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REVIEW

Regulation of Amino Acid Transport in *Saccharomyces cerevisiae*

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SUMMARY We review the mechanisms responsible for amino acid homeostasis in *Saccharomyces cerevisiae* and other fungi. Amino acid homeostasis is essential for cell growth and survival. Hence, the *de novo* synthesis reactions, metabolic conversions, and transport of amino acids are tightly regulated. Regulation varies from nitrogen pool sensing to control by individual amino acids and takes place at the gene (transcription), protein (posttranslational modification and allostery), and vesicle (trafficking and endocytosis) levels. The pools of amino acids are controlled via import, export, and compartmentalization. In yeast, the majority of the amino acid transporters belong to the APC (amino acid-polyamine-organocation) superfamily, and the proteins couple the uphill transport of amino acids to the electrochemical proton gradient. Although high-resolution structures of yeast amino acid transporters are not available, homology models have been successfully exploited to deter-

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mine and engineer the catalytic and regulatory functions of the proteins. This has led to a further understanding of the underlying mechanisms of amino acid sensing and subsequent downregulation of transport. Advances in optical microscopy have revealed a new level of regulation of yeast amino acid transporters, which involves membrane domain partitioning. The significance and the interrelationships of the latest discoveries on amino acid homeostasis are put in context.

KEYWORDS amino acid homeostasis, solute transport, *Saccharomyces cerevisiae*, trafficking, membrane partitioning, regulation of transport, plasma membrane, systemic fungi

INTRODUCTION

Cells and their organelles are enclosed by biological membranes, which are selectively permeable structures that allow for different conditions to be maintained "inside" and "outside." The regulated movement of molecules over these membranes is required for numerous processes that are essential to cells, organisms, and populations; these include the uptake of essential nutrients, regulation of the concentration of metabolites and ionic species, extrusion of toxic substances, genetic exchange of nucleic acids, cell-cell signaling, and defense (1). This is mediated by transport systems, both specific and broad range, which transport molecules of various sizes and chemical properties, including ions, sugars, amino acids, lipids, nucleic acids, and even folded proteins.

Biological membranes consist of two major components: lipids and proteins. Membrane lipids are amphipathic molecules that in aqueous solutions self-organize into a continuous bilayer structure where the hydrophobic tails point toward an imaginary central plane and the hydrophilic heads orient outward. Lipid bilayers are semipermeable barriers due to their chemistry; small nonionized molecules, e.g., oxygen or carbon dioxide but also many weak acids and bases (M. Gabba, J. Frallicciardi, J. van't Klooster, R. K. Henderson, Ł. Syga, R. Mans, A. J. A. van Maris, and B. Poolman, submitted for publication), are able to pass through easily, while ionized and hydrophilic molecules, e.g., salts, carbohydrates, or amino acids, cannot. Specialized proteins termed integral membrane proteins are able to associate with both the hydrophilic surface and the hydrophobic core of the lipid bilayer, and a large subset are the membrane transport systems.

Transport systems can be broadly classified into channels (a subset present in the outer membrane of bacteria and mitochondria is referred to as pores or porins) and carriers (also called transporters, porters, or permeases) (Fig. 1). Channels contain a translocation path that, when open, is accessible simultaneously to both sides of the membrane. Substrate selectivity occurs through the chemical properties of the amino acid residues lining this path, and transport can be regulated by mechanisms that control the opening and closing of the channel. Carriers typically operate via an alternating-access mechanism (2, 3); that is, they transit between different conformational states in which the binding site is accessible from only one side of the membrane at a time. This allows for regulation by mechanisms that control the transition between the inward-facing and outward-facing states.

Contrary to enzymes, transport systems facilitate a change in the location of a molecule rather than a modification of its chemical structure (discussed in references 4 and 5). The kinetics of transport and enzymatic catalysis are described by similar models, and in its simplest form, solute transport follows the Michaelis-Menten equation, where v is the rate of translocation of a solute from out to in or from in to out. K_m (the Michaelis constant) refers to the solute concentration at which the rate of translocation is half-maximum. When multiple solutes are co- or countertransported, the rate of transport will depend on the degree of occupancy of the different ligand-binding sites in the protein, which allows cooperativity (6).

