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ABC Transporters in *Saccharomyces cerevisiae* and Their Interactors: New Technology Advances the Biology of the ABCC (MRP) Subfamily

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INTRODUCTION

Members of the ATP-binding cassette (ABC) superfamily catalyze the ATP-dependent transport of chemically diverse compounds across cellular membranes, including the plasma membrane or intracellular organellar membranes (27, 29, 51, 61). In humans, ABC transporters are clinically important for maintaining the blood-brain barrier, which excludes cytotoxic drugs from the brain, and for mediating cellular resistance to chemotherapeutic drugs (52, 58, 153). Loss-of-function mutations in ABC transporter genes are implicated in a diverse and ever-increasing number of inherited human diseases, including the lung disease cystic fibrosis, the cholesterol transport disor-

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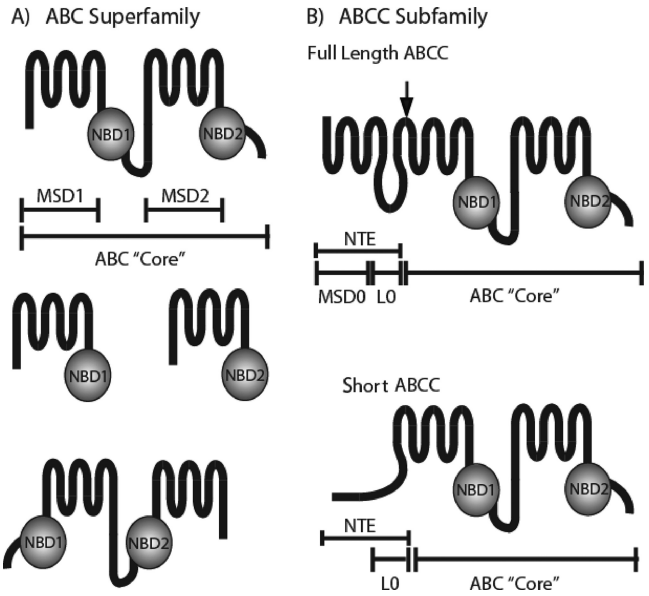

FIG. 1. (A) Overall architecture of the ABC superfamily. ABC transporters (top) have an "ABC core" region consisting of two MSDs (MSD1 and MSD2) containing six transmembrane spans and two cytosolic NBDs connected by a linker region (not labeled). ABC transporters can also be expressed as half-molecules (middle), with each half containing a single MSD and NBD; the halves can homo- or heterodimerize to form a functioning transporter. Some ABC transporters are encoded in "reverse" (bottom), where the NBDs precede the MSDs. (B) Overall architecture of the ABCC subfamily. Members of the ABCC subfamily of ABC transporters contain a characteristic NTE in addition to the "ABC core." In full-length ABCCs (top), the NTE contains an MSD (MSD0) with five transmembrane spans and a cytosolic loop (L0). In short ABCCs (bottom), an L0 domain, but no MSD0, is present. The arrow over the full-length ABCC indicates the site of Ycf1p posttranslational processing in L6 (discussed in the text).

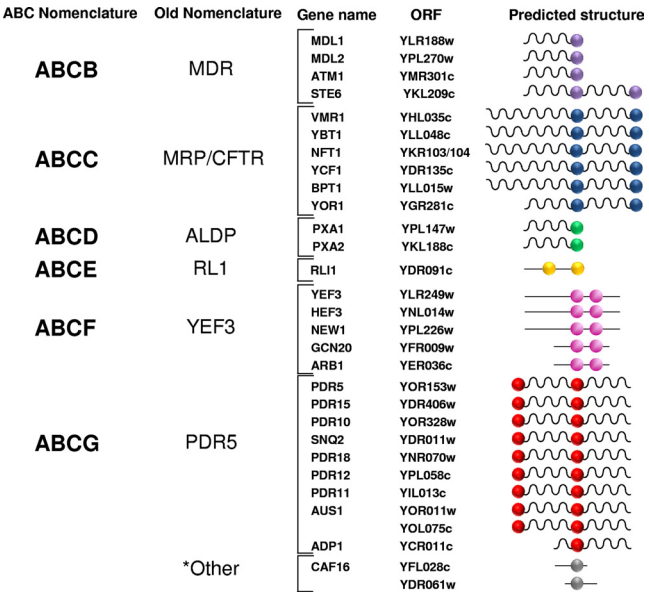

FIG. 2. Assignment of yeast ABC proteins into subfamilies ABCB to ABCG using the mammalian nomenclature. Yeast ABC proteins are divided into subfamilies according to the sequence similarity within their NBDs. The ABCB to ABCG (left) (http://nutrigene.4t.com/humanabc.htm) (25–27) and traditional (right) (107, 155) yeast subfamilies are shown. We propose here to rename the yeast subfamilies using the standard ABC nomenclature employed for human ABC transporters. Each subfamily is separately colored: purple, ABCB; blue, ABCC; green, ABCD; yellow, ABCE; magenta, ABCF; red, ABCG. Note that the mammalian ABCA subfamily is absent in yeast. Two of the yeast ABC proteins, Caf16p and Ydr061w, are not closely homologous to any of the mammalian ABC transporter subfamilies and are labeled "other" (gray). Balls indicate NBDs, wavy lines indicate MSDs, and straight lines signify nonmembrane sequences. Only four of the subfamilies contain members with membrane spans (ABCB, ABCC, ABCD, and ABCG) and are thus likely to function as transporters.

der Tangier's disease, the retinal syndrome Stargardt's disease, the elastic tissue disorder pseudoxanthoma elasticum, and many others. A comprehensive list of the diseases that map to ABC genes is available (25–27; see also http://nutrigene.4t.com/humanabc.htm). In contrast to diseases that are associated with a loss of transporter function, the overexpression of ABC proteins such as the human MDR1 or MRP1 protein can enhance multidrug resistance in mammalian cells (3, 13, 58). Thus, an understanding of the mechanistic principles and substrate selectivity determinants of ABC transporters has important biological and medical implications. To gain insight into the workings of ABC transporters, investigators are currently employing methodologies ranging from X-ray crystallography to genetic analysis in model organisms. The latter approach is exemplified by the yeast studies discussed in this review.

Nucleotide-Binding Domains Are the Diagnostic Features of ABC Proteins

Members of the ABC superfamily share a conserved overall architecture (Fig. 1A). The ABC "core domain" consists of two homologous halves, each containing a membrane-spanning domain (MSD) with multiple transmembrane spans (generally, but not always, six) and a nucleotide-binding domain (NBD), which couples nucleotide hydrolysis to substrate transport (26, 61, 62, 97, 98). In eukaryotes, these homologous halves are encoded as a single polypeptide or as half-molecules that form homo- or heterodimers. The NBDs can be located N or C terminally to the MSDs (as shown in Fig. 1A) (13, 26, 61).

Nucleotide hydrolysis is critical for ABC protein function. Each NBD is ~200 residues in length with several conserved regions, including the Walker A and B motifs (separated by ~90 to 120 residues) and a "signature" motif with the consensus sequence LSGGQ (also called the C motif and located just upstream from the Walker B region) (28, 61, 97, 98). X-ray crystallographic analysis of model ABC transporters from bacteria indicates that the two NBDs of a single transporter interact in a head-to-tail fashion, with the Walker A and B motifs of one NBD interacting with the C motif of the other (24, 64, 97, 98). It is notable that a subset of ABC proteins are comprised solely of NBDs and lack membrane spans entirely (ABCE and ABCF subfamilies) (Fig. 2 and see Fig. 4); these ABC proteins do not function as transporters but instead are likely to couple ATP hydrolysis to other processes such as DNA repair and protein translation (78, 174, 175).

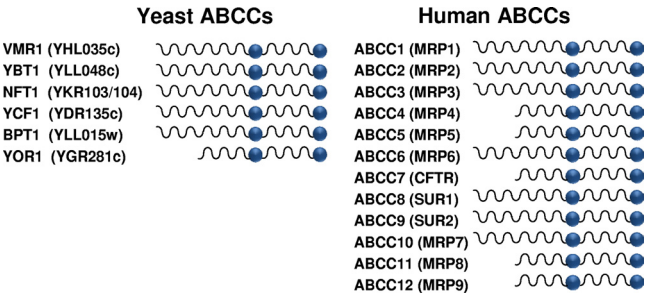

FIG. 3. Yeast and human ABCC subfamily members. Yeast ABCC proteins are shown at left with their common names and ORF names (in parentheses). Human proteins are shown at right along with their ABCC names and common names (in parentheses) (http://nutrigene.4t.com/humanabc.htm). The diagram indicates whether the particular subfamily member is full length or short.

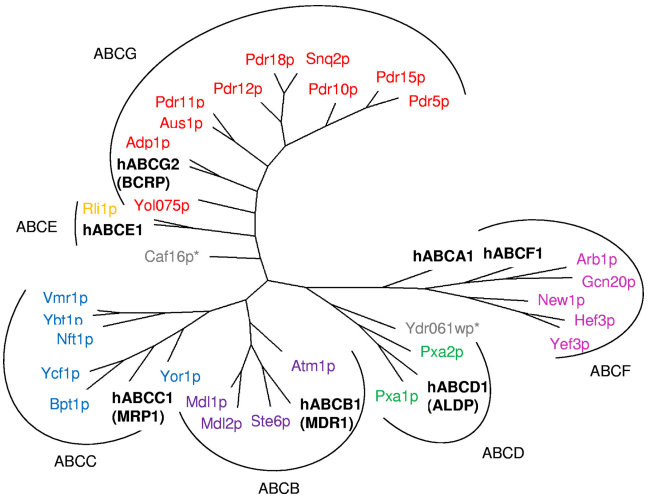

FIG. 4. Yeast ABC phylogenetic tree. The protein sequences of the yeast ABC transporters have been subjected to a multiple-sequence alignment using CLUSTALW and phylogenetic analysis, and the resulting data are depicted in a radial-tree format (PHYLO). Subfamilies have been highlighted and grouped by black lines and arcs. As in Fig. 2, the nomenclature ABCB to ABCG is used to assign the yeast ABC proteins to their homologous subfamilies. Colors are as defined in the legend of Fig. 2. For each subfamily, a mammalian member (boldface type) was included in the analysis as a point of reference.

