lines ending with an arrow indicate positive regulation.

the membrane, ending in cytoplasmic NBDs, which are in close proximity and asymmetrically organized [13]. This is contrasting earlier genetic data suggesting that Pdr5p is active as a monomer [14]. Notably, the shape and structure of Pdr5p TMS10 and its neighbouring extracellular loop 6, perhaps through interaction with other TMSs, determines both specificity and susceptibility to inhibitors [15], as well as proper transporter folding [16].

The domain structure of Pdr5p appears established, but this is not true for other PDR family members, including Pdr12p, Pdr15p and Pdr10p. Notably, Pdr12p does not transport hydrophobic drugs, but confers resistance to weak organic acids. Pdr12p, the closest homologue of Snq2p, mediates energy-dependent extrusion of water-soluble carboxylate anions, such as those used for food preservation, including benzoate, sorbate and propionate [17].

The full-size ABC transporter Pdr15p is another plasma membrane protein whose function is not well understood, although it confers resistance to chloramphenicol and polyoxyethylene-9-laurylether [18], as well as herbicides (Mamnun et al., in preparation). Finally, two additional plasma membrane PDR-transporters, Aus1p and Pdr11p, may be involved in sterol uptake when endogenous sterol biosynthesis is impaired, as for example during anaerobic growth [19]. *AUS1* is also upregulated in response to azole treatment [20], implying that it could mediate drug efflux. Alternatively, Aus1p may somehow counteract aberrant membrane lipid organization as caused by the absence of ergosterol, a direct consequence of azole treatment.

Although *S. cerevisiae* harbors several MRP/CFTR members, the Yor1p oligomycin transporter appears as the only one in the plasma membrane. The phenylalanine residue at position 670 of NBD1 in Yor1p is critical for function; its deletion results in an unstable Yor1p variant retained in the endoplasmatic reticulum (ER) and unable to confer oligomycin resistance [21]. Moreover, mutagenesis of N-terminal and C-terminal Yor1p regions suggest that multiple signals drive proper Yor1p trafficking [22]. Finally, the only full-size ABC protein of the MDR-subfamily, Ste6p, was the first ABC transporter identified in yeast, mediating the export of the farnesylated **a**-factor lipopeptide pheromone, which is essential for mating of haploid yeast cells [23–25].

4.2. Vacuolar ABC transporters – the intracellular defense line

A major task of yeast MRP/CFTR proteins seems to be vacuolar sequestration [7,8] via transport of a variety of toxic compounds, a function quite similar to the mammalian orthologues operating in liver detoxification of conjugated xenobiotics [1]. The yeast cadmium factor (Ycf1p) mediates vacuolar detoxification of heavy metals and glutathione-S conjugates (GS-conjugates) [26–29], as well as red pigment accumulating in *ade2* mutant cells [30]. Ycf1p is a yeast orthologue of mammalian MRPs, which have been implicated in MDR phenotypes of tumor cells, as well as in prominent genetic defects in hepatobiliary transport processes [1]. The domain organisation of Ycf1p also includes a putative regulatory domain (R-domain) as present in CFTR [31], as well as the NTE present only in the MRP subfamily [32,33]. The NTE appears necessary for efficient membrane localization of Ycf1p [34]. Phosphorylation of two residues, Ser^{908} and Thr^{911}, within the putative Ycf1p R-domain is essential for cadmium detoxification [35]. Furthermore, a unique region within lumenal loop 6 in the first transmembrane domain of Ycf1p, designated loop 6 insertion ($L6_{ins}$), is necessary and sufficient for proteolytic processing, and, in addition, appears to regulate substrate specificity [36]. Interestingly, recent studies imply a possible contribution of Ycf1p in cellular aging processes. Absence of *YCF1* results in a dramatic loss of viability during chronological aging. This effect is due to increased apoptosis, indicating that Ycf1p might play an important role in apoptotic cell death (Jungwirth et al., unpublished results).

The closest homologue of Ycf1p, Bpt1p [37], also mediates cadmium detoxification, resistance to acetaminophen, *ade2* pigmentation and catalyzes the transport of glutathione conjugates, as well as free glutathione [30,38,39]. Another related vacuolar ABC transporter, Ybt1p/Bat1p, mediates ATP-dependent bile acid transport. While the normal function of Ybt1p/Bat1p remains unknown, it is conceivable that Ybt1p/Bat1p sequesters fungal detergent-like molecules equivalent to bile acids to prevent breakdown of organelle membranes [40].

4.3. Mitochondrial and peroxisomal ABC transporters

Three ABC proteins, Atm1p, Mdl1p and Mdl2p, reside in the inner mitochondrial membrane. The ABC protein Atm1 [41] performs an essential function in the generation of cytosolic but not mitochondrial iron–sulfur (Fe/S) proteins by exporting Fe/S cluster precursors from mitochondria [42]. Atm1p acts as a homodimer, mutations in the NBD affect dimer stability [43], and conserved residues in Walker A and B motifs are essential for function [44].