Several types of transport can be defined based on the driving force for the movement of molecules across the membrane. The simplest process is facilitated

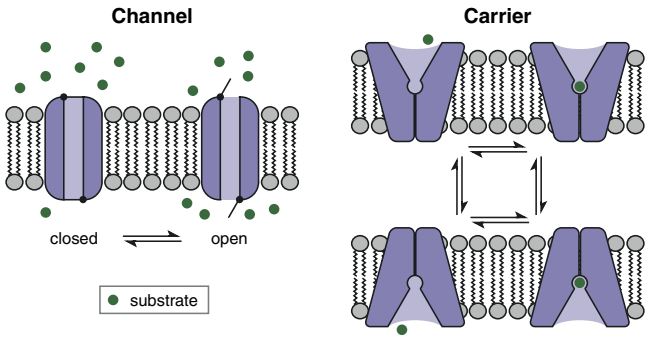

FIG 1 Simplified models of the two major types of transporters responsible for the movement of molecules across membranes.

diffusion, whereby the transport system merely provides a pathway through the membrane that allows molecules to move down their concentration gradient (downhill flux). By definition, this kind of transport is nondirectional and does not allow for accumulation of the solute. Facilitated diffusion can occur via channels or carriers (the latter are referred to as uniporters). Solute transport by facilitated diffusion can be enhanced by "metabolic trapping," in which chemical modifications such as phosphorylation prevent the substrate from moving out of the cell or its organellar compartments (7). In some cases, the proteins responsible for transport and for chemical modification have coevolved and are classified as belonging to a single transport system (e.g., group translocation systems) (8), but these are not found in yeast or other fungi. Active transporters are carriers that can achieve uphill flux of molecules by coupling their movement across the membrane to a source of free energy. Primary active transporters use chemical, electrical, or light energy, i.e., the breaking of covalent bonds, the transfer of electrons from donor to acceptor molecules, or the absorption of photons (9). Secondary active transporters make use of the energy contained in electrochemical gradients (generated at the expense of primary energy sources) by coupling the movement of two or more molecules, at least one of which is moving down its concentration gradient (10). This movement can be in either the same direction (symport) or the opposite direction (antiport). In this review, we focus on amino acid transporters in fungi, in particular those of *Saccharomyces cerevisiae*, the type of transporters, where they function, and the way in which they achieve amino acid homeostasis.

AMINO ACID TRANSPORT AND METABOLISM

Amino acids are major building blocks of any cell, and regulation of the corresponding pool sizes is crucial for cell growth and survival. The term "amino acids" is most commonly used to refer to the 20 standard proteinogenic (protein-forming) amino acids. In fact, an amino acid is any organic compound containing both amine (-NH_2) and carboxyl (-COOH) functional groups. Over 500 naturally occurring amino acids have been identified (11). Many of these are important metabolites. In this review, we focus on the transport of the 20 proteinogenic amino acids as well as analogs such as citrulline, ornithine, γ-aminobutyric acid (GABA), *S*-adenosylhomocysteine (SAHC), *S*-methylmethionine (SMM), and *S*-adenosylmethionine (SAM) (Fig. 2), which are typically transported by similar systems. Other reviews have covered the transport of more exotic amino acids (12, 13).

Not only do amino acids act as the building blocks of proteins, but *S. cerevisiae* can also use most proteinogenic amino acids, as well as citrulline, ornithine, and GABA, as a sole nitrogen source (14). The exceptions are cysteine, histidine, and lysine, although other yeasts, including some *Saccharomyces* strains, are able to use lysine as a sole N source (15–18). The catabolism of most amino acids also provides a source of carbon, except for those that feed into the Ehrlich pathway (19). Amino acids are involved in

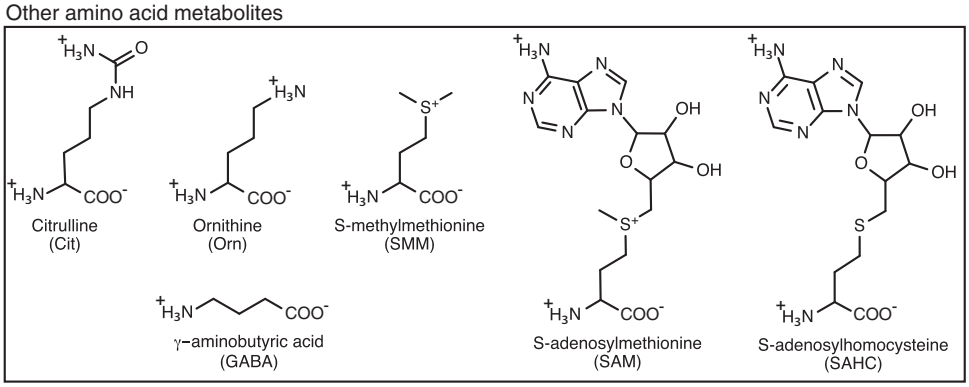

FIG 2 Structures of the most common amino acid analogs in fungi.