ABCA to ABCG Subfamilies

Through the efforts of ABC researchers and the Human Genome Organization (HUGO), the mammalian ABC superfamily has been divided into seven subfamilies (designated ABCA to ABCG) based on the relatedness of sequences within their NBDs (http://nutrigene.4t.com/humanabc.htm) (25–27). Members of each subfamily tend to have partially overlapping physiological and biochemical functions. This principle is well exemplified by studies of ABCC subfamily members in yeast, plants, and mammals (Fig. 3). ABCC transporters generally transport xenobiotic compounds or toxic metabolites that have first been conjugated to glutathione (GSH), glucuronide, or sulfur (3, 15, 33, 58, 66, 82, 125, 160).

Below, we discuss the complete inventory of yeast ABC proteins and then focus on members of the ABCC subfamily in yeast, particularly Ycf1p, which has been proven an excellent model for improving our understanding of the shared features of ABCC subfamily members throughout the eukaryotes.

INVENTORY OF YEAST ABC PROTEINS

The genetic, biochemical, and cell biological tractability of yeast makes it a particularly well-suited model system for investigating protein function and protein interaction networks and for defining cellular pathways (12, 45, 67, 87, 140, 152). This has proven to be the case for many aspects of ABC transporter biology, particularly because upon the completion of the yeast genome sequence project in 1996, *Saccharomyces cerevisiae* became the first organism for which a glimpse of the complete inventory of ABC transporters was available (30, 48, 107, 155). Because ABCC transporters exhibit significant functional redundancy, the ability to easily knock out multiple genes in yeast has greatly facilitated our understanding of ABCC transporter function.

The yeast genome contains 30 ABC proteins (Fig. 2 and 4 and Table 1) originally identified based on BLAST searches for homologs of NBD1 of STE6 (30, 107, 155). Of these proteins, 22 are predicted to contain multiple membrane spans and are thus considered to be true ABC transporters, while the remaining 8 have no predicted spans and presumably carry out nontransport functions in the cell (Fig. 2). The localization and the function of the 22 yeast ABC transporters are shown in Table 1 and Fig. 5. Previous phylogenetic analyses established the existence of six ABC subfamilies in yeast, and each was assigned a subfamily name, with the name being based on a prominent mammalian or yeast representative of that subfamily (147, 155). Here, we propose to redesignate the old subfamily names in accordance with the now commonly accepted HUGO nomenclature for ABC proteins (ABCA to ABCG) (http://nutrigene.4t.com/humanabc.htm) (Fig. 2 and 4 and Table 1). Thus, we propose that the former yeast subfamily names MDR, MRP/CFTR, ALDP, RLI, YEF3, and PDR5 be redesignated ABCB to ABCG, respectively (Fig. 2 and 4 and Table 1). It should be noted that the mammalian ABCA subfamily is entirely absent in yeast. In addition, two yeast ABC open reading frames (ORFs) (CAF16 and YDR061w) cannot be classified into any of the HUGO subfamilies and are categorized here as "other" (Fig. 2 and 4). The redesignation of yeast subfamilies that we propose here should facilitate a better correlation of experimental findings between yeast and mammalian ABC transporters.

Over the years, studies of yeast ABC transporters have made important contributions to our understanding of many areas of cell biology and drug resistance. Examples include the identification of physiological ABC transporter substrates (notably, the lipopeptide mating pheromone **a**-factor for Ste6p) (11, 88, 104, 106), the elucidation of ubiquitin as a trafficking signal for the endocytosis of certain membrane proteins (Ste6p being one of the first examples) (10, 77, 86), the analysis of endoplasmic reticulum (ER) quality control machinery and the identification of ER-associated compartments (34, 68, 69, 112), the discovery of new roles of ABC half-transporters in organellar function (96, 142), and the elucidation of pleiotropic drug resistance networks that are transcriptionally coregulated and that may contribute to the growing problem of drug resistance of fungal pathogens such as *Candida albicans* or *Candida glabrata* (37, 57, 85). The reader is referred to several compre-

TABLE 1. Classification, localization, and function of *Saccharomyces cerevisiae* ABC transporters[a]

Family	Transporter	ORF	Localization	Description	Reference(s)
ABCB	MDL1	YLR188w	Mitochondrial inner membrane	Involved in export of peptides from mitochondria, plays a role regulating oxidative stress; function may overlap with that of ATM1; homodimerizes	20, 172
	MDL2	YPL279w	Mitochondrial inner membrane	Unknown; homodimerizes	96
	ATM1	YMR301c	Mitochondrial inner membrane	Essential for cytosolic iron-sulfur clusters and iron homeostasis; homodimerizes	80
	STE6	YKL209c	Plasma membrane	Exporter of **a**-factor pheromone in *MAT***a** cells	79, 106
ABCC	VMR1	YHL035c	Vacuole	Unknown	30
	YBT1	YLL048c	Vacuole	Transports bile acid in vitro; transports Ade2 pigment in vivo	113, 144
	NFT1	YKR103/104	Vacuole	Unknown	102
	YCF1	YDR135c	Vacuole	Glutathione *S*-conjugate transporter involved in cellular detoxification; transports heavy metals such as cadmium, mercury, lead, and arsenite; transports Ade2 pigment; bile pigments and MRI contrast agents are in vitro substrates and unconjugated bilirubin	94, 120, 154
	BPT1	YLL015w	Vacuole	Glutathione *S*-conjugate transporter involved in cellular detoxification; transports cadmium and Ade2 pigment in vivo; function overlaps with that of Ycf1p	83, 120
	YOR1	YGR281c	Plasma membrane	Multidrug transporter; exports oligomycin, organic anions, and many other compounds	23, 76
ABCD	PXA1	YPL147w	Peroxisome	Implicated in transport of acyl coenzyme A esters across the peroxisomal membrane; heterodimerizes with Pxa2p	60, 142, 143
	PXA2	YKL188c	Peroxisome	Implicated in transport of acyl coenzyme A esters across the peroxisomal membrane; heterodimerizes with Pxa1p	60, 142, 143
ABCG	PDR5	YOR153w	Plasma membrane	Multidrug transporter involved in resistance to xenobiotic compounds (mutagens and anticancer drugs) and cations and in steroid transport	7, 100, 109
	PDR15	YDR406w	Plasma membrane	Multidrug transporter involved in general stress response for cellular detoxification	168, 169
	PDR10	YOR328w	Plasma membrane	Multidrug transporter involved in the pleiotropic drug resistance network	6, 168
	SNQ2	YDR011w	Plasma membrane	Multidrug transporter involved in multidrug resistance and resistance to singlet-oxygen species	141, 161
	PDR18	YNR070w	Plasma membrane	Putative transporter implicated in pleiotropic drug resistance	9, 132
	PDR12	YPL058c	Plasma membrane	Multidrug transporter involved in weak organic acid resistance	65, 121
	PDR11	YIL013c	Plasma membrane	Multidrug transporter involved in multidrug resistance and mediating sterol uptake	134, 167
	AUS1	YOR011w	Plasma membrane	Involved in sterol uptake	167
	YOL075c	YOL075c	Plasma membrane	Unknown	30
	ADP1	YCR011c	Plasma membrane	Unknown	30

[a] The ABC transporters are listed by formal name and ORF name and are grouped into their respective subfamilies as described in the text and in the legends of Fig. 2 and 4.

hensive reviews that have catalogued the contributions of yeast ABC transporter studies in discovering basic cell biological principles and in providing clues to the roles of ABC transporters in human health and disease (9, 74, 107, 147, 155).

The remainder of this review will focus on one of the best-studied subfamilies in yeast, the ABCC subfamily. We highlight the many advances that have been made in our understanding of the role of ABCC transporters in a variety of cellular processes and discuss the role of these transporters in heavy metal detoxification, fungal multidrug resistance, and the development of integrated membrane yeast two-hybrid (iMYTH) technology to identify membrane protein interactors. Because of the functional and structural similarities between yeast and mammalian ABC transporters, advances derived from the yeast studies have impacted our understanding of all ABCC transporters, as discussed in further detail below.

