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REVIEW ARTICLE

Protein-facilitated transport of hydrophobic molecules across the yeast plasma membrane

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In yeasts, the plasma membrane forms the barrier that protects the cell from the outside world, but also gathers and keeps valuable compounds inside. Although it is often suggested that hydrophobic molecules surpass this checkpoint by simple diffusion, it now becomes evident that protein-facilitated transport mechanisms allow for selective import and export of triglycerides, fatty acids, alkanes, and sterols in yeasts. During biomass production, hydrophobic carbon sources enter and exit the cell efficiently in a strictly regulated manner that helps avoid toxicity. Furthermore, various molecules, such as yeast pheromones, secondary metabolites and xenobiotics, are exported to ensure cell–cell communication, or increase chances of survival. This review summarizes the current knowledge on how hydrophobic compounds interact with protein-facilitated transport systems on the plasma membrane and how selective import and export across the yeast plasma membrane is achieved. Both the model organism *Saccharomyces cerevisiae*, as well as unconventional yeasts are discussed.

Keywords: alkane; drug; fatty acid; hydrophobic compound; mating factor; plasma membrane; sterol; transport; xenobiotic; yeast

This review tries to answer a – at first sight – straightforward question: 'how do lipidic molecules cross the cytoplasmic membrane of the yeast cell?'. For a long time, it was assumed that passive diffusion over biological membranes was the preferred means of transport for small molecules, but meanwhile it became clear that specific mechanisms and transporter proteins play a major role. For instance, glycerol uptake is governed by the Stl1p symporter in *Saccharomyces cerevisiae* [1]; and even in the efflux of the smaller ethanol molecule active transport is involved, although one could not yet identify the specific transporter(s) [2,3]. For hydrophobic compounds that interact with membrane components, the assumption of passive diffusion is even stronger; simple intercalation with the lipidic membrane compounds is expected to lead to intracellular accumulation [4]. Yet, 20 out of the 48 human ATP-Binding Cassette (ABC) transporters were proven to facilitate lipid-related transport [5]. Quite often, the lipophilicity characteristics of a compound are used to predict their accumulation in biological cells or tissues (e.g. LogP values). Again, this approach ignores the importance of carrier-mediated transport, which can lead to discrepancies of 100-fold or more [6].

Spontaneous crossing of hydrophobic molecules over the lipid bilayer can indeed be observed when studying artificial liposomes. A proportion of the lipidic molecules that come in contact with the

Abbreviations
ABC, ATP-binding cassette; DHA, drug/H^+ antiporter; FABP, fatty acid-binding proteins; FATP, fatty acid transport proteins; LCB, long-chain bases; MDR, multidrug resistance; PDR, pleiotropic drugs resistance; PRY, pathogen-related yeast.

phospholipid bilayers do diffuse past the polar head region. Two mechanisms allow overcoming the increased order of water molecules around the polar lipid head groups originating from increased-affinity hydrogen bonding and the reduced diffusion rate that goes along with it: the transient channel; and the solubility diffusion mechanism [7]. However, artifacts leading to transport need to be considered with this artificial liposome system; for example, the residual solvent reservoirs that provide a vehicle for transport or the aqueous pore defects which do not occur in biomembranes [8]. Hence, the relevance of artificial liposomes in the study of transport across biomembranes should be questioned. When looking at biomembranes, one recognizes that the majority of the hydrophobic molecules remain in the extracellular milieu if not for the action of a transport protein [6]. In biological membranes, the protein content is not to be underestimated since the protein : phospholipid mass ratio is as high as 4:1. This discrepancy clearly indicates that extrapolation from liposome studies to genuine biomembranes is not that straightforward [9]. Several studies recognize specific proteins with the dedicated function to regulate lipid crossing, proving that it is not a matter of simple equilibrium [10]. The artificial liposome model also ignores composition variation in lipid membranes itself; whereas cells invest energy to maintain asymmetry in the two leaflets of the phospholipid bilayer [11]. This observation is not consistent with a purely passive role for the membrane border. Furthermore, the importance of transport proteins is illustrated by several human disorders where lipidic transport anomalies are the root cause, such as Tangier disease, Stargardt syndrome, progressive familial intrahepatic cholestasis, pseudoxanthoma elasticum, adrenoleukodystrophy or sitosterolemia [12].

The examples above demonstrate the significance of protein-mediated transport of small molecules across the cytoplasmic membrane, including the import and export of hydrophobic or lipidic compounds. Although membrane transporters are involved in one-third of the reconstructed metabolic networks in yeast, they are still widely neglected [13]. Even in well-studied model organisms, (predicted) transporters remain orphans in terms of experimental assessment [14]. So far, only 26 crystal structures of the model yeast *S. cerevisiae* transporters have been obtained although they are predicted to make up 5% of the 6237-members counting proteome (Transport Classification Database consulted for crystal structures at January 6, 2019) [15,16]. The reason for this experimental lacuna is multifold. Producing, purifying, and crystallizing these highly dynamic membrane proteins to obtain high-resolution structures is extremely difficult. Functional studies are also not legion because often, passive diffusion is presumed to be the primary process and the analytical methods to measure fluxes across a biological membrane are complex. The fact that no actual biochemical conversion takes place (compound A remains compound A, and hence the conversion into product B cannot be measured) requires different approaches compared to the classical biocatalytical ones.

Hydrophobic substrates entail in practice extra difficulty to perform transport studies. Their aqueous insolubility necessitates the use of detergents or lipidic environments. These can differ substantially in experimental set-up, affecting dose–response behavior of the studied transporter. The detergents in turn, could affect the mode of interaction between substrate and transporter [17]. Furthermore, the activity of ABC transporters is influenced by the presence or absence of certain membrane lipids; various lipids and detergents used in ATPase assays to reconstitute artificial membranes are (putative) substrates for the transporter under study [18]. In some cases, lipids are needed for structural stabilization either as integral part of the transporter or more indirect, by forging the membrane fluidity and lateral membrane pressure [19]. All of the above makes transport assays with hydrophobic substrates extremely difficult and hard to reproduce.

In this review, transport of hydrophobic substrates over the yeast plasma membrane will be described. For details on intracellular transport of these compounds, we direct the reader to other dedicated reviews [20]. A division is made based on the direction of flux, 'inward' and 'outward' systems respectively. However, it should be noted that this division is strictly artificial; theoretically every reaction is thermodynamically reversible. Transport systems are divided here according to their natural function in standard physiological conditions.

Adaptation at the interphase

As a consequence of the poor solubility of lipophilic molecules in water, their concentration in extracellular environment is very low. This makes growth on them as sole carbon source not a simple undertaking and further adjustments by hydrocarbon-degraders are necessary. One strategy to promote the accessibility of these substrates is the secretion of emulsifying agents, although this strategy is not prevalent in all hydrocarbon-degrading yeasts [21]. In the oleaginous yeast *Yarrowia lipolytica*, for example, the production of the bioemulsifier liposan was described [22,23]. Another

strategy to improve direct contact with hydrocarbons for assimilation is to modify the cell adhesion properties. This can include the formation of protrusions at the cell surface; a decrease in the thickness of the cell wall and periplasmic space; changes in the membrane fatty acid composition and, therefore, fluidity; invagination of the membrane; changes in the membrane protein content; and formation of electron-dense channels to the ER [24].