numerous metabolic pathways, including the synthesis of NAD^+, folate, glutathione, nucleotides, polyamines, and phospholipids. It is thus not surprising that although wild-type yeast cells can synthesize amino acids *de novo*, they also actively import them from the extracellular environment. This allows cells to respond to different environments and take advantage of available resources. It is also energetically less expensive; the estimated cost of *de novo* amino acid synthesis under aerobic conditions (expressed in activated phosphate bonds or ATP equivalents) is between 9.5, for glutamate, and 75.5, for tryptophan (20). The uptake of amino acids has been shown to contribute to the virulence of the mammalian pathogens *Cryptococcus neoformans* (21) and *Candida albicans* (22, 23), and it is also believed to play a role in infection by plant pathogens such as rust fungi (24).

Within the cell, amino acids are also actively transported to and from both the vacuole and mitochondria (Fig. 3 and 4). The efflux of amino acids from yeast cells has been demonstrated under specific conditions (25, 26), but much less is known about the transport mechanisms involved.

Yeast vacuoles (reviewed in references 27 and 28) are acidic compartments bound by a membrane with distinct lipid and protein compositions. They share properties with plant vacuoles and mammalian lysosomes and play roles in protein degradation, storage, and detoxification. Amino acids are not evenly distributed between the cytosol and vacuole: 80 to 90% of the cellular pool of histidine, lysine, and arginine is in the vacuole, while a similar fraction of glutamate and aspartate is present in the cytosol (29–31). Assuming that the vacuole(s) occupies approximately 25% of the cell volume, this would mean a 10- to 30-fold difference in concentration. These concentration gradients require the active import of amino acids into the vacuolar amino acid pools. In addition, breakdown of proteins, targeted to the vacuole as part of the quality control mechanisms of the cell (32, 33), further contributes to the vacuolar pools. Export from the vacuole allows for the recycling of amino acids. For example, protein synthesis and survival under nitrogen starvation conditions require not only autophagy (the delivery of cytosolic components to the vacuole for degradation) but also efflux of the resulting amino acids via specific transporters (34, 35).

Mitochondria accommodate a number of metabolic pathways in addition to the tricarboxylic acid (TCA) cycle and respiratory chain components. In some cases, e.g., arginine biosynthesis, parts of the pathway are physically separated by means of locating specific enzymes in either the cytosol or the mitochondrial matrix (the space bound by the inner mitochondrial membrane) (Fig. 3). Low-molecular-weight solutes, including amino acids, are able to permeate the outer mitochondrial membrane, but the inner membrane is selective and uses so-called mitochondrial carriers to translocate amino acids (36–38).

Amino Acid Transporters from *Saccharomyces cerevisiae*

The different protein classification and nomenclature systems can lead to confusion.