THE ABCC (MRP) SUBFAMILY

ABCC (MRP) Transporters Are Glutathione Conjugate (GS-X) Pumps and Can Contain an N-Terminal Extension

Members of the ABCC (also called MRP) subfamily play key roles in the efflux of xenobiotic compounds in eukaryotic cells. Human MRP1 (ABCC1) is the founding member of the ABCC subfamily and was discovered based on its ability, when

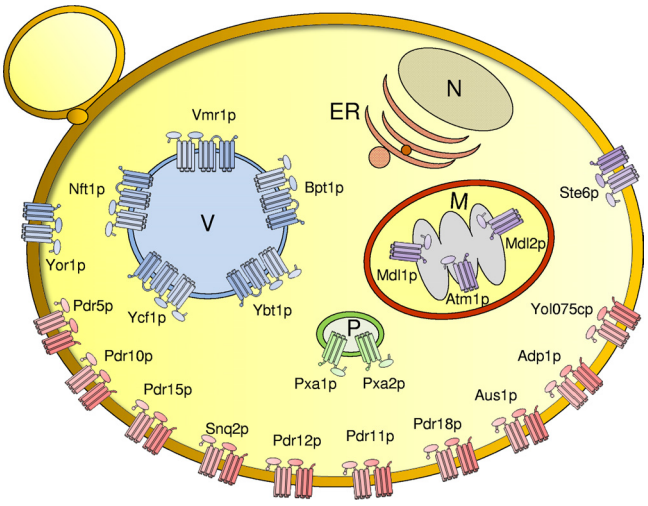

FIG. 5. Subcellular localization of *S. cerevisiae* ABC transporters. The 22 yeast ABC proteins containing membrane spans are colored by their subfamily, ABCB (purple), ABCC (blue), ABCD (green), and ABCG (red), and are localized to the indicated intracellular organelles (P, peroxisomes; V, vacuole; M, mitochondria) and the plasma membrane (not labeled). No ABC proteins localize to the nucleus (N) or ER. The three mitochondrial ABC transporters are localized to the inner mitochondrial membrane. Because they are sequestered from the cytosol by the outer mitochondrial membrane, iMYTH studies cannot be performed with these three ABC transporters. Cylinders indicate MSDs. Full-length transporters and half-transporters are depicted, as are the full-length and short MRPs. NBDs are represented by ellipses.

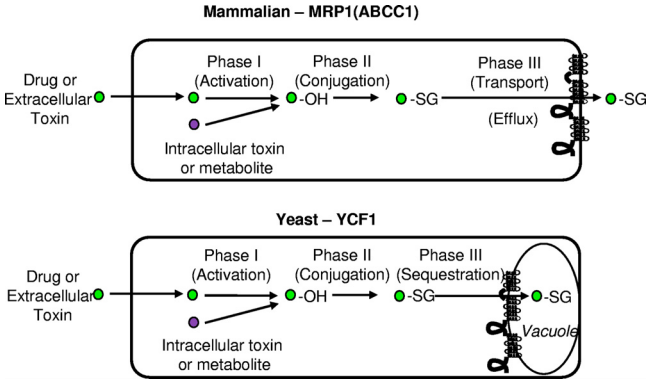

FIG. 6. Cellular detoxification, phases I, II, and III. The cellular detoxification of intracellular and extracellular toxins in yeast and mammalian cells generally utilizes the phases of detoxification shown here and described in the text. Toxins or metabolites acted upon by this system are indicated by a circle. For the phase I "activation" step, the addition of OH is indicated. For phase II, the conjugation of GSH (−SG) to the toxin is shown. Phase III is mediated by an ABC transporter to move the conjugated toxin across a cellular membrane, either the plasma membrane for MRP1 or the vacuole membrane for yeast Ycf1p, as discussed in the text.

overexpressed in a human cancer-derived cell line, to confer multidrug resistance to a variety of chemotherapeutic cancer drugs (21). The discovery of other mammalian, yeast, and plant MRP1 homologues soon followed (3, 13, 33, 74, 76, 124, 125, 154, 155). Generally, ABCC subfamily members are distinguished from other ABC transporters by two striking hallmarks, one structural, the other functional: (i) in addition to the "ABC core" domain, they contain a characteristic "N-terminal extension" (NTE), which comprises an MSD (MSD0) with five transmembrane spans and a short cytosolic loop (L0) (Fig. 1) (although it should be noted that a subset of ABCCs have only an L0 and no MSD0), and (ii) ABCC proteins transport most substrates in the form of GSH conjugates and, in some cases, glucuronide or sulfate conjugates, rather than transporting the unmodified substrates themselves (14, 22, 58, 125). The connection between the NTE and GSH remains unclear at present. However, as discussed below, the discovery that human MRP1 transported GSH conjugates (53, 63, 89, 90) helped answer a long-standing question in the field of pharmacology, namely, which class of cellular pumps is responsible for the efflux of GSH-conjugated toxins out of the cell (33, 70).

MRP1 (ABCC1) Is the Long-Sought-After GS-X Pump

Pharmacologists had appreciated for many decades, even before the identification of the corresponding machinery, that cells possess an effective cellular detoxification system involving three phases that act to detoxify and ultimately remove exogenous toxins that have gained entry into mammalian cells (Fig. 6) (72, 160, 173). In phase I, the chemical "activation" of a toxin, for instance, by the addition of an epoxide mediated by cytochrome P-450, results in its increased chemical reactivity. Phase II involves the enzymatic conjugation of GSH, glucuronide, or sulfate to the activated toxin, which further increases its solubility. Finally, in phase III, the ATP-dependent efflux of activated toxins out of the cell occurs via a transporter (170). While the biochemical activity of the phase III GSH conjugate transporters, also referred to as the GS-X pumps, was well established for decades, their molecular identity remained elusive until MRP1 was discovered in 1994 and shown to be a major GS-X pump in human cells (21, 90, 123). Later, other ABCC subfamily members were also shown to be GS-X pumps (3, 13, 33, 66).

In addition to xenobiotic toxins, ABCC transporters also efflux endogenous physiological substrates, including the signaling molecule leukotriene C_4 (LTC_4) (mediated by MRP1) and metabolites destined for bile, including bilirubin, glucuronide conjugates, and sulfated bile salts (mediated by a variety of ABCC transporters) (Fig. 6) (3, 13, 33, 66). Taken together, it has become clear that the ABCC subfamily plays an important protective role in the cell, in processes ranging from cellular assault by externally derived chemicals to the physiological transport of signaling molecules or endogenously generated toxic products.

The Mammalian ABCC Subfamily Contains Full-Length and Short Members

Human ABCC subfamily members are shown in Fig. 3 (25, 26). Although most of these proteins are likely to function as transporters, three are not: CFTR (ABCC7) is an ion channel, and SUR1 (ABCC8) and SUR2 (ABCC9) are ion channel regulators. However, the possibility that these ABCCs also have undiscovered transport functions should be considered.

The ABCC subfamily of ABC transporters consists of two structurally distinct classes of proteins. The first class of human ABCC proteins (7 of 12) possesses a complete NTE (containing both MSD0 and L0) (Fig. 1B and 3) and are designated

"full-length" ABCC proteins. The second class of proteins (5 of 12) contains only a partial NTE (L0 is present, but an MSD0 region is not). The latter proteins are designated "short" ABCC proteins (Fig. 1B and 3). The MSD0 portion within the NTE of "full-length" ABCCs is implicated in the proper trafficking of several ABCC proteins, while L0 is required for substrate transport (but not localization) in all cases where it has been tested, but whether the NTE also contributes to substrate specificity is not known (8, 41, 103, 165, 166).

A complete understanding of the role of the NTE in "full-length" transporter function remains to be established and is a major subject of current research. An issue under study is whether the NTE, so distinctive for the ABCC subfamily, also plays a special role in the unique ability of this subfamily to specifically recognize GSH-conjugated substrates (123). Because the weight of current evidence suggests that GSH-binding sites of ABCC transporters lie in the core domain and not the NTE, the role of the NTE in the recognition of GSH conjugates remains questionable (22).

MEMBERS OF THE YEAST ABCC SUBFAMILY

The ABCC subfamily in yeast is shown in Fig. 2 to 4 and Table 1. Five members are "full length" (Ycf1p, Bpt1p, Ybt1p, Nft1p, and Vmr1p), and one is "short" (Yor1p) (107, 155). Interestingly, while Yor1p localizes to the plasma membrane, the others all appear to be localized to the vacuolar membrane (Table 1 and Fig. 5) (75, 83, 94, 105, 113, 145). However, it should be noted that the localization data for Nft1p and Vmr1p are preliminary, and a punctate pattern in addition to a vacuolar membrane pattern have been observed for these transporters (105). Both the efflux of harmful compounds out of the cell and their transport into the vacuole have the same benefit of removing dangerous toxic molecules from the cytosol and, thus, away from intracellular targets. In many cases, yeast ABCCs exhibit overlapping substrate specificity (144, 145). The fact that both Ycf1p, a "full-length" ABCC transporter, and Yor1p, the plasma membrane-localized "short" transporter, can confer cadmium resistance (discussed in detail below) indicates that there is not a critical functional distinction between full-length and short ABCCs and that their distinct cellular locations may not necessarily confer unique properties (111). The yeast system is ideal for further dissecting these issues.

Below, we discuss what is currently known about each of the yeast ABCC transporters, discussing "full-length" ABCC transporters first, with a focus on Ycf1p, the best-studied member of the family. In the last section, we provide a comprehensive discussion of the newly developed split-ubiquitin MYTH system, which is specifically designed to identify membrane protein interactors and has been used with great success to identify functional interactors of Ycf1p.

Ycf1p

Ycf1p is the prototypical yeast ABCC transporter with a broad range of xenobiotic substrates. (i) Discovery of Ycf1p as a GSH-cadmium transporter and complementation of yeast *ycf1*Δ by human and plant MRPs. Ycf1p is the first member of the ABCC subfamily identified in yeast and remains the best-characterized member to date. The *YCF1* gene was discovered in 1994 based upon its ability to confer cadmium resistance when overexpressed (hence the name yeast cadmium factor) (154). Conversely, the deletion of *YCF1* results in cadmium hypersensitivity. A surprising early finding was that Ycf1p is not localized to the plasma membrane, where efflux pumps had previously been found. Instead, Ycf1p was shown to reside on the vacuolar membrane (the vacuole in yeast being equivalent to the mammalian lysosome) (94).