Protrusion-forming structures have first been observed during growth on *n*-alkanes of *Candida tropicalis* and *Candida maltosa* by Osumi and colleagues in 1975 [25]. Also, in *Y. lipolytica*, it was shown that the number of protrusions increased with time of incubation with fatty acids (oleic acid) or alkanes (decane, hexadecane), but decreased in the presence of glucose [26,27]. Simultaneously, the number of surface-bound lipid droplets increased during growth, whereas the size of the droplets decreased, proving the direct interfacial transport and importance of protrusions for a good interaction between the cell and the hydrophobic substrates. The protrusions are 'slime-like' outgrowths of 50 nm high by 150 nm wide that reach the cell membrane through electron dense channels. Directly underneath the cell membrane surface beneath these outgrowths, the ER is located. This led to the hypothesis that the protrusions serve as docking platform for lipid droplets, from which the *n*-alkanes adhere and are subsequently transported to the ER, where they are hydroxylated to fatty alcohols. The actual uptake by endocytosis and protrusions might still require the coordinated action of transporter proteins tethering in these regions, or the occurrence of channels [23]. Hydrocarbon droplets could no longer adhere to the cells after pronase treatment, which demonstrates the involvement of polysaccharide-fatty acid complexes on the surface [28].

During protrusion formation in *Y. lipolytica*, a decrease in cell wall thickness from 40 to 25 nm occurs [26]. This adaptation is necessary to overcome its main function as barrier; it acts as a highly charged molecular sieve that is closely connected to the lipid bilayer and shields the cell from drastic changes in the intracellular homeostasis [29]. The mannoproteins that are mostly found in the outer layer of the cell wall, carry negatively charged phosphate and carboxyl groups which give the cell wall the virtue of acting as ion exchanger [30]. Hence, the cell is safeguarded from the entrance of toxic molecules with this first selection based on size and charge. A fine example of this biosorption is the protection against toxicity caused by heavy metals [31]. The cell wall armor provides the cell with some -literal- elasticity against environmental changes or extracellular product accumulation e.g. high ethanol production [32]. The intimate relation between cell wall and plasma membrane governed by GPI-anchored glycoproteins may explain that both the cell wall integrity and the Pleiotropic Drugs Resistance (PDR) pathway are upregulated after exposure to organic solvents [33]. The cell wall proteins Wsc3p, Pir1p, Ynl90wp, and Pry3p, for example, provide aid in the *n*-decane and *n*-undecane relief in *S. cerevisiae* [34]. They are all involved cell wall maintenance; Wsc3p and Pir1p in a direct manner by signaling to cell wall integrity pathways. Ynl190wp in its role as a hydrophilin, potentially creating a more hydrophilic environment and therefore fortifying the cell wall barrier against organic solvents [34]. In the case of Pry3p, the protective function is an indirect result of its role in yeast mating, which also requires remodeling of the cell wall and fusion of the plasma membranes [35].

The plasma membrane lipid composition is far from homogenous and several transporters are deployed to maintain asymmetry in the phospholipid bilayer of biological membranes (as reviewed in ref. [36]). Primary active transporters such as ABC transporters 'flip-flop' phospholipids in the bilayer. Secondary active transporters belonging to the multidrug, oligosaccharidyl-lipid, polysaccharide superfamily transfer a range of molecules in exchange for H^+ or Na^+. Third, scramblases function bidirectionally without energy requirement [17,37]. Experimental studies for transport systems in yeast are less abundant, despite the clear importance of phospholipid distribution in terms of plasma membrane fluidity and the impact it has on transport. The overabundance of phosphatidylethanolamine, for example, increases membrane rigidity. This kind of lipid imbalance was illustrated by the plasma membrane-localized transporter *CDR6/ROA1* null mutants of *C. albicans* resulting in an increased resistance to azoles [38]. Furthermore, the so-called 'lipid rafts' are formed where significant quantities of glycosphingolipids, sterols, and glycosylphosphatidylinositol-anchored proteins pile up. Embedded in the biological membrane, these highly specialized domains compartmentalize cellular functions, such as the processing of surface proteins. Here, Pdr10, for example, one of the major *S. cerevisiae* multidrug resistance (MDR) efflux pumps is associated with lipid raft-like domains [39]. The same goes for Aus1p, Pdr11p and their associative proteins needed for sterol uptake in *S. cerevisiae* in anaerobic conditions [40].

Uptake of lipophilic compounds

Triglycerides and fatty acid uptake

Triglycerides are the predominant hydrophobic carbon source for organisms ranging from bacteria to yeast and multicellular eukaryotes. Even more, they are important intracellular storage components for many of these organisms. Remarkably however, no direct triglyceride import mechanism is known. Access to this carbon source relies on the secretion of extracellular lipases, which hydrolyze the triglycerides to glycerol and free fatty acids [41]. Subsequently, the separate components are taken up by the cell. Growth on triglycerides is no evident property among yeasts. The model yeast *S. cerevisiae,* for instance, cannot catabolize these oils because it does not secrete lipases [42]. By contrast, the oleaginous yeast *Y. lipolytica* produces as many as 16 cell wall-bound lipases or lipases that are secreted once the carbon source becomes scarce in the medium [43]. Higher multicellular organisms have developed more sophisticated methods for triglyceride trafficking among tissues, which involve binding to proteins such as albumin and apolipoproteins. However, also in these organisms, triglycerides must be converted into free fatty acids prior to cell entry [44].

As opposed to the fatty acids entangled in triglycerides, exogenous free fatty acids are more readily taken up. Yet, how they traverse the plasma membrane is not universally agreed upon. The uptake is widely considered to be dual, both in yeasts and mammalian cells. On the one hand, there might be a passive process based on diffusion that predominates at high concentrations. On the other hand, there might also be an active protein-mediated process [45–47]. However, as mentioned in the introduction, recent reviews were published that contradict this notion quite strongly, asserting that membrane passage is only carrier-mediated [14]. The concept of active protein-mediated transport of free fatty acids is supported by several facts: (a) fatty acid transport is inducible; (b) it is protease sensitive, or can be blocked with antibodies; (c) the 'flip' between the two surfaces of the membrane is rate-limiting; and (d) cell types with high levels of fatty acid metabolism transport at higher rates [48,49].

In mammalian cells, this protein-facilitated process involves at least four different protein systems, including CD36, caveolin, plasma membrane fatty-acid-binding protein (FABPpm), and fatty acid transport proteins (FATP) [50]. The exact mode of action is not fully understood and is complicated by the proteins' other biochemical activities that are distinct from their role in transport. In the yeast model organism *S. cerevisiae*, orthologs to one of these protein systems are found: the fatty acid transport proteins Fat1p and Fat2p. Fat1p is no traditional transporter, but combines the uptake of long-chain fatty acids with an acyl-CoA-synthetase activity required for activation of very long chain-fatty acids (>C22), which are generally not transported into the cell but are synthesized *de novo* [48]. ΔScfat1 cells are still able to grow on medium-chain fatty acids, suggesting that multiple length-specific systems are involved in fatty acid import [51]. Fat2p is located in the peroxisome, and its role in fatty acid import is less clear. Once imported, the fate of fatty acids is diverse. In general, activation by acetyl-CoA is required. This obtained acyl-CoA pool can be applied to generate energy by means of β-oxidation; provide building blocks for other hydrophobic compounds, such as phospholipids, sterol esters, etc.; or be, again, converted into the storage component triglycerides [44,52]. Acetyl-CoA activation is performed mainly by *FAA1* and the partially redundant *FAA4* gene products in *S. cerevisiae* immediately after uptake of exogenous fatty acids [53]. For *Escherichia coli*, it was shown that acyl-CoA synthetase operates in conjunction with the membrane-bound uptake protein FadL. In this regard, the fatty acid activation is the main driver in the uptake and described as 'vectorial acylation'. The acyl-CoA synthetase activity influences the transport across the membrane, proposedly by abstraction [54]. It converts a hydrophobic substrate into a water-soluble CoA ester concomitantly trapping the substrate in the cytoplasm and influencing its subcellular distribution. *S. cerevisiae* has a comparable system where import is coupled to activation, as indicated by the dual function of Fat1p, as well as by the concerted function of Fat1p and Faa1p [48]. Zou *et al.* [55] further substantiated this hypothesis by confirming the co-localization of Fat1p and Faa1p at the yeast plasma membrane. Furthermore, just as in mammalian cells, fatty acid uptake in yeasts relies at least partially on protein-mediated endocytosis. *S. cerevisiae* strains Δypk1, Δend3, Δvrp1 or Δsrv2, all exhibiting defects in actin remodeling and therefore endocytosis, display reduced levels of fatty acid import. A working model for fatty acid uptake is proposed by Jacquier and colleagues in which fatty acids are first internalized in an energy- and Ypk1-dependent endocytotic step before they are activated by Faa1p, Faa4p or Fat1p (see Figure 1) [50].