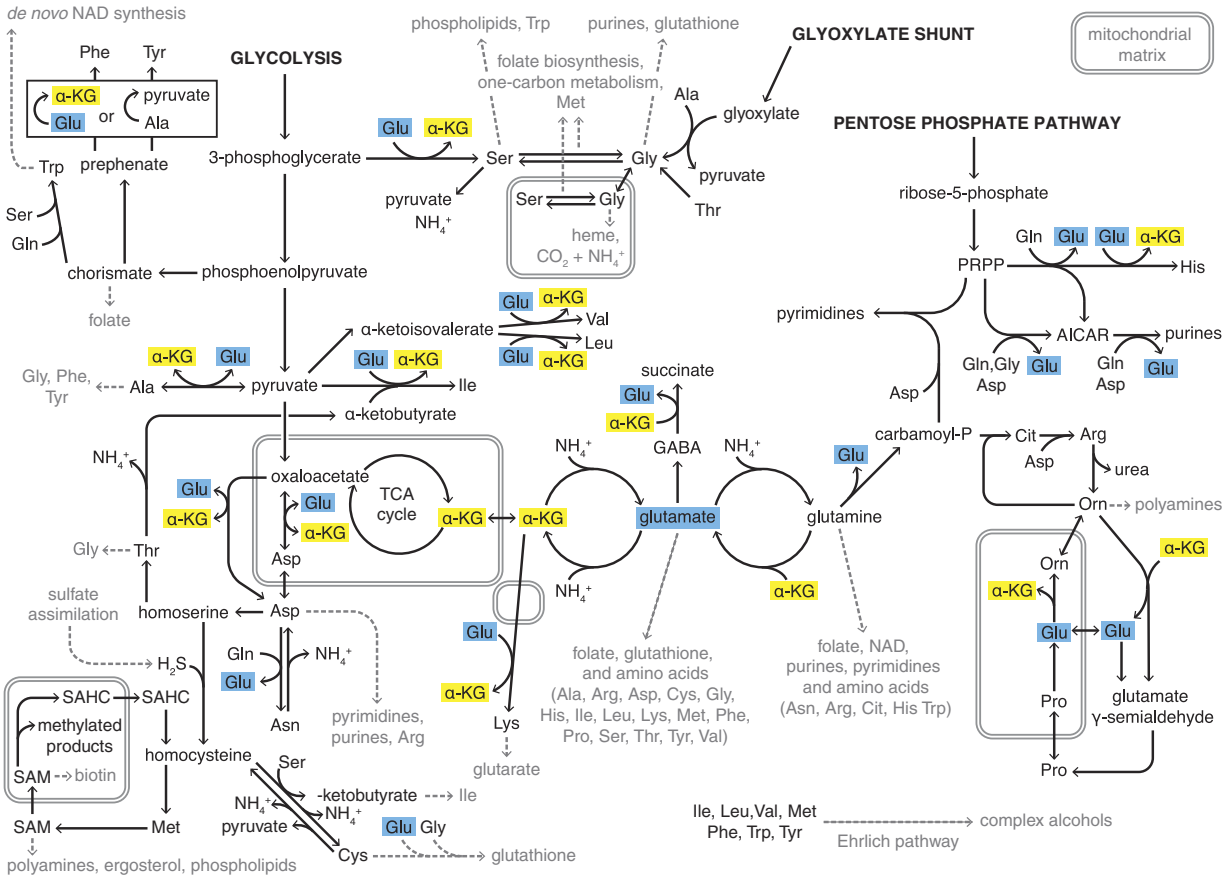

FIG 3 Overview of *S. cerevisiae* metabolic pathways involving amino acids. (Based on figures in reference 13 and expanded using information from the YeastPathways database [https://pathway.yeastgenome.org/] and the KEGG pathway database [http://www.genome.jp/kegg/pathway.html].) Not all reactions and intermediates are shown. Boxes with double gray lines indicate reactions known or predicted to take place within the mitochondrial matrix. Proteinogenic amino acids are indicated by their three-letter codes. α-KG, α-ketoglutarate; AICAR, aminoimidazole carboxamide ribonucleotide; Cit, citrulline; GABA, γ-aminobutyrate; Orn, ornithine; PRPP, phosphoribosylpyrophosphate.

Here, we use the definitions given in the Transporter Classification Database (TCDB) (www.tcdb.org) (39). To date, the proteins shown to mediate amino acid transport in fungi belong to five families: APC (amino acid-polyamine-organocation) (TCDB 2.A.3), AAAP (amino acid/auxin permease) (TCDB 2.A.18), MC (mitochondrial carrier) (TCDB 2.A.29), LCT (lysosomal cystine transporter) (TCDB 2.A.43), and MFS (major facilitator superfamily) (TCDB 2.A.1). They are all either proven or predicted to be secondary active transporters that catalyze solute:proton symport or antiport, with the exception of the mitochondrial carriers, which mediate both solute:solute and solute:proton/ion antiport (40, 41). The activity of the proton-coupled transporters relies on the electrochemical proton gradient generated by the plasma membrane (PM) and vacuolar membrane (VM) ATPases (Fig. 4). The plasma membrane ATPase Pma1 transports 1 proton per ATP molecule hydrolyzed, while the vacuolar membrane ATPase Vma1 is thought to translocate 2 to 4 protons per ATP molecule (42, 43). The electrochemical proton gradient across the mitochondrial inner membrane is generated by the components of the electron transport chain and is for the greater part used to synthesize ATP by the F_oF_1-ATP synthase.