The ABC protein Mdl1p forms dimeric and maybe even homo-oligomeric complexes in the presence of ATP, assembling in a nucleotide-dependent manner with monomeric F_1F_0-ATP synthase [45]. Mdl1p exports mitochondrial peptides generated by proteolysis of inner-membrane proteins in the mitochondrial matrix [46]. Moreover, Mdl1p may also play a role in the regulation of cellular resistance to oxidative stress [47]. Notably, Mdl1p is the closest homologue of the mammalian transporter associated with antigen presentation (TAP), which drives transport of antigenic peptides into the ER lumen [48]. Interestingly, the second mitochondrial ABC transporter with high sequence similarity to TAP, Mdl2p, does not affect peptide transport across the inner membrane and its function escaped discovery so far.

In *S. cerevisiae*, two peroxisomal ABC transporters, Pxa1p (also known as Pat2p, Pal1p, Ssh2p) and Pxa2p (also known as Pal1p, Pal2p), are members of the ALDp-subfamily [49,50]. Both are half-size transporters of the peroxisomal membrane, sharing a MDR-like TMS_6-NBD membrane topology. Pxa1p/Pxa2p are yeast orthologues of the human Pmp70 and ALDp-like peroxisomal transporters associated with the fatal neurodegenerative disease adrenoleukodystrophy [1]. The yeast null mutants fail to grow on fatty acids such as palmitate or oleate as the sole carbon source [51–53], implying that Pxa1p and Pxa2p mediate peroxisomal import of long chain fatty acids (LCFA) for β-oxidation [49,51,54]. Alternatively, Pax1p/Pax2p might function as a peroxisomal acyl-CoA flippase [55].

5. Regulation of ABC gene expression in *S. cerevisiae*

The binuclear $Zn(II)_2Cys_6$ zinc finger regulators [56] Pdr1p [57] and Pdr3p [58] are among the master regulators of the

PDR network (Table 2) they form homo- and heterodimers [59] and regulate transcription in the promoter of PDR target genes through *cis*-acting elements, known as PDREs (pleiotropic drug resistance elements) [60–62]. Transcription of *PDR5*, *SNQ2*, *PDR10* and *PDR15*, as well as *YOR1* (Table 2) is controlled by Pdr1p/Pdr3p and Yrr1p, the latter modulating expression of both *SNQ2* and *YOR1* [63,64]. *PDR3* and *YRR1* are also autoregulated via PDREs in their own promoters [63–65]. Remarkably, Pdr1p and Pdr3p can positively or negatively regulate expression of target genes, implying that additional factors modulate their activity [66,67]. For example, the $Zn(II)_2Cys_6$ regulator Rdr1p, acts as a repressor of *PDR5* in a PDRE-dependent manner. Heterodimers of Rdr1p with Pdr1p or Pdr3p compete with Pdr1p/Pdr3p for binding to PDREs [68]. The zinc cluster protein Stb5p also acts through PDREs. Stb5p acts predominantly within a Pdr1p heterodimer, while no interactions occur with Pdr3p or Yrr1p, the latter is only present as a homodimer [56,69].

Genomic approaches uncovered another zinc finger regulator, Pdr8p, as involved in the PDR network [70]. Pdr8p mediates resistance to ketoconazole and oligomycin, operating mainly through Yrr1p and its respective target genes. *YRM1* (yeast reveromycin resistance modulator) encodes another $Zn(II)_2Cys_6$ regulator acting as a specific inhibitor of Yrr1p. In the absence of Yrr1p, Yrm1p activates the transcription of most genes regulated by Yrr1p, reflecting the high level of complexity of the regulatory processes controlling drug resistance phenotypes in yeast [71].

Recently, yet another novel zinc finger regulator, War1p, was identified as the main modulator of weak acid stress adaptation through transcriptional control of *PDR12* [72]. War1p controls a rather small regulon [73], forms homodimers, is activated by stress-triggered hyper-phosphorylation (Frohner et al., in preparation), and decorates a *cis*-acting weak acid response element in the *PDR12* promotor [72,73].

Ecm22p and Umc2p are members of the $Zn(II)_2Cys_6$ transcription factors involved in regulation of membrane sterol homeostasis [74,75] through control of ABC transporters. Based on genome-wide transcriptional profiling of a *Upc2-1* gain-of-function mutant strain that exhibits aerobic sterol influx, *PDR11* and *AUS1* were identified as major determinants required for uptake of free sterols under conditions of impaired ergosterol biosynthesis [19].

The ABC transporter Pdr15p is induced upon various stresses, including heat shock, high osmolarity and weak acid stress in an Msn2p-dependent manner [18]. The Cys_2His_2 zinc finger protein Msn2p, a master regulator of general stress response pathway, modulates a large set of genes in response to a variety of different environmental stimuli [76]. Interestingly, Pdr15p stress induction requires Msn2p [18], but bypasses upstream components of the HOG pathway [77], suggesting the existence of a novel as yet undisclosed HOG signaling branch converging at the downstream Msn2p regulator [18]. Moreover, Pdr15p levels are strongly elevated in cells undergoing diauxic shift, while expression of Pdr5p almost completely disappears [12,18]. Thus, Pdr15p and Pdr5p may perform non-overlapping functions related to detoxification in different growth phases or under certain metabolic conditions.