Motivated by the structural similarity between human MRP1 and yeast Ycf1p, investigators proposed that like MRP1, Ycf1p might be a GSH conjugate transporter. Using an in vitro transport assay, this was shown to be the case. A radiolabeled substrate, GS-conjugated dinitrophenyl (^{3}H-DNP-GS), was efficiently transported into vesiculated vacuoles prepared from a wild-type (WT) (*YCF1*) strain but not from a *ycf1*Δ strain (94). Notably, it was shown that cadmium is transported by Ycf1p as a cadmium–bis-GSH complex (Cd-GS_2), providing the first evidence for the involvement of GSH in heavy metal transport (93). Those early studies indicated that Ycf1p, like MRP1 (ABCC1) in mammalian cells, functions as a phase III export pump. However, rather than excreting GSH conjugates into the extracellular space, Ycf1p transports conjugated substrates across the vacuolar membrane, sequestering them within the vacuolar lumen (Fig. 6) (1, 93, 145, 154).

Strikingly, human *MRP1*, heterologously expressed in a yeast *ycf1*Δ mutant, was shown to complement its cadmium sensitivity in vivo and to restore GSH *S*-conjugate transport activity into yeast vacuoles in vitro (157). Furthermore, the expression of a plant MRP from *Arabidopsis thaliana*, AtMrp1, also complements a *ycf1*Δ mutant (158). This remarkable functional interchangeability of yeast, human, and plant MRPs suggests that ABCC activities are likely to be similar in all organisms, reflecting an impressive level of cross-organismal conservation in function. As discussed below, attempts are currently being made by researchers to harness this property for the purpose of phytoremediation, in which plants are used to remove harmful toxins from the soil and water.

(ii) Transcriptional regulation of the *YCF1* gene by Yap1p. Shortly after its discovery, *YCF1* was shown to be regulated by Yap1p, a transcription factor which belongs to the b-Zip class of DNA-binding proteins and is related to mammalian Ap-1 and yeast *GCN4* (164). The overexpression of Yap1p confers resistance to cadmium in yeast that is dependent on Ycf1p (50, 114, 164). Yap1p binds to a Yap1p response element located upstream of the *YCF1* ORF and promotes increased levels of expression of *YCF1*. A number of studies have implicated Yap1p as being the single most critical transcriptional regulator in defending cells against oxidative stress (57, 133).

(iii) Ycf1p transports a broad range of xenobiotic substrates: in vivo studies. A series of reports have shown that Ycf1p can transport many different heavy metals in addition to cadmium, as evidenced by the inability of a *ycf1*Δ strain to grow in media containing these metals. These metals include lead (Pb), antimony (Sb), mercury (Hg), and arsenite (As) (46, 56, 122, 149). Gueldry and coworkers pointed out that care must be taken in carrying out such growth tests, as the composition of the medium can influence the apparent efficacy of heavy metals or other compounds to inhibit yeast growth (56). In particular, certain supplements, such as the amino acid histi-

dine, can chelate heavy metals, reducing their effective concentration. It is likely that all heavy metal substrates of Ycf1p are transported in the form of a GS-metal complex, as shown for Cd-GS_2 and Hg-GS_2 (56, 93). However, this has yet to be experimentally confirmed. Results of a number of studies examining As^{3+} resistance in yeast, bacteria, and mammalian cells have shown that As^{3+} forms multiple metabolites, including both GSH and methyl conjugates (135, 136).

In addition to heavy metals, a number of other cytotoxic compounds have been shown to require Ycf1p for their detoxification. Examples include the antioxidant/oxidizing agents diamide and 1-chloro-2,4-dinitrobenzene (94, 163). A potentially useful Ycf1p substrate that has yet to be fully exploited is monochlorobimane, which can be added to living yeast cells and visualized by fluorescence microscopy. In a WT *YCF1* strain, monochlorobimane is concentrated in the vacuole, whereas in a *ycf1*Δ mutant, it is excluded (94). Thus, this fluorescent compound may serve as a sensitive in vivo indicator of Ycf1p function, or the lack thereof, facilitating the analysis of mutant forms of Ycf1p and Ycf1p-protein interactions.

Clearly, Ycf1p can transport a range of exogenous compounds, many of which are cytotoxic. However, a potential role of Ycf1p in sequestering endogenous cellular toxins, possibly arising from normal metabolic processes, has remained unclear. With the increasing ability of researchers to carry out large-scale metabolic systems biology studies of yeast, one tantalizing avenue of research is to identify candidate endogenous substrates for Ycf1p and other yeast MRPs by high-throughput metabolomics, comparing *ycf1*Δ deletion or *YCF1*-overexpressing strains to a WT strain.

(iv) Ycf1p transports many different xenobiotic compounds in vitro. Ycf1p can transport GSH conjugates (e.g., DNP-GS), glucuronate conjugates (e.g., E217βG), and reduced GSH, all three of which are human MRP1 substrates (94, 126–128, 145). The transport of these substrates by Ycf1p indicates that Ycf1p is a phase III transporter in yeast, similar to its human counterpart, MRP1 (Fig. 6). Transport assays are performed utilizing right-side-out-vesiculated yeast vacuoles prepared from WT (*YCF1*) and mutant (*ycf1*Δ) yeast strains (93, 94, 119). In general, these assays are designed to measure the transport of radioactively labeled substrates into the vesiculated vacuoles. Ycf1p has also been expressed in Sf21 insect cells, and membrane vesicles from these cells show Ycf1p-dependent transport properties similar to those of yeast vacuole preparations (131).

In addition to the "standard" MRP substrates noted above, additional substrates for Ycf1p have been demonstrated by assays of in vitro transport into yeast vacuoles. These substrates include unconjugated bile pigment and several magnetic resonance imaging (MRI) contrast agents (117, 120). As discussed below, for nearly all substrates tested to date, Ycf1p exhibits overlapping substrate specificity with its closest relative, Bpt1p (Fig. 2 and Table 1), and, in some cases, other yeast ABCCs as well (117, 120, 145). One exciting potential use for yeast Ycf1p transport assays is for the identification of inhibitors that ultimately might also be useful for inhibiting human MRP activity.

In addition to examining particular substrates, transport assays in vitro have also been important for examining the energy requirements for ABC transporter activity. We have demonstrated that Ycf1p can use either ATP or GTP to power substrate transport in vitro, as has also been reported for several other ABC transporters (49, 119, 162). The significance or universality of alternative nucleotides is not clear, although it has been proposed that Pdr5p increases its multidrug transport specificity by using more than one nucleotide as an energy source (49).

(v) Potential of harnessing Ycf1p for phytoremediation. There has been significant interest in harnessing Ycf1p's ability to transport heavy metals for phytoremediation, which refers to the use of plants to reduce the levels of environmental contaminants (54, 149, 159). Heavy metals such as Pb, Cd, Hg, and As represent some of the most significant toxic soil and dust contaminants in industrialized countries. Phytoremediation has been proposed to be a low-cost, effective way to remove heavy metal contaminants from soil. Notably, Ycf1p expressed in the model plant *Arabidopsis* promotes the improved tolerance of these plants to Pb(II) and Cd(II) and results in a high intracellular concentration of the metals, presumably due to their sequestration in the vacuole (149, 159). An extension of this work involving the expression of yeast Ycf1p in poplar, a tree that grows rapidly and to a high mass, making it suitable for bioremediation, was recently suggested (146). Although not yet accomplished, it has been proposed that ultimately, full-grown transgenic poplars, replete with vacuolar heavy metals resulting from Ycf1p-mediated transport, could be cut and removed from a contamination site, proving an effective mechanism by which soil can be decontaminated relatively inexpensively and in an environmentally friendly way.

Ycf1p transports endogenous metabolites and could be involved in metabolic "quality control" in yeast. Ycf1p plays an important role in the striking pink/red pigmentation of yeast colonies that arise from adenine biosynthetic mutants in yeast (144, 145). Specifically, strains carrying *ade1* or *ade2* mutations accumulate an intense red pigment in their vacuoles when they are grown under conditions of adenine limitation. The formation of this pigment involves a series of steps, including the conjugation of the adenine biosynthetic intermediate phosphoribosylaminoimidazole (AIR) to GSH, the Ycf1p-dependent transport of AIR-GS into the vacuole, and further metabolism within the vacuole, ultimately giving rise to the familiar red pigment associated with an *ade2* yeast strain (18, 144, 145). In a *ycf1*Δ mutant, the pigmentation of an *ade2* mutant colony is greatly diminished due to the significant lack of transport of AIR-GS into the vacuole. The residual pigmentation in a *ycf1*Δ mutant is due to a low level of transport activity mediated by two other ABCC transporters, Bpt1p and Ybt1p (144, 145). The "red *ade2* assay" has been used experimentally as a sensitive in vivo assay for the function of Ycf1p and/or its functional interactors (119).

Importantly, the cytosolic elimination of the *ade2* red intermediate may reflect an important cellular role for Ycf1p and other ABCC transporters in a "metabolic quality control" system that prevents the aberrant cytosolic accumulation of potentially toxic biosynthetic intermediates. A role for Ycf1p in metabolic quality control is being experimentally sought but is complicated by the redundancy of other yeast ABCCs. To most effectively determine the role of Ycf1p or any ABCCs, human or yeast alike, in metabolic quality control, the optimal model would be cells that are missing multiple ABCC proteins. This

type of analysis could be carried out using strains with a deletion of any number and combination of yeast ABCCs (F. Roth, Harvard University, personal communication).