Table 1 lists the characterized *S. cerevisiae* amino acid transporters. Members of each family have homologs in higher eukaryotes, and the members of the APC superfamily and the MFS also have homologs in prokaryotes. Whole-cell proteome analyses in *S. cerevisiae* have allowed the determination of the abundance levels for amino acid transporters, which range from tens to tens of thousands of molecules per cell; the collective data were recently unified in a single data set (44). These studies show that

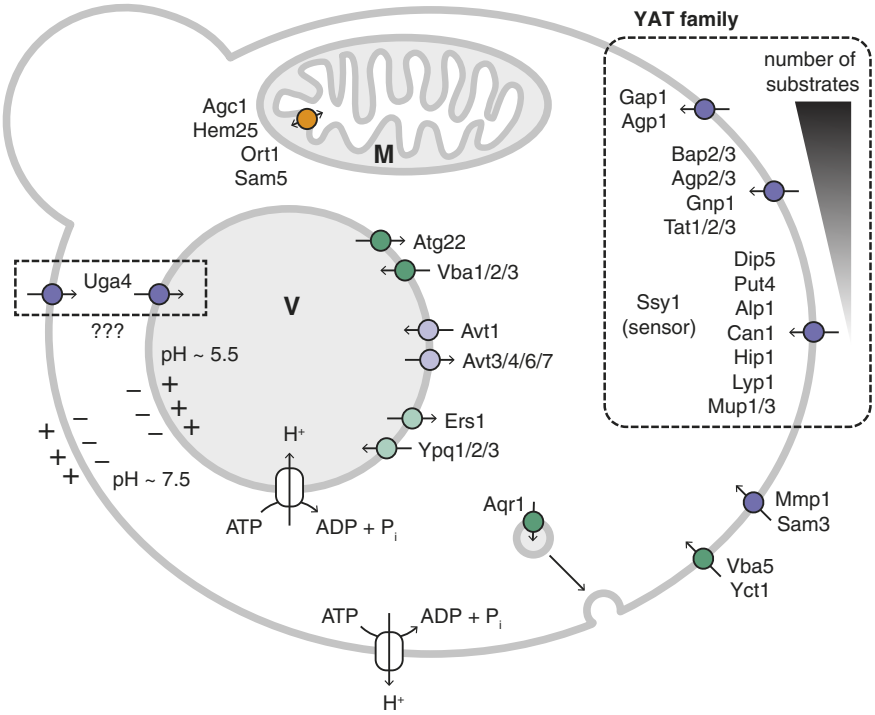

FIG 4 Amino acid transport systems in *S. cerevisiae*. Agc1, Hem25, Ort1, and Sam3 are solute:solute antiporters. All other amino acid transporters are proton-coupled symporters or antiporters, and their activity thus relies on the electrochemical proton gradient. The specificity range of the members of the yeast amino acid transporter (YAT) family is schematically indicated, the gradient colored triangle indicates the number of substrates; details are given in Table 1. Uga4 has been described as a vacuolar transporter (120), but the protein is consistently found in the plasma membrane (119, 342). Transporters are colored according to their family (dark purple, APC; light purple, AAAP; orange, MC; pale green, LCT; dark green, MFS). M, mitochondrion; V, vacuole.

high-affinity transporters such as Gap1, Lyp1, and Mup1 are upregulated in minimal media (thousands to tens of thousands per cell) relative to rich (yeast extract-peptone-dextrose [YPD]) media (tens to hundreds per cell) (45, 46), which is consistent with the known mechanisms of expression regulation (see below).

Specificity of Transporters

Studying transporter activity. Transporter energetics, kinetics, thermodynamics, and substrate specificity can be studied by performing transport assays, typically using radiolabeled substrates to trace the translocation of molecules across membranes. Molecules transported into a cell or membrane vesicles are separated from external substrates by ultrafiltration, using a vacuum pump. The amount of the accumulated radiolabeled substrate is determined by liquid scintillation counting or a Geiger counter directly (lower sensitivity) (47). Typically, ^{14}C- or ^{3}H-labeled substrates are used, and many radiolabeled biomolecules are commercially available.