Fat1p of the sophorolipid-producing yeast *Starmerella bombicola* seems to have a similar function as the

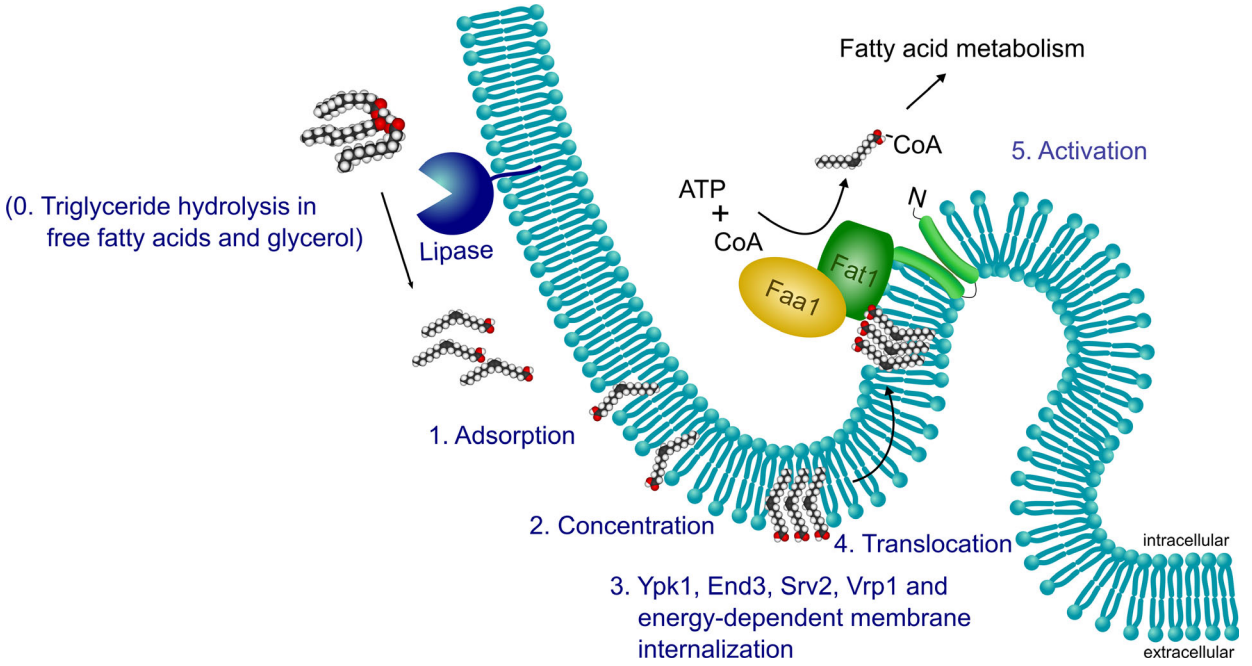

Fig. 1. Working model for fatty acid uptake as proposed by Jacquier et al. [50]. Triglycerides are hydrolyzed by cell wall-bound or secreted lipases in selected yeasts. Free fatty acids adsorb to the plasma membrane and concentrate. In an energy and actin-organizing protein (Ypk1, End3, Srv2, and Vrp1)-dependent step, endocytosis occurs before fatty acids are translocated and then CoA-activated by the concerted function of Fat1p and Faa1/Faa4.

one in *S. cerevisiae*; deletion-mutants display impaired growth on minimal medium containing fatty acids and a significant reduction in the uptake of fluorescent fatty acid analogs [56]. However, FATP function does not seem to be unconditionally conserved. The oleaginous yeast *Y. lipolytica*, for instance, possesses a clear Fat1p homolog, but this protein is not essential for growth on fatty acids. Rather a task in mobilization of fatty acids from lipid bodies was suggested. This could be explained by a more restricted localization pattern, caused by the absence of a second N-terminal transmembrane region in Ylfat1 [57]. In this yeast, two carrier systems are suggested to be responsible for active fatty acid uptake; one specific for C12 and C14 fatty acids, and a second one, specific for C16 and C18 fatty acids [45]. Both systems display a higher affinity to unsaturated fatty acids as compared to saturated ones [58]. Nevertheless, the corresponding genes are yet to be identified. YlFaa1 is the principal player in vectorial acylation, but also executes fatty acid elongation and desaturation, functions not associated with ScFaa1 [59]. Notably, the fatty acid chain length should be at least C9, because growth of *Y. lipolytica* is no longer observed with shorter-chain fatty acids, due to toxicity [60]. Evidence for *Y. lipolytica* points toward a similar endocytosis working model as in *S. cerevisiae*; changing the medium from a glucose to oleic acid-based carbon source resulted in a shift in protein expression toward upregulation of proteins involved in targeting and fusion processes of transport vesicles [61].

Alkane uptake

The assimilation of alkanes is no common feature attributed to the fungal kingdom in general, or yeasts specifically. Nevertheless, some yeast species adapted to survival in alkane-enriched environments. These are not necessarily associated with oil-contaminated sites, but can also relate to the wax layer of plant or insect cuticles. Hence, degradation of alkanes can be an important first step in colonization. The alkane degrading pathway in yeasts is characterized by the presence of cytochrome P450 monooxygenase genes of the CYP52 family; only yeasts possessing these enzymes are able to thrive on alkanes as the sole carbon source. The pathway is absent in *S. cerevisiae*, but well-developed in several species from the Candida clade, including *C. tropicalis* and *C. maltosa*, as well as in the above-mentioned *Y. lipolytica* [62]. In the 1960s, the alkane assimilating capacities of this latter species were exploited for the production of single-cell protein

generated from cheap mineral oil byproducts. As described above, alkane uptake is linked to protrusion formation [27], and cell contact can be facilitated by the secretion of biosurfactants [21,24]. Nevertheless, proteins are very likely to be required for the crossing of the plasma membrane. In *Y. lipolytica* the ABC transporter *ABC1*, similar to the yeast pleiotropic drug resistance transporter *PDR5*, was identified as essential for alkane uptake. The Δ*ABC1* strain is unable to grow on alkanes with a chain length of 14 till 16, shows slow growth on C12 and C13 and grows normally when C10 and C11 are provided as the sole carbon source. This illustrates the chain-length specificity of the ABC-transporter and suggests the involvement of other transporters to cover to full carbon chain range. Three highly homolog genes *ABC2-4* are prime suspects [63,64]. Furthermore, one can accept that *ABC1* orthologs fulfill similar tasks in other alkane assimilating yeasts. When running a protein BLAST with *Yarrowia ABC1* (XP_503953.2) proteins displaying the highest amino acid similarity (53–56% AA identity) are putative transport proteins belonging to *Aspergillus* species, which are also known alkane degraders. Furthermore, homologs from *C. tropicalis* and *C. maltosa* sharing 45–52% amino acid identity can be retrieved as well.