Ycf1p proteolytic processing and trafficking. Studies of Ycf1p trafficking and processing have relied on antibodies that recognize Ycf1p or a C-terminally green fluorescent protein-tagged version of Ycf1p that retains normal function and trafficking properties (102, 103). An unusual aspect of Ycf1p biology is that this transporter is posttranslationally processed to yield N- and C-terminal cleavage products that are stable and remain tightly associated with one another (102, 163). Cleavage occurs within luminal loop 6 (L6) in a region representing an insertion that is specific to Ycf1p (Fig. 1). The "L6 insertion" appears to be functionally transplantable in that it can promote processing when moved to new luminal locations in Ycf1p or another transporter, Bpt1p (102). Cleavage occurs only after Ycf1p reaches its final vacuolar destination and is dependent on the vacuolar proteases Pep4p and Prb1p. Pep4p, the master vacuolar protease, and Prb1p have reciprocal roles in activating each other. Surprisingly, the processing of Ycf1p is not required for its activity (103). However, certain mutations within the Ycf1p "L6 insertion" appear to alter substrate specificity, suggesting that both cytosolic and luminal domains can influence substrate binding or, possibly, substrate release (102). The recent publication of the crystal structure for the mammalian ABC transporter MDR1 (ABCB1; also called P-glycoprotein) and the bacterial ABC transporter Sav1866 suggests the possibility that luminal L6 of Ycf1p may be in very close proximity to the predicted pore from which substrates might exit; however, it should be noted that such a model has yet to be tested (2, 24, 64).

Mutant forms of Ycf1p also appear to hold promise for gaining insight into the ER quality control processes that handle misfolded membrane proteins, such as the mutant form of CFTR (CFTR-ΔF508) that results in cystic fibrosis. Such misfolded membrane proteins are not permitted to exit the ER and undergo degradation by the ubiquitin-proteasome system. Most ABCC proteins, including Ycf1p, have a phenylalanine analogous to CFTR-F508. When mutated in Ycf1p (Ycf1-Δ713), the mutant form of Ycf1p is retained in the ER, in structures called ER-associated compartments, and degraded by the proteasome, providing an excellent model for studying ER quality control components (101, 163). Current studies in our laboratory are focused on using Ycf1p and the ABCB transporter Ste6p as models to study protein misfolding and ER-associated degradation (68, 101, 105).

Mutational structure-function analysis of Ycf1p: partial-molecule studies and ABC mutants. Ycf1p can be subdivided into two "partial molecules" containing the NTE (MSD0 plus L0) and the "core" domain that upon coexpression can reconstitute functional Ycf1p in the vacuole membrane. Studies with mutant forms of these Ycf1p partial molecules have provided evidence that the L0 portion of the NTE is absolutely required for Ycf1p transport function (103). Furthermore, the deletion of a highly conserved 17-amino-acid amphipathic helical region within L0 is sufficient to abolish Ycf1p function (102). In contrast, data from partial-molecule studies indicate that MSD0 is required for the proper localization of Ycf1p (103). A functional role for MSD0 could not be assessed, because when MSD0 is compromised, Ycf1p mislocalizes. Similar to Ycf1p, the L0 region in mammalian ABCC transporters is critical for the function of human MRP1 and MRP2, and likewise, MSD0 is required for proper MRP1 and MRP2 localization to the plasma membrane (4, 41, 165, 166). Because analysis of the yeast Ycf1p NTE has provided generalizable insights into this intriguing module that sets ABCC transporters apart from other ABC subfamilies, its further analysis in Ycf1p is warranted.

To perform mutational structure-function analyses of Ycf1p, investigators have carefully analyzed an extensive series of site-directed mutants within NBD1, NBD2, a region within the linker called the R domain, and the so-called intracellular loop 4 (CL4) positioned between membrane spans 15 and 16 of Ycf1p (which has a total of 17 spans). Residues chosen for mutagenesis are conserved in MRP1 and CFTR, and some of the mutations were analogous to cystic fibrosis-associated mutations (39, 40, 163). Mutants were assessed for Cd(II) resistance in vivo and vacuolar transport activity in vitro (transport of radiolabeled LTC_4 into vacuolar vesicles). Most of the mutations were found to be deleterious, due either to a loss of function or to biogenesis defects (the latter class manifests as a lack of protein due to degradation). However, a few of the mutants showed differential effects on the ability of yeast to grow on Cd(II) and Ycf1p-dependent LTC_4 transport, potentially suggesting alterations in substrate binding (39, 40, 163). Follow-up studies using these mutants may be useful for elucidating the basis of Ycf1p substrate specificity.

In addition to the primary mutational analysis described above, intragenic suppressor studies were carried out, starting with mutations that inactivate Ycf1p function. One of these was D777N in the Walker B motif of NBD1, which inactivates Ycf1p function (39). Gain-of-function suppressor mutations that restored Ycf1p function to *ycf1-D777N* were identified in multiple regions of Ycf1p, including NBD1 itself, NBD2, MSD1, and MSD2 (39). These results suggest that NBD1 may functionally interact with NBD2, MSD1, and MSD2 for proper Ycf1p function. Ultimately, an analysis of the ATPase catalytic cycle of Ycf1p will be an important area for further research and would be invaluable for assessing the mutants discussed above. However, in light of the low ATPase activity of most ABC transporters in vitro and the lack of substrate stimulation of ATPase activity in most cases, analysis of the ATPase catalytic cycle of Ycf1p promises to be challenging (79, 137).

Posttranslational modulation of Ycf1p function by phosphorylation and interaction partners. Ycf1p appears to be strongly positively regulated by phosphorylation. Two residues (S908 and T911) within the linker of the core domain of Ycf1p are important for its positive regulation by phosphorylation (36, 118). The alteration of either residue to alanine (S908A and T911A) lowers cadmium resistance in vivo and LTC_4 transport in vitro. In the double mutant (S908A T908A), cells show an even more severe phenotype in both assays, and this mutant form of Ycf1p exhibits a marked gel mobility shift, reflecting diminished phosphorylation (36). Furthermore, the mutation of these residues to aspartic acid (S908D T911D), which mimics phosphorylation, restores WT Ycf1p-dependent function. To date, the kinases responsible for phosphorylating these residues are not known (36).

Recently a number of large-scale proteomic studies aimed at identifying the yeast "phosphoproteome" have been reported.

In several of those studies, phosphopeptides derived from Ycf1p were observed among a vast repertoire of total yeast phosphopeptides (19, 92, 148). The phosphorylated residues in the Ycf1p core domain include S908 and T911 (discussed above) as well as S869, S870, S872, S873, S903, and S914 (19, 92, 148). One additional phosphorylated residue, S251, lies within the L0 domain of Ycf1p. Recent studies from our group suggest that phosphorylation at S251 may negatively regulate Ycf1p function, since an S251A mutant exhibits increased resistance to cadmium in vivo and increased Ycf1p transport activity in vitro compared to WT *YCF1* (118). Both activities are restored to the WT level in an S251E mutant. Notably, through interactor studies (discussed below), a kinase gene, *CKA1*, that encodes a subunit of the protein kinase, CKII, was identified. Interestingly, a *cka1*Δ mutant is cadmium sensitive, and additional genetic evidence points to the possibility that Cka1p may act by phosphorylating S251 of Ycf1p (118).

In general, the role of phosphorylation in regulating ABC protein function remains unclear. The studies discussed above suggest an important mechanistic role of phosphorylation in both positively and negatively regulating Ycf1p transporter function. Interestingly, many putative Ycf1p phosphorylated residues are highly conserved in human MRPs (118). Thus, findings for Ycf1p phosphorylation at particular sites could pave the way for examining whether mutations that ablate or mimic phosphorylation have a predictable pattern of positive or negative impact on function in mammalian ABCC transporters.

Regulation of Ycf1p function by Tus1p, a guanine nucleotide exchange factor for Rho1p. Multispanning membrane proteins are underrepresented in protein interaction studies because many of the screens employed in those studies are not amenable for use with an integral membrane protein such as Ycf1p. However, interaction partners are just as likely to regulate the activity and function of membrane proteins as they do for soluble proteins. By using iMYTH technology, which is discussed in detail below, we identified a number of potential Ycf1p interactors. Strains bearing deletions of several of these interactors exhibit a decreased level of cadmium resistance compared to the level exhibited by a WT strain (119). One of these is the *TUS1* gene, which encodes a well-characterized cytosolic guanine nucleotide exchange factor for the small GTPase Rho1p (139, 171). We showed that Tus1p is a Rho1p-dependent positive regulator of Ycf1p function (119). Tus1p interacts with Ycf1p via the Tus1p C-terminal region that contains a citron domain, whose function remains uncharacterized.