Transport assays can be performed in intact cells (*in vivo*) or isolated organelles, membrane vesicles, or purified protein-containing lipid vesicles (proteoliposomes) (*in vitro*). *In vivo* measurements probe the transporter in the native environment of the cell but have the disadvantages that, in many cases, the transported substrates are modified and that multiple different proteins contribute to the observed activity. *In vitro* measurements in proteoliposomes do not have this caveat but suffer from the fact that the (lipid) environment is nonnative. Kinetic and thermodynamic measurements and assessment of the driving force(s) for transport are best performed in membrane vesicles or proteoliposomes. Prior to this, indications of the mechanism(s) can be obtained in cells, provided that genetically well-defined strains are used. Ionophores that dissipate the proton motive force (PMF) [e.g., protonophores like carbonyl cyanide-

TABLE 1 *S. cerevisiae* amino acid transporters

Family and protein	Substrate(s) (K_m)[a]	Direction[b]	Reference(s)
Plasma membrane transporters			
APC (YAT)			
Agp1 YCL025C	Broad range (not Lys or Arg); Ile (0.6 mM), Leu (0.2 mM), Phe (0.6 mM), Cys (0.2 mM), Gln (0.79 mM), Asn (0.29 mM)	ext → cyt	55, 56, 60, 64, 65, 234
Agp2 YBR132C	Val, Ile, Leu (0.25 mM), Phe, Thr	ext → cyt	60
Agp3 YFL055W	Val, Ile, Leu (0.49 mM), Phe, Ser, Thr, Glu, Asp	ext → cyt	60, 80
Alp1 YNL270C	Arg	ext → cyt	80, 81
Bap2 YBR068C	Val, Ile, Leu (37 μM), Ala, Phe, Tyr, Met, Cys	ext → cyt	56, 64, 66, 68, 80, 345
Bap3 YDR046C	Val, Ile, Leu, Ala, Phe, Tyr, Trp, Met, Cys, Thr	ext → cyt	67, 80
Can1 YEL063C	His, Lys (150–250 μM), Arg (10–20 μM), Orn	ext → cyt	78, 346
Dip5 YPL265W	Ala, Gly, Ser, Gln, Asn, Glu (48 μM), Asp (56 μM)	ext → cyt	80, 83
Gap1 YKR039W	All natural aa, Cit, Orn, Leu (20 μM)	ext → cyt	53, 60, 347
Gnp1 YDR508C	Leu, Pro, Met, Cys, Ser, Thr, Gln (0.59 mM), Asn	ext → cyt	55, 69, 80
Hip1 YGR191W	His (17 μM)	ext → cyt	70, 77
Mmp1 YLL061W	*S*-Methylmethionine	ext → cyt	84
Lyp1 YNL268W	Met, Lys (10–25 μM)	ext → cyt	78, 79, 348, 349
Put4 YOR348C	Ala, Gly, Pro	ext → cyt	55, 74–76, 80
Sam3 YPL274W	*S*-Adenosylmethionine	ext → cyt	84, 85
Tat1 YBR069C	Val, Leu, Ile, Cys, Thr, Tyr, Trp, His (0.37 mM)	ext → cyt	67, 70, 71, 80
Tat2 YOL020W	Ala, Gly, Cys, Phe, Tyr, Trp	ext → cyt	71, 72, 80
Tat3	Leu, Phe, Tyr (0.16 mM), Trp	ext → cyt	73
APC			
Mup1 YGR055W	Met, Cys	ext → cyt	350, 351
Mup3 YHL036W	Met	ext → cyt	350
MFS			
Yct1 YLL055W	Cys (55 μM)	ext → cyt	90
Vba5 YKR105C	Lys, Arg	cyt → ext	91
Vacuolar transporters			
MFS			
Vba1 YMR088C	His, Lys	cyt → vac	97, 98
Vba2 YBR293W	His, Lys, Arg	cyt → vac	97
Vba3 YCL069W	His, Lys	cyt → vac	97
Atg22 YCL038C	Ile, Leu, Tyr	cyt ← vac	101, 102
AAAP			
Avt1 YJR001W	Neutral aa, His	cyt → vac	92, 95
Avt3 YKL146W	Neutral aa	cyt ← vac	92, 96, 101
Avt4 YNL101W	Neutral aa, His, Lys, Arg	cyt ← vac	92, 96, 101
Avt6 YER119C	Glu, Asp	cyt ← vac	92, 352
Avt7 YIL088C	Pro, Gln	cyt ← vac	35
LCT			
Ers1 YCR075C	Cystine	cyt ← vac	104, 353
Ypq1 YOL092W	Lys, Arg	cyt → vac	105, 106
Ypq2 YDR352W	Arg	cyt → vac	105, 107
Ypq3 YBR147W	His	cyt → vac	105, 107
Mitochondrial transporters			
MC			
Agc1 YPR021C	Glu, Asp	cyt ↔ mit	40
Hem25 YDL119C	Gly	cyt ↔ mit	112, 113
Ort1 YOR130C	Lys, Arg, Orn	cyt ↔ mit	41, 108, 109
Sam5 YNL003C	*S*-Adenosylmethionine, *S*-adenosylhomocysteine	cyt ↔ mit	111
Other			
APC			
Uga4 YDL210W	γ-Aminobutyric acid	ext → cyt or cyt → vac	119, 120
MFS			
Aqr1 YNL065W	Ala, Glu, Asp	cyt → ext	354, 355