Sterol uptake

Unesterified sterols are parts of all eukaryotic membranes, where they influence fluidity, membrane potential and protein localization [65]. They are found in all different cellular compartments with widely varying concentrations. Their esterified counterpart is used mainly for energy storage and is deposited in intracellular lipid droplets. An ingenious system for conversion and intracellular transport is installed to maintain homeostasis, but as this is not the scope if this review, we refer the reader to other articles [20,66]. When grown aerobically, yeasts activate ergosterol biosynthesis instead of uptaking sterols from the environment. This phenomenon is known as 'aerobic sterol exclusion'. However, in oxygen-restrictive conditions, exogenous ergosterol uptake is obligatory, as biosynthesis requires molecular oxygen and yeasts are unable to grow without sterols present in their membranes. Genome-wide transcriptional analysis of wild-type compared to the *UPC2-1* hypoxic transcriptional regulator deficient strain revealed that two ABC transporters, Aus1p and Pdr11p, drive the uptake of ergosterol and other sterols, including cholesterol, from the environment [67]. Aus1p and Pdr11p were found to be intrinsically interchangeable, as only deletion of both proteins led to an anaerobic lethal phenotype. How these two ABC transporters work is unknown; they may allow transfer across the plasma membrane in a conventional manner, or they may navigate the sterol insertion into the outer leaflet and subsequent flip-flop across the bilayer [68]. ATPase activity was not stimulated by cholesterol in Aus1p reconstituted proteoliposomes, which indicates that additional proteins may be required for sterol transport; some candidates are discussed below [69]. Expression of the transcription factor Sut1 is increased 9.6-fold under anaerobic conditions, which leads to a 2.6-fold increase in sterol uptake given that both Aus1 and the cell-wall protein Dan1p are functionally expressed [70]. The Arv1p transmembrane protein that is located in the ER also has a critical role in exogenous sterol uptake [71]. Hence, Aus1p and Pdr11p are speculated to influence sterol uptake indirectly, by altering the distribution of lipids in the plasma membrane and, thereby, allowing sterols to be more easily extracted by intracellular lipid-transfer proteins [72].

The human pathogenic yeast *C. glabrata* behaves similarly to *S. cerevisiae* under hypoxia, and takes up exogenous sterols. In addition, *C. glabrata* that are resistant to azole ergosterol biosynthesis inhibitors, have evolved to use the Aus1p transporter for ergosterol uptake even under aerobic conditions [73]. As a consequence, these strains can import exogenous sterol simultaneously with ergosterol synthesis, whereas *S. cerevisiae* exclusively takes up sterols under hypoxic conditions and not under normoxia. This acquired ability contributes to the increased azole resistance in pathogenic fungal species such as *C. glabrata*, but also *Pneumocystis jirovecii* and the filamentous fungi *Aspergillus fumigatus* [74,75]. A *C. glabrata* Pdr11p ortholog to *S. cerevisiae* Pdr11p has not been found. The human pathogenic yeast *C. albicans* was long believed to be unable to take up exogenous sterols. It does not appear to carry any Pdr11p or Aus1p orthologs, it can grow anaerobically without the need for exogenous sterols, and sterol auxotrophs do not respond to their addition [76]. In fact, it is possible for *C. albicans* to grow without the presence of ergosterol in its membranes and the opposite situation as in *S. cerevisiae* is observed; sterol import has been observed during aerobic growth. This phenomenon was conveniently named 'anaerobic sterol exclusion'; the uptake of sterols increased linearly over time under normoxic conditions, while very little uptake is seen under microaerophilic conditions [77]. A selective transport system deviating from the Aus1/Pdr11 system in *S. cerevisiae* is evidently present, but remains yet to be uncovered. Bilayer crossing by passive

diffusion is highly unlikely as such is not observed in *S. cerevisiae*. More evidence that passive crossing is not possible is found in *Schizosaccharomyces pombe*: it lacks detectable homologs of *S. cerevisiae Aus1/Pdr11* and is unable to import exogenous cholesterol under low oxygen conditions [78].

Uptake of hydrophobic xenobiotics

While the efflux of drugs by ABC-transporters is well studied in the context of multidrug resistance phenomena, protein-mediated uptake is less characterized. Nevertheless, ample evidence confirms a role for transporters in the import of xenobiotics or drugs across biological membranes [79,80]. A fine example was shown by Winter *et al.* [81]: cancer cells lacking the solute carrier SLC35F2 were no longer capable of taking up 99.5% of the hydrophobic drug YM155. Lanthaler and colleagues tested 26 drugs with various properties by exposing them to a collection of yeast strains with deletions of 111 genes encoding plasma membrane transporters. For 18 out of the 26 tested drugs one or more carriers were identified to provide entry into the yeast cell [82]. Of these 18 actively transported compounds, 13 can be considered as hydrophobic and these are listed in Table 1. As one can see, some transporters are redundant, suggesting a rather broad substrate specificity. Certain activities can be expected based on similarities between xenobiotics and the natural substrates. For instance, the high affinity uridine permease fui1 is capable of acting on the structurally related benzbromarone. Nevertheless, the more distinct Bay 11-7985 is recognized as well. Moreover, the high-affinity nicotinamide riboside transporter *NTR1* is capable of transporting the quite diverse structures of methotrexate and diphenyleneiodium chloride. Surprisingly, the well-characterized myo-inositol transporter itr1 is involved in the uptake of the antifungal imidazoles fluconazole, ketonazole, and clotrimazole; molecules quite divergent from the natural substrate. Notably, also the abovementioned fatty acid transporter fat1 has been found to import xenobiotics. Furthermore, biochemical analysis suggested an energy-independent transport mechanism for fluconazole conserved across various yeast species [83].

As one can learn from Table 1, the uptake of hydrophobic drugs relies on the coordinated action of several transporters. Delgado and colleagues confirmed these findings for the antitumor lipid edelfosine [84]. Three carrier proteins were found to be involved: Lem3p, Agp2p and Doc1p. Lem3p had already been reported before to be essential the uptake of alkylphosphocholine type of drugs in yeast, including edelfosine miltefosine and ferifosine [85]. For this, lem3 seems to work in concert with the lipid flippases P4 ATPases dnf1 and dnf2 [86]. It is not clear whether the flippase activity is required for the nonalkylphosphocholine substrates cisplatin and tunicamycin, although co-occurrence of dnf2 in Table 1 might suggest this for the latter one. Furthermore, the aliphatic chain of the antibiotic compound could promote recognition by the flippase complex. The role of the other two edelfosine carriers is less clear; doc1 is no real transporter and takes part in ubiquitination; and also agp2, annotated as an amino acid permease, rather seems to act as a membrane-located sensor and regulator activating other transporters [87]. Notably, the other 'true' amino acid importer agp1, which is known to have a broad substrate range, seems to be responsible for the uptake of protoporphirine, the structure of which is quite distinct from that of an

Table 1. Hydrophobic drugs and carrier proteins responsible for uptake in yeast cells. After [82] and [89].