Previous to our study, cytosolic modulators of the ABC transporters had not been generally considered, because transporter-containing vesicles, nucleotides, and substrate are sufficient to promote ABC transport activity in vitro. However, we showed that the addition of cytosol from a WT *TUS1* strain increases Ycf1p-dependent transport activity by about twofold and requires the presence of GTP. Importantly, this activation fails to occur with cytosol prepared from a *tus1*Δ strain, indicating that Tus1p is required for the observed stimulation of Ycf1p activity (119). In addition, Ycf1p-dependent transport activity was diminished in vacuoles prepared from a *rho1*^{ts} mutant strain and in this case could not be further stimulated by the addition of Tus1p. Taken together, these results suggest that Rho1p stimulates Ycf1p function above the basal level when it is recruited to Ycf1p by Tus1p (119). Rho1p is indeed known to stimulate the activity of two other enzymes in yeast [protein kinase C and β(1-3)-glucan synthase] (16, 17, 91, 110). These studies suggest diverse roles for yeast Rho1p in regulating numerous cellular functions, including Ycf1p activity.

Bpt1p and Ybt1p Functionally Overlap with Ycf1p

Bpt1p and Ybt1p have not been extensively characterized. However, to the extent to which they have been characterized, both appear to have some overlapping function with Ycf1p. As discussed below, both are localized to the vacuole membrane (Fig. 5 and Table 1) and play a role in the cellular detoxification of both endogenous and exogenous compounds (105, 113, 117, 120, 145). However, neither Bpt1p nor Ybt1p undergoes Pep4p-dependent cleavage like Ycf1p (102), and both have dissimilar expression patterns from one another and from that of Ycf1p (145) (see the Saccharomyces Genome Database [SGD] [www.yeastgenome.org/] for expression profiles).

Bpt1p is the closest yeast homolog to Ycf1p and is named for its ability to function as a bile pigment transporter in vitro, an activity which it shares with Ycf1p (120). Bile pigments are degradation products of heme-containing proteins that are excreted by hepatocytes in humans. In vitro transport assays showed that one of these bile pigments, unconjugated bilirubin, can be transported into yeast vacuolar vesicles. Bpt1p and Ycf1p make roughly equal contributions to unconjugated bilirubin transport (120). In addition, gadolinium (Gd)-based MRI contrast reagents, which are also excreted by hepatocytes, can be transported into yeast vacuoles. Both Bpt1 and Ycf1p can mediate their transport, but Bpt1p plays the major role for some contrast agents, while Ycf1p does so for others (117). Finally, Bpt1p also shares the ability to mediate the in vitro transport of free GSH, GSH conjugates (DNP-GS and [^{14}C]*S*-2,4-dinitrobenzeneglutathione {DNB-GS}), and a glucuronide conjugate (E217βG) with Ycf1p, although in all of these cases, the activity of Ycf1p predominates (5, 83, 145). In vivo, Bpt1p contributes to a small extent to cadmium resistance and the formation of the red Ade2 pigment that is associated with Ycf1p (144, 145). A similar pattern of overlapping but nonidentical substrate specificity as seen for yeast Ycf1p and Bpt1p has also been documented for mammalian MRPs, reflecting a recurring theme within the ABCC subfamily in different organisms (5, 13, 33, 66, 125).

Bpt1p does not appear to be regulated by the oxidative stress regulator Yap1p, as is the case for Ycf1p (145). However, the Bpt1p expression level was shown to be strongly elevated upon the entry of yeast into stationary phase (83). Since yeast faces a number of metabolic challenges during high-density growth, it was suggested that the pattern of expression of Bpt1p is consistent with a direct role for Bpt1p in the detoxification of cellular metabolites (83). However, Bpt1p, like Ycf1p, may also protect cells from the products of environmental challenges such as oxidative stress.

Considerably less is known about Ybt1p than Bpt1p. Ybt1p was first named Bat1p (for bile acid transporter) based on the ability of WT yeast vacuoles, but not vacuoles prepared from a *bat1*Δ mutant, to transport bile acids such as taurocholate (113). However, due to a nomenclature conflict, the gene name

was changed to *YBT1* (for yeast bile acid transporter) (see the SGD [www.yeastgenome.org/]). In vivo, Ybt1p has been shown to provide a minor (less than 5%) contribution to the formation of red Ade2 pigment, along with Ycf1p and Bpt1p, indicating that it is capable of transporting GSH conjugates (144, 145). However, other Ybt1p substrates have not been reported.

Vmr1p and Nft1p

Vmr1p and Nft1p are the least characterized of the yeast ABCC transporters. No substrates are known for either one. Preliminary data suggest that both proteins appear to be localized to the vacuolar membrane (Fig. 5 and Table 1) (105), yet both proteins show an additional "punctate" pattern, which may reflect Golgi apparatus localization, endosomal localization, or some other organellar site of localization. The name Vmr1 stands for vacuolar multidrug resistance transporter (D. Wawrzycka, SGD [www.yeastgenome.org/], personal communication); however, no Vmr1p substrates are known to date (56). However, interestingly, recent high-throughput proteomic studies of yeast have found Vmr1p to be associated with both ribosomal complexes and the mitochondria (43, 129, 130).

Nft1p originally presented a confusing picture because in the major laboratory strain of *Saccharomyces cerevisiae*, S288c, it is present as two contiguous ORFs, YKR103w and YKR104w, separated by a nonsense codon. S288c is the strain that was used for the sequencing of the yeast genome. However, in other laboratory strains of *Saccharomyces cerevisiae* and in most other fungal species, YKR103w and YKR104w comprise a single continuous full-length ORF, which we named *NFT1*, for new full-length transporter (102). One potential explanation for the apparently mutated form of *NFT1* in S288c is that the manner in which this strain was cultivated in the laboratory led to unintentional selection against full-length *NFT1* (102). We considered the possibility that the mutation of *NFT1* might provide lithium resistance, which is potentially advantageous for cells subjected to LiCl during transformation experiments. However, no evidence that Nft1p plays a role in LiCl resistance was found. Indeed, no function has yet been ascribed to Nft1p. One way to learn about the activities of the *NFT1* and *VMR1* gene products would be to challenge strains overexpressing these genes with an arsenal of toxic compounds and determine if resistance occurs in any case.

Yor1p, a Short ABCC at the Plasma Membrane That Mediates Pleiotropic Drug Resistance

In vivo studies of Yor1p activity. Yor1p is the second-most-studied yeast ABCC. Yor1p is atypical among the yeast ABCCs in that it is the only "short" ABCC and the only one thus far that localizes to the plasma membrane (Fig. 2 and 3 and Table 1) (75, 76). Whether these two characteristics are related is unclear. The name Yor1p (for yeast oligomycin resistance) reflects that fact that the *YOR1* gene was discovered based on its ability to confer resistance to the mitochondrial poison oligomycin when overexpressed and to confer sensitivity when deleted (76). Early on, Yor1p overexpression was also shown to confer resistance to the anionic antifungal drug reveromycin A and the anionic fluorescent compound rhodamine B, leading to the suggestion that Yor1p was a multispecific organic anion transporter (23). However, a large-scale study of several hundred compounds identified many more Yor1p substrates and indicated that Yor1p could transport an impressive variety of compounds with quite distinct chemical features (85, 134). Thus, Yor1p is now more appropriately considered to be a pleiotropic drug transporter.

Indeed, Yor1p is now viewed as being one of the three major pleiotropic drug transporters in yeast (Yor1p, Pdr5p, and Snq2p) that comprise the pleiotropic drug resistance network (57, 84, 85, 147). Yor1p is coregulated with Pdr5 and Snq2 by the positively acting transcription factors Pdr1p and Pdr3p, and it exhibits overlapping substrate specificity with Pdr5p and Snq2p but also has distinct substrate preferences (134). It has become quite common to delete these three transporters, Yor1p, Pdr5p, and Snq2p, yielding a strain called AD1-3 (also called AD123), to sensitize strains to various drugs for the purpose of conducting high-throughput drug screens (95, 134). One such example involved using a strain with deletions of these three genes to screen the effects of estrogenic compounds on signaling through the estrogen receptor (59). In other cases, it has been advantageous to use a strain with deletions of eight of the major ABC transporters, called AD1-8 (or AD12345678) (31, 111, 134). Both AD1-3 and AD1-8 have been used to examine the cellular uptake of the yeast vital stain diS-C3(3), a substrate for Yor1p, Pdr5p, and Snq2p. The cellular uptake and efflux of this dye permit the analysis of pump function in vivo by simple measurements of fluorescence intensity. In this assay, strains AD1-3 and AD1-8 show a high level of accumulation of dye in comparison to their WT counterparts due to a diminished efflux capacity (44).

An interesting issue is whether Yor1p, like Ycf1p, is a GS-X transporter. Data from a recent report showed that Yor1p confers resistance to Cd^{2+} but only at 23°C and not at higher temperatures (111) (although it should be noted that an early study implicated Yor1p in Cd^{2+} tolerance even at 30°C [23]; however, this finding could not be repeated by others). In contrast, Ycf1p confers Cd^{2+} resistance at all temperatures. Interestingly, the overexpression of Yor1p can suppress a *ycf1*Δ Cd^{2+} sensitivity phenotype at 23°C (111). Furthermore, the Yor1p-mediated efflux of cadmium is accompanied by the efflux of GSH, suggesting that Yor1p transports both together as a Cd-$(GS)_2$ complex, just as Ycf1p does. Thus, because Yor1p lacks MSD0, it is tantalizing to suggest that the MSD0 of ABCC transporters is not required for GSH conjugate recognition.