Finally, basic-leucine zipper (b-ZIP) transcription factors such as Yap1p have also been implicated in ABC gene regulation, although their precise role in PDR or stress response has not been unraveled so far [78,79]. Yap1p decorates promoters of *SNQ2* and *YCF1* to modulate their expression [80,81]. Notably, another member of the b-ZIP family, Yap8p, also participates in arsenite detoxification by regulating Ycf1p expression [82,83]. Interestingly, recent studies revealed a link between Yap1p and the aging process, because overexpression of *YAP1* significantly improves survival during chronological aging in yeast cells [84], possibly through Ycf1p expression regulation (Jungwirth et al., unpublished data).

6. The physiological role of ABC proteins in *S. cerevisiae*

For ABC transporters such as Ste6p, Atmp1p, and Pxa1p/Pxa2p, the physiological substrates have been identified [23–25,42,51]. As for PDR transporters, detoxification remains as one of the most plausible physiological function, since they

Table 2
Regulators of ABC proteins or ABC genes in the yeast *S. cerevisiae*

Regulator	Cellular function	ABC target genes	Reference
$Zn(II)_2Cys_6$			
Pdr1p	Regulation of PDR	*PDR5*, *PDR10*, *PDR11*, *PDR15*, *SNQ2*, *YOR1*	[57]
Pdr3p	Regulation of PDR	*PDR5*, *PDR10*, *PDR15*, *SNQ2*, *YOR1*	[58]
Yrr1p	Regulation of PDR	*YOR1*, *SNQ2*	[63]
Rdr1p	Negative regulation of *PDR5*	*PDR5*	[68]
Stb5p	Regulation of PDR, Pdr1p-heterodimer	*PDR5*, *SNQ2*	[56]
Pdr8	Regulation of PDR	*PDR15*, *YOR1*	[70]
Yrm1p	Specific inhibitor of Yrr1p	*YOR1*, *SNQ2*	[71]
War1p	Regulation of weak acid stress response	*PDR12*	[72,73]
Ecm22p and Upc2p	Regulation of sterol biosynthesis	*PDR11*, *AUS1*	[74,75]
Cys_2His_2			
Msn2p	Regulation of general stress response	*PDR15*	[77]
b-ZIP			
Yap1p	Regulation of oxidative stress response	*YCF1*, *SNQ2*, *PDR5*	[80]
Yap8p	Modulation of arsenite resistance	*YCF1*	[83]
General TF and other			
Ngg1p	Inhibition of Pdr1p activity		[66,67]

PDR, pleiotropic drug resistance; TF, transcription factor.

eliminate hundreds of structurally and functionally unrelated cytotoxic compounds, as well as potentially toxic metabolites [85]. Furthermore, Ycf1p and Bpt1p drive vacuolar sequestration of heavy metals as well as GS-conjugates [26–29,39]. Although yeast is a unicellular organism, vacuolar sequestration of heavy metal ions, rather than their extrusion, might be explained by a beneficial effect on immediate neighbouring cells.

Notably, membrane phospholipids and sphingosine long chain bases (LCBs) are suspected substrates of yeast ABC transporters such as Pdr5p [86,87]. Indeed, there is accumulating evidence for a role of eukaryotic and mammalian ABC proteins in membrane lipid transport [88]. Like certain mammalian ABC transporters [89,90], Pdr5p, Yor1p, Pdr10p, Aus1p and Pdr10p may translocate at least some membrane phospholipids or even steroles [19,91]. Hence, at least some ABC transporters may function in controlling or modulating membrane lipid homeostasis, regulation of membrane permeability and phospholipid bilayer distribution. ABC pumps could also remove oxidized or damaged membrane lipids. Indeed, membrane-damaging agents such as detergents and lysophospholipids, all of which exert massive membrane stress, strongly induce Pdr15p levels (Mamnun et al., in preparation).

7. Conclusions and perspectives

In summary, in addition to cellular detoxification, other hypothetical physiological function(s) of ABC pumps might be a role in the dynamic regulation of the membrane lipid compositions or bilayer asymmetry. However, facts are modest at best, as most studies addressing membrane lipid asymmetry employed short-chain fluorescent lipids as transport or "flippase" substrates, which may be recognized as "drugs" rather than natural membrane lipids. Hence, reconstituted in vitro systems [92], as well as natural lipid substrates [89], will be necessary to unequivocally demonstrate functions for ABC transporters in phospholipid homeostasis. During the past years, intensive research efforts led to a better understanding of the molecular mechanisms of members of the ABC protein family. Nevertheless, the cellular substrates and physiological functions of many eukaryotic ABC pumps remain enigmatic. We can thus anticipate many exciting discoveries on fungal ABC transporters, particularly when considering the broad variety of ABC proteins uncovered in the genomes of other yeasts and especially in those from fungal pathogens.

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