Drug	Function	Involved plasma membrane transporter(s)
Bay 11-7985	Anti-inflammatory	fui1
Benzbromarone	Uricosuric agent	fui1
Cantharidin	Toxic compound from the Spanish fly, anticancer potential	fen2, snq2
Cisplatin	Chemotherapeutic	fcy2, lem3 (fat1, fps1, hnm1)ctr1
Clotrimazole	Antifungal	itr1
Diphenyleneiodium chloride	Inhibitor of several oxidases	ntr1
Fluconazole	Antifungal	itr1, fcy2
5-fluorouracil	Anticancer	fcy2
Ketoconazole	Antifungal	itr1, fat1
Methotrexate	Chemotherapeutic	ntr1
Protoporphyrin	Photodynamic anticancer agent	agp1
Tirapazamine	Anticancer	agp3, alp1, hnm1
Tunicamycin	Antibiotic	lem3, dnf2, qdr2

amino acid (Table 1). A third amino acid permease, agp3, seems to also be able to expands its natural substrate range toward the anticancer drug tirapazamine [88]. Similarly, arginine permease alp1, can also pump tirapazamine into the cell.

The promiscuous nature of several amino acid permeases toward different types of hydrophobic drugs could be related to the aromatic nature of several amino acids. Most of the permeases display wide substrate adaptability toward all types of amino acids ranging from hydrophilic to hydrophobic, small to aromatic, and with different charges. Similarly, the activity of the high affinity copper transporter ctr1 toward platinum-containing drugs could be based on general recognition of the bivalent ion [89,90]. This would seem adequate for the small compound cisplatin (*cis*-diamminedichloridoplatinum), but is less likely for the more bulky compounds carboplatin and oxaliplatin; so probably other recognition mechanisms are involved here [91]. As in many examples, the activity of yeast ctr1 toward platinum drugs is conserved in mice and humans; rendering yeast cells interesting model systems to study drug effects and uptake [89].

In conclusion, it is clear that several different types of hydrophobic drugs do not pass the cytoplasmic membrane by simple diffusion, but by hijacking permeases which normally perform another function. Some of these permeases have a quite broad substrate specificity, such as the promiscuous amino acid permeases, while others seem to have specific functions, such as the uptake of copper. For these reasons, prediction of which transport system will act on a specific drug is difficult, further complicated by the fact that many drugs are taken up by the coordinated action of several proteins.

Efflux of lipophilic compounds

The thermodynamics in a transport process, dictate that if no source of energy is provided, transport remains passive, following Fick's law of diffusion [92]. In other words, the diffusive flux of each molecule goes from regions of high concentration to low concentration with a magnitude that is proportional to their concentration gradient. This becomes problematic in the case of hydrophobic compounds. They tend to intercalate into the membrane and alter the membrane integrity. Ultimately, this can lead to loss of function or even cell death [64]. In the struggle to survive, microorganisms have evolved several defense mechanisms: they can alter their membrane/cell wall composition, or they can activate excretion mechanisms based on energy-consuming transporters to keep the internal concentration as low as possible, thereby avoiding cytotoxicity. In 1993, Smit and colleagues linked the murine *MDR2* to phospholipid export [93]. Since then, it has been recognized that cells actively excrete not only exogenous compounds, but also lipophilic endogenous compounds. Notwithstanding more than 20 years of research, the mechanics underlying outward lipid transport remain speculative. Four processes have been proposed to mediate efflux of lipophilic compounds (Figure 2): (a) according to the 'flip-flop' model, substrates intercalate into the inner

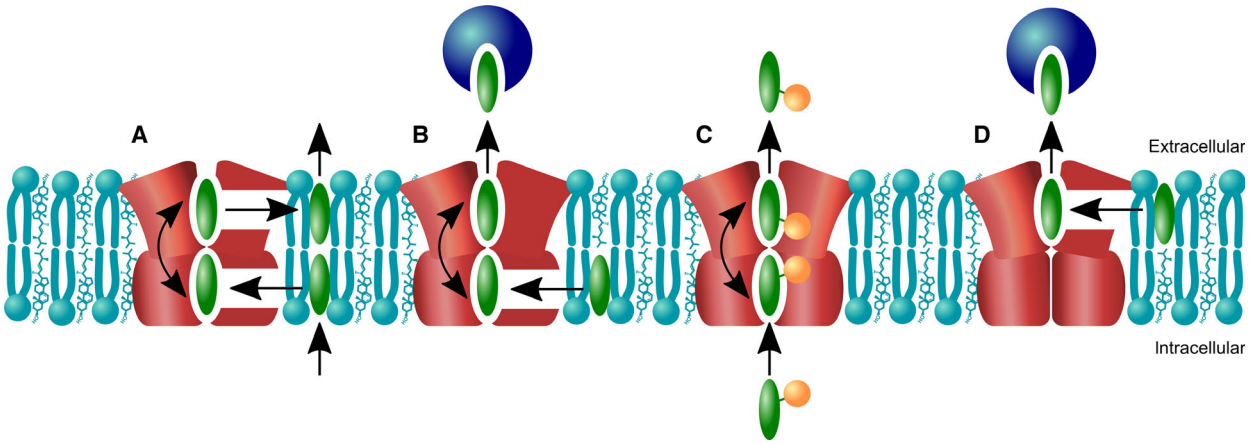

Fig. 2. Four putative hydrophobic molecule export mechanisms: (a) the substrate intercalates in the inner leaflet, 'flip-flops' by transporter action to the outer leaflet and exits the cell; (b) the substrate enters the transporter from the inner leaflet, 'flips' and exits the cell directly from the transporter possibly with help from an extracellular facilitating protein to avoid re-entering into the outer leaflet; (c) an amphiphilic substrate enters and exits the transporter without direct contact with the lipid bilayer; (d) the substrate enters the transporter from the outer leaflet, exits the cell, possibly with help from an extracellular facilitating protein. Figure reproduced from Ref. [94].

leaflet, 'flip' by virtue of the transporter and intercalate in the outer leaflet where they can exit the cell; (b) substrates that are intercalated in the inner leaflet, 'flip' and exit the cell, possibly with help from an extracellular protein that prevents substrates from re-entering in the outer leaflet; (c) amphiphilic substrates may enter and exit through a transporter without dissolving in the bilayer; and (d) substrates intercalated in the outer leaflet, enter a transporter and exit the cell, possibly assisted by an extracellular facilitating protein [94]. Additionally, expulsion of compounds can occur via extracellular membrane vesicle secretion, analogous to mammalian exosomes [95]. Following content loading and vesicle assembly in the cytoplasm, vesicles are transferred to the cell surface and secreted to the extracellular environment. How the vesicles traverse the yeast cell wall is still a matter of debate: secretory vesicles may be forced through the cell wall by turgor pressure after release from the plasma membrane; cell wall-modifying enzymes may be involved; or the vesicles may transit through channels [96]. These three hypotheses are considered to be the most likely and are not mutually exclusive. Following secretion, the internal content of the vesicles is released through the lytic activity of extracellular lipases. The phenomenon of secretory vesicle secretion in fungi was discovered only a decade ago and therefore awaits more insights.