Biochemical analysis of Yor1p. For biochemical studies, a strain called SUPERYOR has been useful, in which Yor1p comprises roughly 10% of the total protein in the plasma membrane due to its overexpression (in strain AD1-8), so that Yor1p is the major ABC transporter present (31, 55). In membrane preparations from this strain, Yor1p could be photolabeled by azido-ATP and exhibits ATPase activity that is inhibited by vanadate, as is the case for other ABC transporters. Furthermore, mutations in NBD2 of Yor1p reduced the level of activity to that measured for substrates derived from a *yor1*Δ mutant (55). However, unlike some other ABC transporters, Yor1p ATPase activity was inhibited, rather than stimulated, upon the addition of substrates, an observation also made for yeast Pdr5p (31, 38, 55, 83). It was speculated that an unknown

Yor1p substrate might be present in membrane preparations and that an added substrate could act as a competitor, leading to a reduction in the rate of ATP hydrolysis (55). Alternatively, some of the Yor1p substrates tested may have dual binding sites within the pore and the NBDs, and therefore, the binding of the substrate to the transporter would also inhibit the ATPase activity of the NBDs (55). An interesting yet unclear finding is that Yor1p can be phosphorylated in vitro, as observed upon the incubation of Yor1p-containing membranes with [γ-^{32}P]ATP (32). Whether phosphorylation occurs in vivo, and, if so, whether it is stimulatory or inhibitory, has not been examined.

Trafficking, folding, and assembly of Yor1p. Yor1p has proven to be an excellent model protein for analyzing the processes of ER exit and ER-associated degradation (75, 116). Yor1p has two diacidic (DxE) ER exit motifs, one within the NTE and the second at the C terminus (35). The N-terminal DxE is the dominant one of the two motifs. When this DxE is deleted, Yor1p is retained in the ER in vivo and cannot be packaged into CopII-coated ER-to-Golgi membrane transport vesicles in vitro (116). The DxE motif of Yor1p is needed to interact with the "B"-site cargo recognition domain of the CopII subunit Sec24p (116).

The Yor1p-ΔF670 mutant (equivalent to the cystic fibrosis mutation CFTR-ΔF508) results in ER retention followed by ER-associated degradation (75). The ER retention of Yor1p-ΔF670 is due to its misfolding, as assayed by limited-proteolysis, cross-linking, and gel migration assays (116). Ultimately, ER-retained Yor1p-ΔF670 is degraded by the ubiquitin-proteasome system. Interestingly, when Yor1p-ΔF670 is stabilized, by preventing its degradation in a mutant (*ubc7*Δ) defective for its ubiquitination, it still cannot exit the ER and is precluded from entering CopII vesicles in an in vitro packaging assay. It is possible that chaperones bound to misfolded Yor1p-ΔF670 may block the access of its DxE ER exit motif to the B site of Sec24p and, hence, inhibit its ER exit (116). An understanding of precisely how Yor1p-ΔF670 engages the ER quality control checkpoint may eventually shed light onto mechanisms for releasing ER-trapped CFTR-ΔF508 into the plasma membrane, which in turn could potentially provide an important clue to treatments that might reduce the symptoms of cystic fibrosis.

In addition to providing insights into protein trafficking and quality control, an understanding of how Yor1p is folded and assembled is likely to provide principles that are generalizable for all ABC proteins. To this end, Pagant and coworkers (115) have been attempting to genetically and biochemically define the intramolecular interactions that contribute to the tertiary assembly of Yor1p. Using chemical cross-linking, they defined interactions between the NBDs of Yor1p and between the NBDs and specific intracellular loops (CLs) that are consistent with current structural models of bacterial ABC transporters. Furthermore, starting with a *yor1* mutation that alters an intracellular loop (CL2) and causes oligomycin sensitivity due to the misassembly of Yor1p, intragenic suppressors that restored oligomycin resistance were found. These suppressing mutations were shown to restore multiple interdomain interfaces (115). In addition to providing a greater understanding of ABC transporter architecture, the continued application of this approach is also expected to reveal the molecular components and mechanisms that govern ER retention and quality control for ABC proteins, which, at this point, are largely unknown.

iMYTH TECHNOLOGY, A POWERFUL TOOL FOR IDENTIFYING ABC INTERACTORS IN YEAST AND POSSIBLY HUMANS: YCF1 AS A TESTING GROUND FOR THE VALIDITY OF iMYTH TECHNOLOGY

Considering the importance of the ABC transporter family in diverse diseases and multidrug resistance, obtaining an understanding of the function and mechanism of action of these proteins is of crucial importance. One of the major steps toward obtaining this understanding is through the elucidation of the complete ABC transporter interactome. The identification of the interaction partners of various ABC transporters will provide valuable information about the specific pathways in which these proteins are involved as well as the elements involved in the regulation and mediation of their function. Constructing such an interactome, however, is particularly challenging since the hydrophobic nature of these multispanning membrane proteins makes them recalcitrant to analysis using conventional interaction assays, particularly those which are amenable to use in a high-throughput format.

A powerful tool traditionally used for performing large-scale in vivo analysis of protein-protein interactions is the YTH system (42, 152). The YTH system exploits the modular nature of eukaryotic transcription factors. A "bait" protein is fused to the DNA-binding domain and a "prey" protein is fused to the activation domain of a transcription factor. Neither portion is functional on its own, but a functional transcription factor can be reconstituted upon physical interactions between the bait and prey, resulting in the activation of a reporter system and typically yielding the expression of β-galactosidase or His3p. Major advantages of this classical YTH system include the ability to rapidly screen for interactions within the natural environment of a cell as well as facile determinations of the DNA encoding any detected interaction partners. Because the assay relies on the reconstitution of a protein-protein interaction inside the nucleus of the cell, however, it requires that the proteins be both soluble and targeted to the nucleus. As such, it is poorly suited for the screening of membrane proteins, which, due to their hydrophobic nature, tend to form insoluble aggregates outside of the membrane environment (150). Thus, a different approach is required for the meaningful analysis of membrane protein interactions.

One such approach is the MYTH system, which has recently been developed into a powerful alternative to the traditional YTH system, retaining all of the latter method's advantages yet being suitable for the analysis of proteins localized to a membrane environment (47, 71, 81, 119, 138, 151, 156) (Fig. 7). The MYTH system is based upon the concept of "split ubiquitin." Ubiquitin is a highly conserved, 76-amino-acid protein that is covalently linked to the lysine residues of substrate proteins, thereby regulating their function and/or targeting them for degradation (99). Research has shown that ubiquitin can be expressed as two separate N- and C-terminal fragments (termed N_{ub} and C_{ub}, respectively) capable of interacting and reconstituting a quasinative molecule (73). This "pseudoubiquitin" is similar enough to native ubiquitin that it can be recognized by cytosolic deubiquitinating enzymes (DUBs), a class