[a]Numbers in parentheses are the reported (apparent) K_m values for the given substrate. aa, amino acids.
[b]ext, extracellular; cyt, cytosol; vac, vacuolar lumen; mit, mitochondrial matrix.

4-(trifluoromethoxy)phenylhydrazone (FCCP) and carbonyl cyanide *m*-chlorophenylhydrazone (CCCP)], the membrane potential (K^+ ionophore valinomycin), or the pH gradient (K^+/H^+ ionophore nigericin) are used to manipulate the driving forces of secondary active transporters (e.g., all the amino acid permeases [AAPs] in yeast), but

they indirectly also affect the ATP pool of the cell; the combination of valinomycin and nigericin is very effective in dissipating the electrochemical proton gradient (Δp).

One can also use the ionophores to (transiently) generate a membrane potential ($\Delta\Psi$) or pH gradient (ΔpH), provided that the corresponding ion gradient is known, which is typically the case in *in vitro* experiments in vesicles or proteoliposomes. Thus, by diluting vesicles containing 100 mM K^+ ions into a medium with 1 mM K^+, the K^+ gradient is used to generate a $\Delta\Psi$ of -120 mV (see the Nernst equation for conversion of the ion gradient into a voltage difference). Similarly, by imposing gradients of weak acids or bases, a ΔpH inside alkaline or acidic can be generated. In our previous study (48), we used this approach to vary the magnitude of the $\Delta\Psi$ and/or ΔpH across the membrane and determine the driving-force dependence of the lysine-H^+ symporter Lyp1.

Sample preparation both *in vivo* and *in vitro* involves the expression of the transporter (or a genetically engineered variant), using the native organism or a suitable expression host. The vast majority of studies on amino acid transport in yeast have been done in intact cells, and in most cases, more than one transport protein is responsible for the observed uptake, which obscures the data. In a number of studies, sets of well-defined mutants have been used, and by using the appropriate substrate(s) concentrations, one can obtain detailed information about the substrate specificity of the transporters, e.g., as reported in Table 1. Finally, in some studies, pools of amino acids inside cells or specific organelles are analyzed by LC (liquid chromatography) coupled to fluorescence detection or MS (mass spectrometry).

Plasma membrane transporters. Most of the amino acid transporters in the *S. cerevisiae* PM belong to the APC family (reviewed in references 49–51). They are sometimes called the yeast amino acid transporter (YAT) family or AAPs (12, 13, 52). Mup1, which transports methionine and cysteine, and Mup3, which transports methionine, are more distantly related but still belong to the APC superfamily.

The YAT family includes transporters with both broad- and narrow-range substrate specificities as well as membrane proteins predicted to act as sensors. Many of the transport specificities overlap, although this apparent redundancy is less pronounced when the conditions under which the proteins are expressed are considered. There are two known broad-range transporters: Gap1, the general amino acid permease with a high affinity for all amino acids which is present mainly under poor-nitrogen conditions (53, 54), and the broad-specificity transporter Agp1, which imports all natural proteinogenic amino acids except arginine and lysine (55, 56). Gap1 functions not only as a transporter but also as a nutrient sensor (see "Additional Functions of Amino Acid Transporters," below), signaling the availability of specific amino acids to the protein kinase A (PKA) pathway (57–59).