Triglyceride and fatty acid efflux

Uptake, synthesis and degradation of fatty acids is tightly regulated in microbial cells because free fatty acids exert detergent-like properties that result in cell lysis, stress responses and impairment of basic physiological processes [97]. In general, fatty acids are only present at low concentration, as most are bound to proteins or cofactors [98]. Efflux of fatty acids results in loss of metabolic energy and is therefore in general avoided. Some mutant yeasts do have fatty acid secretion capability, showing that the classification of transport processes in the inward and outward direction is indeed inaccurate and the direction of transport is influenced by environmental conditions. This is, for example, nicely illustrated by the first ever reported palmitic and stearic acid secreting mutant of *Y. lipolytica* and its further optimized progeny [99,100]. Scharnewski *et al.* [54] could similarly engineer *S. cerevisiae*: they knocked out *FAA1* and *FAA4* to disconnect vectorial acylation, which resulted in fatty acid secretion. According to the endocytosis working model proposed by Jacquier *et al.* [50], the lack of abstraction by CoA activation results in export from the cell through membrane recycling. Recently, members of the cysteine-rich secretory proteins, antigen 5, and pathogenesis-related proteins (CAP) superfamily were identified as required for the survival of yeast cells with increased intracellular FFA concentration because of the Δ*FAA1*, Δ*FAA4* double knockout genotype. More specifically, the Pathogen-Related Yeast (PRY) proteins – the secreted glycoproteins Pry1, Pry2 and cell wall-associated Pry3 – are essential for export of free fatty acids and therefore evasion of the toxicity [101]. Given that Pry1 is secreted and the fact that it appeared crucial to escort FFA to the extracellular environment, the authors suggested the notion that fatty acids are exported as complexes bound to Pry1. Computational modeling in comparison to the CAP protein tablysin-15 suggests substrate binding buried deep inside the protein's hydrophobic channel. The authors stress the mechanistic difference with extracellular fatty acid-binding proteins (FABP) such as albumins that are believed to bind and solubilize the fatty acids once they are outside the cell [35].

Apart from *S. cerevisiae,* oleaginous yeasts such as *Y. lipolytica* are deployed for large-scale fatty acid production because of their natural ability to accumulate lipids on average to 40% of their biomass with maximal levels exceeding 70% [42]. In *Y. lipolytica*, disruption of *FAA1* also gave the fatty acid secretion phenotype [99]. Surprisingly, the direction of transport in the *Y. lipolytica* mutant appeared to be dependent on the growth phase; re-import was observed in the stationary phase independent of their activation by any cellular acyl-CoA synthetase. Conclusively, fatty acid activation is wanted for uptake, but not strictly required. Moreover, addition of raffinose to the cells in stationary phase instantaneously resulted in shift of the direction back to export. This strongly supports an active mechanism for fatty acid export [54].

Fatty acids packed in the form of triglycerides are also secreted. Nojima *et al.* isolated a *S. cerevisiae* mutant secreting triglycerides in the culture medium. The molecular mechanism of this phenotype could not be elucidated, nevertheless it was confirmed that autolysis did not occur and the mutant had acquired a lipid-transport mechanism deviating from the wildtype [102]. Moreover, the oleaginous yeast *Cryptococcus curvatus* was shown to discharge its lipid content extracellularly when cultured in high acetic acid conditions [103].

Alkane efflux

Studies of transporters with hydrophobic substrates often involve toxicity phenotypes. This is advantageous, as it helps the identification of toxicity relief

channels based on transcriptome analysis. As such, it was discovered that efflux pumps Snq2p, Pdr10p and Pdr5p are involved in alkane export in *S. cerevisiae*, reducing intracellular levels and at the same time enhancing tolerance levels of C10 and C11 [34,104]. In addition, the more specific transporters *ABC2* and *ABC3* of *Y. lipolytica* can be applied to improve tolerance of *S. cerevisiae* to the same C10/C11 alkanes, confirming their role as efflux pumps [105].

Sterol efflux

The genetic cause of Tangier disease, a disorder that manifests as defective cholesterol efflux in mammals, was identified in mutations of the *ABCA1* transporter that has the task to promote cholesterol efflux to lipid-poor apolipoproteins [106]. Comparable sterol exporters have not been assigned in yeast due to unfavorable metabolic economics, though some ABC family members in *S. cerevisiae* (Pdr5p, Snq2p, and Pdr16p) are speculated to be associated with sterol homeostasis because of their ability to export steroids [107,108]. The same holds true for the major MDR transporters Cdr1p and Cdr2p in *C. albicans* [109]. Sterol excesses in yeasts are usually stored in lipid droplets after esterification.

Yet another system dealing with detoxification of sterol overabundance was discovered; free sterols are reversibly converted into their acetylated counterpart by the acetyltransferase Atf2 and subsequently excreted. They are deacetylated by the membrane-anchored lipase Say1p (Figure 3). Deletion of *SAY1* resulted in secretion of the sterol acetate indicating that the acetylation is key to their secretion [110]. In normal circumstances, deacetylation by Say1p enables the cell to retain the sterol if needed. This acetylation cycle is, an example, how cells protect themselves from too hydrophobic environments. Indeed, both *ΔSAY1* and Δ*ATF2* mutants are sensitive to the small, hydrophobic plant compound eugenol, a member of the alkylbenzene class, which accords with its possible function of this acetylation–deacetylation cycle in lipid detoxification [111]. Curiously, acetylated sterols are even more hydrophobic than free sterols, which make intracellular traffic or the presence of acetylated sterol in the aqueous culture medium rather contradictive. Choudhary *et al.* [111] identified the CAP superfamily members, and more specifically PRY proteins as potential carriers of acetylated sterols. However, PRY proteins bind sterols through a separate and independent-binding site from the hydrophobic tunnel, where they accept free fatty acids (see section on fatty acid efflux) to solubilize the hydrophobic compound and facilitate excretion [101]. Computational modeling studies identify the flexible loop within the CAP domain, which harbors the caveolin-binding motif (a short stretch of aromatic amino acids) as responsible for sterol binding [35]. This putative mode of sterol binding diverges from what is seen in cytosolic sterol-binding proteins, such as Osh4, StAR/STARD1, or NPC2, where substrate-binding site is buried in a hydrophobic tunnel inside the protein. A variety of sterols, steroids and sterol precursors were shown to bind Pry1 with competitive efficiency [112]. For a complete block in cholesterol acetate secretion, a double knockout of Pry1 and Pry2 was necessary, which indicates their redundant activity. The function of Pry3 in lipid export is less clear. In the presence of functional

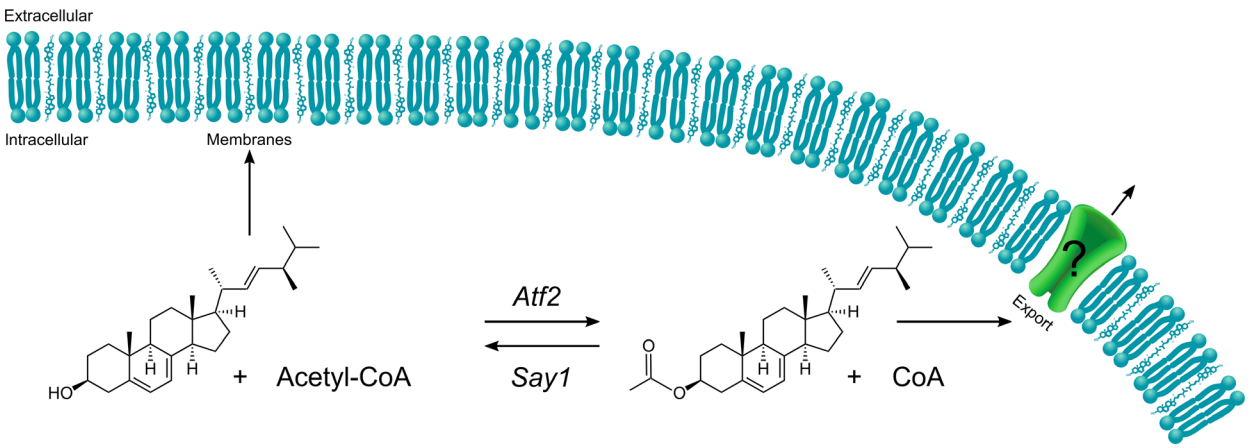

Fig. 3. Detoxification of sterols by the reversible activity of an acetylation/deacetylation cycle. Sterol acetylation is executed by the acetyltransferase ATF2, resulting in the export of the acetylated compound. Deacetylation and therefore retention in the cell requires the activity of the deacetylase SAY1.