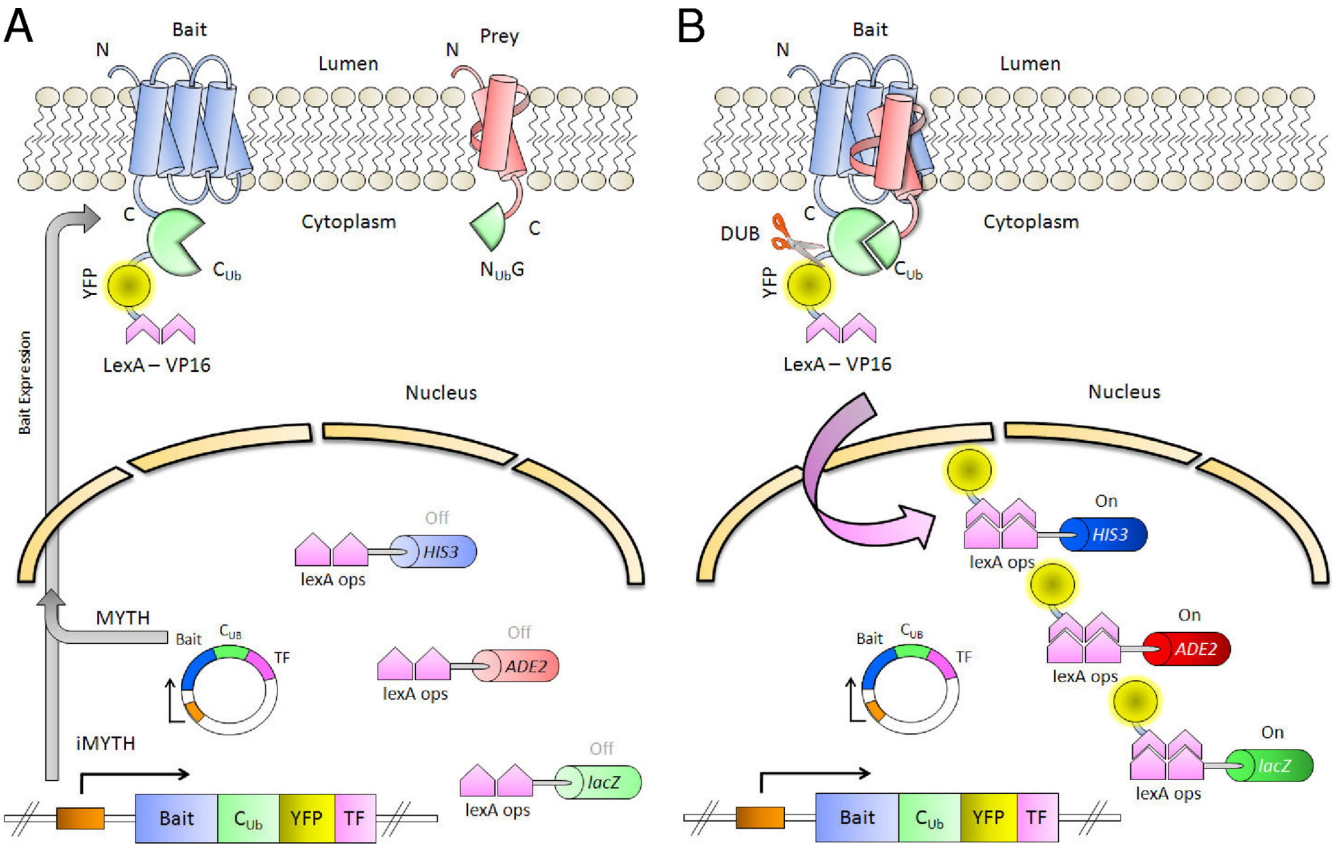

FIG. 7. Outline of the iMYTH system. (A) The bait, a membrane protein of interest (in blue), is fused to the C-terminal moiety of ubiquitin (C_{Ub}), yellow fluorescent protein (YFP), and transcription factor LexA-VP16. The prey protein (in red) is fused to the N-terminal moiety of ubiquitin ($N_{Ub}G$). Both traditional MYTH (cassette on an exogenous plasmid) and iMYTH (genomically integrated cassette) systems are illustrated. The promoter is shown as an orange box. (B) If the bait and prey interact, the half-ubiquitin moieties reconstitute into a "pseudo-ubiquitin." Cytosolic DUBs recognize this "pseudoubiquitin" and cleave its C-terminal end, releasing the transcription factor into the nucleus. The transcription factor binds to the LexA operator sites (lexA ops) and activates the reporter genes *HIS3*, *ADE2*, and *lacZ*.

of proteases responsible for deconjugating ubiquitin from modified proteins via the hydrolysis of the amide bond formed between the protein and the C-terminal residue of ubiquitin (73, 99). The spontaneous reconstitution of N_{ub} and C_{ub} can be disrupted by a mutation of the isoleucine 13 residue in the N_{ub} fragment to glycine, forming a fragment referred to as $N_{ub}G$. An interaction between proteins covalently attached to the $N_{ub}G$ and C_{ub} fragments, however, is sufficient to allow the reconstitution of the quasinative ubiquitin, thus allowing the system to be adapted for use as a "sensor" of protein-protein interactions (73).

In the MYTH system, the C_{ub} moiety is fused to a reporter molecule, "TF," consisting of the *Escherichia coli* DNA-binding protein LexA fused to the transcriptional activation domain of VP16 from herpes simplex virus. This TF molecule is capable of activating the transcription of reporter genes (typically *HIS3*, *lacZ*, and *ADE2*) under the control of promoters containing LexA-binding sites (Fig. 7) (71, 81, 151). "Baits" are generated by the fusion of C_{ub}-TF to the C terminus of a membrane-anchored protein of interest, which prevents TF from diffusing into the nucleus and activating the transcription of the reporter genes (151). "Preys," in the form of either specifically selected proteins or entire libraries, are generated by the fusion of the $N_{ub}G$ moiety to their N or C termini (71, 81). Yeast cells expressing the bait are transformed with prey constructs and plated onto selective medium. Cells in which particular bait and prey proteins interact reconstitute the "pseudoubiquitin," leading to DUB recognition and the proteolytic release of TF, which can then enter the nucleus and activate reporter gene transcription, thereby allowing growth on selective medium and subsequent verification using an X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) screen (71, 81, 151). There are currently two major forms of the MYTH system, which are distinguished primarily by whether the bait is expressed ectopically from a plasmid (traditional MYTH system) or endogenously under the control of its native promoter (iMYTH screening). The latter is suitable only for the screening of yeast proteins but provides the advantage of maintaining WT expression levels, which helps reduce the number of false positives, a problem inherent in any YTH assay (71, 81, 119). The iMYTH system is also currently configured with the option of using a C_{ub}-yellow fluorescent protein-TF (CYT) tag instead the traditional C_{ub}-TF tag, allowing easier verification of proper bait localization within the cell (81, 119). A detailed overview of iMYTH and traditional MYTH systems is provided in Fig. 7. A recent study demonstrated the utility of using the iMYTH approach to detect interacting proteins of ABC transporters. In that work, the

yeast ABC transporter Ycf1p was screened using an iMYTH approach with endogenous CYT tagging. We found that the use of iMYTH was necessary in order to prevent the strong, nonspecific self-activation of the Ycf1p bait (presumably a result of its mislocalization and misfolding) previously observed when this transporter was overexpressed from a plasmid in a traditional MYTH screen (108, 119). We showed that when expressed from its endogenous promoter, this transporter was present at WT levels and was properly localized to the vacuolar membrane. Additionally, the ability of Ycf1p-CYT to confer cadmium resistance to yeast cells was also measured and was shown to be unaffected by the presence of the C-terminal tag.

A full iMYTH screen using Ycf1p-CYT as a bait and a genomic DNA prey library consisting of genomic DNA fragments (500 to 2,000 bp in length) inserted upstream of an $N_{ub}G$ sequence detected a total of six candidate proteins, which interacted specifically with Ycf1p and not with an unrelated vacuolar membrane bait (Vps55-CYT). These proteins included Tus1p (a cytosolic guanine nucleotide exchange factor for Rho1p), Fab1p (a vacuolar membrane kinase involved in vacuolar sorting), Psa1p (a cytosolic GDP-mannose pyrophosphorylase needed for normal cell wall structure), Num1p (a cytosolic protein involved in nuclear migration and mediating microtubular interactions), Kin4p (a cytosolic protein kinase), and YDR115w (unknown function). We then attempted to establish the biological relevance of the observed interactions. Intriguingly, deletion mutants of Tus1p, Num1p, and Fab1p displayed reduced resistance to cadmium and arsenite, providing strong evidence that they work in concert with, or positively regulate, Ycf1p function (119). Additional experiments with Tus1p also showed that it appeared to be involved in the formation of the red color in *ade2*Δ cells, a phenotype known to be associated with the Ycf1p-mediated uptake of an adenine biosynthetic intermediate into the vacuole (18, 118, 119, 145), as discussed above. Vacuolar uptake experiments using a radiolabeled substrate also revealed that the addition of cytosolic extracts from cells expressing Tus1p stimulated Ycf1p-mediated uptake in a GTP-dependent manner. No such stimulation was observed when cytosolic extracts from *tus1*Δ cells were used. Purified Tus1p was also observed to enhance Ycf1p-mediated uptake into vacuoles (119). This enhancement was dependent upon the presence of functional Rho1p, a membrane-bound GTPase known to be activated by Tus1p, in cells from which the vacuoles were isolated, indicating that Tus1p exerts its effect on Ycf1p via Rho1p GTPase (119, 139). A physical interaction between Tus1p and Ycf1p was also demonstrated by using immunoprecipitation experiments with the unrelated vacuolar ABC transporter Ybt1p as a noninteracting control. The iMYTH approach was also used with a variety of Tus1p truncation mutants as baits, mapping the Ycf1p interaction site to the C-terminal citron-like domain (119).

It is therefore readily apparent that the iMYTH system represents a powerful tool for the detection of membrane protein interactions and appears to be particularly well suited for the study of ABC transporters. Work using Ycf1p as a bait protein clearly illustrates the robustness of the iMYTH system in detecting interactions of biological relevance as well as its adaptability for use in other in vivo interaction experiments, such as for the determination of specific interaction regions between proteins. Our laboratory is currently in the process of using the iMYTH approach to determine the ABC transporter interactome in the yeast *Saccharomyces cerevisiae*. Of the 22 membrane-bound ABC proteins in yeast, 19 are predicted to be suitable for iMYTH analysis (the remaining 3 proteins are reportedly localized to the mitochondrial inner membrane, where they are not accessible to cystolic DUBs) (Table 1 and Fig. 5). Currently, we have constructed endogenous, C-terminally-tagged C_{ub}-TF and C_{ub}-CYT baits from all 19 of these transporters in a total of three different strain backgrounds. Localization studies and screening of these baits, using both genomic and cDNA $N_{ub}G$ libraries, have recently been completed in our laboratory. Preliminary results have already detected a number of intriguing interactions, and the final, completely constructed interactome promises to provide a host of valuable insights into the function and regulation of ABC transporter proteins as a whole.

YEAST ABCs, AN IMPORTANT ROLE IN ABC TRANSPORTER RESEARCH

The study of the yeast ABC transporters has played a integral part in expanding our current understanding of the cellular function of ABC transporters and the mechanisms by which their function is regulated (expression, posttranslational modification, trafficking, and/or localization). Methodological advances in yeast genetics and biochemistry, such as iMYTH, will keep yeast, as a model organism, at the forefront of all aspects of ABC transporter research, from cellular biology and biochemistry to pharmacology and metabolism. To this end, yeast will continue to be an important tool in aiding us in our understanding of the role of ABC transporters in human health and disease.

In this review we have discussed the current state of ABCC research in *Saccharomyces cerevisiae* and have described our current understanding of the biochemistry and cellular biology of each member of the ABCC subfamily. We have also described how a new YTH system called iMYTH, which is designed specifically to identify protein interactors for membrane-bound proteins, has resulted in the identification of new functional regulators of the yeast ABCC transporter, Ycf1p. The application of this technology to other members of the ABC transporter family will potentially prove extremely useful in identifying protein interactors that may regulate protein function and/or protein localization. We believe that our work and the work of others using yeast as a tool to determine the role of ABC transporters in cellular metabolism and human health and disease are only in their infancy. It is reasonable to assume that the research carried out on the yeast ABC transporters will continue to make important contributions and will play a major role in directing current and future studies.

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