Pry1 and Pry2, the absence of Pry3 led to decreased cholesterol export presumably by a plasma membrane protective function of Pry3. Deletion of any one of the *PRY* genes, including *PRY3,* resulted in hypersensitivity toward eugenol [111]. Computational modeling and auxiliary competitive assays suggest an overlapping eugenol-binding site with sterol rather than the free fatty acids, located on the flexible loop of the CAP domain [35].

Communication through hydrophobic molecules

Apart from ensuring tolerance against toxic (hydrophobic) substances, yeasts employ efflux pumps for a completely different function: cell signaling. Indeed, lipophilic signaling molecules are common among fungi, primarily acting as mating pheromones. The a-factor secreted by *S. cerevisiae* is probably the best-known example in this class of extracellular signaling molecules. It is a docapeptide post-translationally modified with carboxylmethyl and prenyl moieties that render it highly hydrophobic [113]. Protein prenylation is a ubiquitous feature among eukaryotes and is assumed to facilitate association with the plasma membrane and other signaling proteins [114]. Yeast cells of the *MAT*a haploid mating type synthesize a-factor, which then goes through a series of modifications including C-terminal processing and two sequential N-terminal cleavage steps [115]. Once it is fully matured, the ABC transporter Ste6p mediates a-factor export [116]. Considering its hydrophobic nature, it has been proposed that a-factor is presented to the transporter in association with the inner leaflet of the plasma membrane. It is then 'flipped' to the outer leaflet after ATP hydrolysis [117]. This is an exceptional secretory path for peptides as they are usually secreted by vesicular transport from the ER through the Golgi apparatus. Interestingly, the a-factor counterpart pheromone α-factor is hydrophilic and is secreted by the 'classical' eukaryotic protein secretory pathway [118]. Ste6p is one of the few ABC transporters with a specific physiological substrate whereas the large majority of ABC transporters export chemically distinct xenobiotic compounds [115]. Despite their considerable evolutionary distance, *S. cerevisiae* and the fission yeast *Schizosaccharomyces pombe* have remarkably similar pheromone communication systems. The hydrophobic part of it concerns 'M-factor', a nonapeptide that is both S-farnesylated and carboxymethylated. The ABC-transporter responsible for its export is identified as *MAM1* [119].

Members of the Ascomycetes group secrete both hydrophilic and hydrophobic pheromones, representing opposite mating types, whereas Basidiomycetes exclusively secrete hydrophobic pheromones, representing all mating types ranging from two to thousands [120]. The pioneering study by Kamiya *et al*. in 1979 about rhodotorucine A secreted by the oleaginous yeast *Rhodotorula (Rhodosporidium) toruloides* was the first to report the protein prenylation and carboxylmethylation process leading to lipophilic peptides [121]. Yet, knowledge about export of pheromones from the Basidiomycetes class is scarce and somewhat contradictory. Though all pheromones of this class are stated to be diffusible [122], radiolabeling of tremerogen A-10, the mating factor of the heterobasidiomycetous yeast *Tremella mesenterica*, showed that it initially appeared in a membrane-bound form and subsequently converted into a more soluble form prior to secretion, suggesting a more complex form of transport [123]. Furthermore, basidiomycete pheromones all carry CaaX motifs for C-terminal farnesylation, which in turn aids membrane localization, guiding the pheromone to a transmembrane transporter protein [124,125]. The similarity with the *S. cerevisiae* a-factor again suggests the involvement of active transporter proteins. Kovalchuk *et al*. [126] undertook the identification and initial cataloging of ABC proteins of basidiomycetes, and identified pheromone transporters in all analyzed species, most bearing a single copy. This fits within the hypothesis that Ste6p homologs for secretion of prenylated lipopeptides are evolutionarily highly conserved [127].

Another example of a lipidic signaling molecule is farnesol, used as the quorum-sensing molecule in pathogenic fungi. It was the first quorum-sensing molecule discovered in a eukaryote, more specifically in *Candida albicans*, and was speculated to be potentially produced by one third of the Ascomycetes [128]. Having such a wide spectrum of producers suggests that the excretion mechanism is a very common mechanism or proceeds via diffusion. The Cdr4p, Rta3p and Gnp3p plasma membrane proteins of *C. albicans* were implicated in inter- and intraspecies communication and are therefore potential candidates for export and/or recognition [129]. Interestingly, Rta3p is a member of the lipid-translocating exporter family (Transporter Classification Database TC 9 A.26.1) with potential function in multidrug resistance and membrane phospholipid homeostasis [130]. Its closest homologs and family members *RSB1* and *RTA1* in *S. cerevisiae* show seven transmembrane spanning segments and an ATP-binding domain and are involved in movement of sphingolipid long-chain bases (LCB) from the cytoplasmic side to the extracellular side

conferring plasma membrane asymmetry. The mechanistic details of this intriguing family are hitherto unclear; a direct 'flippase' activity was suggested as well as a more indirect action based on their receptor-like topology [131,132].

In 2007, it was discovered that virulence factors and bioactive fungal lipids such as glucosylceramides, ergosterol, and an obtusifoliol-like molecule are secreted by the fungal pathogen *C. neoformans* through the use of extracellular vesicles [133]. A year later, the same group described that this secretory mechanism is not necessarily associated with virulence, but is rather a common mechanism of extracellular delivery in fungi by showing its presence in *Histoplasma capsulatum*, *C. albicans, Candida parapsilosis, Sporothrix schenckii,* and *S. cerevisiae* [95]. The phospholipids phosphatidylcholine, phosphatidylethanolamine, and phosphatidylserine were characterized as the major structural component of the vesicles, similar as the mammalian exosomes. A bioactive role in intercell communication as was shown for exosomes is supposed for yeast vesicles as well [134]. Hence, a concrete distinction must be made between the lipids as surface constituents stemming from the plasma membrane and lipids as actively secreted components [135].

Efflux of hydrophobic xenobiotics

The *S. cerevisiae* genome harbors 31 distinct ABC protein-encoding genes, though only three of them are essential for growth. What is more, none of those three have a role in transport [126]. Consequently, the physiological role of the majority of the ABC proteins is dispensable under normal growth conditions and they display a great deal of functional redundancy. Out of the five phylogenetic ABC protein subfamilies, the PDR subfamily is relevant for discussion here, because they protect the cells against several hundreds of chemically distinct xenobiotic compounds conferring pleiotropic resistance against xenobiotics, which very often are hydrophobic [136].

Speculation about the reason for the relative broad specificity of ABC exporters in contrast to the narrow substrate range of ABC importers came forth from structural studies; their different transmembrane architecture might play a dominant role [137]. The most renowned transporter related to MDR is the *S. cerevisiae* Pdr5p, which is responsible for flushing drugs, including cycloheximide, azoles and mycotoxins. Though first discovered almost 25 years ago [138], the mechanism of substrate interaction with the transporter is still unclear and seems to be highly dynamic [92]. The substrate volume appears to play a pivotal role; optimal efflux is obtained with substrate volumes of 200–225 $Å^3$, coming to a standstill down to 90 $Å^3$ [139]. Remarkably, substrate recognition is completely free of any requirement for hydrophobicity despite its importance in the recognition by mammalian multidrug resistance transporters such as P-glycoprotein [140]. Clustered together with *PDR5* because of their sequence homology are *PDR10* and *PDR15*, also efflux pumps mainly for cationic amphiphilic drugs and other chemically unrelated hydrophobic drugs [141,142]. Pdr12p is involved in general stress and weak acid response [143,144]. It is strongly induced by a large intracellular pool moderately lipophilic carboxylic acids, such as sorbic acid and benzoic acid, that partition to a certain degree in the inner leaflet of the plasma membrane. This suggests an active mechanism of Pdr12p picking the organic acids from the inner membrane and actively shuttling them outside. Other well-characterized transporters found in the plasma membrane of *S. cerevisiae* include Snq2p and Pdr18p [145,146]. The former was the first MDR transporter of the ABC-type identified in *S. cerevisiae* and confers hyper-resistance to the mutagens 4-nitroquinoline-N-oxide, triaziquone as well as sulphomethuron methyl and phenanthroline. The latter has a role in anionic amphiphilic drug resistance and indirectly a range of other drugs by its function in the control of nonvesicular transport of ergosterol to the plasma membrane [146]. All PDR subfamily members share the 'reverse' topology following the $(NBD\text{-}TMD)_2$ pattern in their domain structure unlike all other ABC subfamilies [142]. Strikingly, they do mediate the efflux of distinct subset of drugs with little overlap in substrate specificity [107]. Furthermore, they have distinct roles in phospholipid translocation and therefore lipid homeostasis [147]. It is interesting to mention that all of the above-stated transporters influence each other, some even via robust physical interaction though functional mechanism remains largely unexplored [148]. It was suggested that the interaction might be important in maintaining stability; Pdr18p is only indirectly connected through physical interactions with intermediate ABC transporters and is nonetheless pivotal for the stability of Snq2p.

Notorious for its resistance, the yeast *Candida albicans* overproduces various efflux pumps in its plasma membrane. A thorough overview of the pumps involved in this MDR phenotype is given in the book chapter 'Candida Efflux ATPases and Antiporters in Clinical Drug Resistance' [149]. One particularly well-studied example is the major multidrug transporter Cdr1p included in the same phylogenetic cluster as *S. cerevisiae* Pdr5/10/15p [109]. It exports a myriad of compounds

including antifungal drugs, herbicides, antibiotics, steroids, and lipids. Thorough systematic mutagenesis of the transmembrane segment primary sequence revealed that Cdr1p possesses several overlapping drug-binding sites within a large, central poly-specific-binding pocket [150]. The majority of amino acid residues taking part in the recognition are of hydrophobic nature, which coincides with the greater affinity of Cdr1p toward hydrophobic rather than hydrophilic drugs. High aromaticity, molecular branching and occurrence of an atom-centered fragment are the defining elements for substrates of Cdr1p [151]. Together with the very similar in sequence, though functionally different transporter Cdr2p, most if not all antifungal drug classes are covered [152]. Despite their presence in the same phylogenetic cluster, Cdr3p and Cdr4p do not appear to have a role in antifungal resistance. They do have their function as a phospholipid translocator in common with Cdr1 and Cdr2 albeit with opposite directionality of membrane bilayer exchange [142,153]. The human pathogenic yeasts *C. glabrata*, *Candida krusei* and *Candida dubliniensis* all have homologs to the *CDR1*/*CDR2* duo providing resistance [154–156]. Knowledge of the ABC transporter type involved in the MDR phenomenon for basidiomycetous yeasts is limited to only one species: *Cryptococcus neoformans* bears *MDR1* and *AFR1* [157,158].

ABC proteins are not the only transporter family involved in xenobiotic efflux. In *S. cerevisiae,* a total of 22 transporters of the other major superfamily, the MFS transporters, have been associated with the expulsion of an array of drugs [159]. *ATR1*, for example, was the first *S. cerevisiae* Drug/H^+ antiporter (DHA) gene to be characterized and found to confer resistance to azoles [160]. Altogether, almost 300 DHA transporters were predicted and clustered in two groups (DHA1 and DHA2) among the *Candida*, *Cryptococcus* and *Aspergillus* species. Only 12 of them have been characterized [159]. The most important are *MDR1, FLU1, NAG3 (TMP1),* and *NAG4 (TMP2)* found in *C. albicans*. They belong to the DHA1 family and find homologs in other pathogenic yeasts such as *C. dubliniensis* and *C. tropicalis* [161]. The DHA transporters *TPO1*/*2* are involved in *C. glabrata* drug resistance and have similar roles in ergosterol and phospholipid homeostasis as Pdr18 in *S. cerevisiae* [162]. Mark that, again, there is a significant functional overlap between them, yet the substrate range is less broad and with lower hydrophobicity index than what is seen in ABC transporters [151,159]. *C. albicans* Mdr1 is, for example, is specific for fluconazole but not for other azoles [163].

Conclusions and perspectives

The importance of solute transport processes in and out of microbial cells is evident. Transporters-mediating uptake of nutrients, fluxes of signaling molecules or the extrusion of toxic compounds has been studied for decades. Yet, the surprisingly widespread belief in the simple diffusion into and out of cells, together with experimental difficulties working with transporters led to significant knowledge gaps. In the case of hydrophobic substrates, it becomes even more complicated considering the interaction with the membrane barrier itself. Unconventional passageways such as vectorial acylation become more important. The relevance of the cell wall to fungal physiology is clear but the mechanism by which molecules -hydrophobic or not- cross this rigid structure remains largely unknown. A profound interplay is proposed between membrane-spanning transporters and vesicle formation. Using this strategy, the yeast cell provides an answer to the small mesh size and high electrical charge of the cell wall. This is illustrated by the protrusion formations that aid uptake of carbon sources in a hydrophobic environment. On the other end, secretory vesicles packed with signaling molecules are secreted in order to bypass the cell wall.

Unfortunately, the discovery of novel small molecule transporters takes time, not in the least due to difficulties with cloning and heterologous expression of membrane proteins [164]. More tools are required for efficient identification of transporter activity and substrate specificity, because investigating the intimate relationship between sequence, structure, substrate, and transport activity will be of essence [165]. In the field of medicine and the fight against multiresistance, this knowledge will boost the development of specific inhibitors with the potency of increasing antifungal effect when used as adjuvants [166]. Furthermore, valid information is gained from drug uptake studies in yeast as a model eukaryote. The focus should not only lie on membrane-spanning channels. The discovery of unconventional passageways as a fundamental biochemical mechanism in the eukaryotic model organism yeast, allows us to extrapolate the basics to the more complex, higher eukaryotes [167]. The way to tackle this enormous task will not be to screen for all possible transport processes, but to use extensive experimental designs integrated in systems biology models with the coordinated activity of molecular, physiological, chemical, and bioinformatical disciplines [80].

Finally, by better understanding fundamental ways, nature created passageways in and out the microbial

cell, biotechnologists can find ways to improve them and in this way create efficient yeast cell factories. Future research in the rational design of high-performance and substrate-specific transporters will help to address the shortcomings originating from intracellular accumulation of the molecule of interest while also preventing loss of carbon fluxes by nonspecific export [168].

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