Neutral amino acids are also imported by transporters with more limited substrate specificities: Agp2, Agp3, Bap2, Bap3, Gnp1, Tat1, Tat2, and Tat3. Agp2 and Agp3 contribute significantly to amino acid uptake only under nutrient-poor conditions and in strains where genes coding for permeases with a higher affinity have been deleted (60). The more important role of Agp2 is to act as a sensor that regulates the expression of importers for carnitine and polyamines (61–63). Bap2 and its paralog Bap3 also have a relatively broad substrate specificity (56, 64–68). Bap2 is important for the import of leucine, which is inhibited by most hydrophobic amino acids (Table 1) except tryptophan. The rate of inhibition of leucine transport correlates linearly with the logP (octanol-water partition coefficient) value of the amino acids except tryptophan (66). Those authors suggest that the substrate affinity of Bap2 is determined by the ability of the amino acid side chain to fit into the hydrophobic binding pocket of the active site.

Gnp1 was first isolated as a high-affinity glutamine permease but is able to transport other amino acids, including cysteine and proline (56, 64, 69). Tat1 and Tat2 were originally identified as importers of tyrosine and tryptophan, although both have been shown to also transport other neutral amino acids (56, 67, 70–72). Tat3 is a tyrosine

permease that is found in the natural *S. cerevisiae* strain RM11-1a but not in the laboratory strain S288c (73).

The remaining transporters have much narrower substrate specificities. Put4 imports proline, alanine, and glycine (55, 74–76). There are several transporters for the specific uptake of basic amino acids: Hip1 (histidine) (70, 77), Can1 (arginine, lysine, histidine, and ornithine) (78, 79) and its paralog Alp1 (arginine) (80, 81), and Lyp1 (lysine) (66, 78, 79, 82). Dip5 catalyzes the high-affinity transport of the acidic amino acids glutamate and aspartate, although there is some evidence that this protein is also able to transport alanine, glycine, glutamine, and asparagine (80, 83).

Two other YAT members catalyze the import of amino acid metabolites and polyamines. Mmp1 imports *S*-methylmethionine, a common plant metabolite which can be used by *S. cerevisiae* as a sulfur source (84). Sam3 imports *S*-adenosylmethionine (SAM) as well as the polyamines putrescine and spermidine (84, 85). Uemura et al. (85) proposed that polyamine transport represents the more important physiological function of Sam3, as SAM is not normally present at high levels in the environment. However, it has been shown that SAM is present at sufficient levels in blood plasma to support the growth of *Pneumocystis*, a parasitic fungus which causes pneumonia and is not able to synthesize SAM *de novo* (86). Loss-of-function mutations in Sam3 confer resistance to the natural-product antibiotic sinefungin, which is a SAM analog (87).

Two members of the major facilitator superfamily (MFS) (reviewed in references 88 and 89) have been reported to transport amino acids across the *S. cerevisiae* plasma membrane. Yct1 is a high-affinity cysteine importer which belongs to the Dal5 subfamily of anion:cation symporters (90). Vba5, whose overexpression increases the import of arginine and lysine into cells (91), is closely related to several vacuolar amino acid transporters and is discussed in the section on vacuolar transporters below.

Vacuolar transporters. The flux of amino acids across the vacuolar membrane is mediated by transporters from the APC superfamily, the MC family, and the MFS, which mediate solute:proton symport or antiport. These systems rely on an electrochemical proton gradient generated by the vacuolar proton-ATPase (V-ATPase), which pumps protons into the vacuolar lumen at the expense of ATP (92–94).

Most vacuolar amino acid transporters in *S. cerevisiae* belong to the AVT (amino acid vacuolar transport) family, which belongs to the APC superfamily. Avt1 transports a broad range of neutral amino acids and histidine into the vacuole (92, 95). Avt3 and Avt4 transport neutral amino acids out of the vacuole, while Avt4 facilitates the export of histidine, lysine, and arginine (92, 96). Avt6 exports the acidic amino acids glutamate and aspartate (91, 92), while Avt7 exports glutamine and proline (35). No activity has been observed for two of the predicted family members, Avt2 and Avt5 (35, 92). It is not known why Avt1 has a different direction of transport (vacuolar import, suggesting proton:amino acid antiport) compared to the other AVT members (vacuolar export, suggesting proton:amino acid symport). At the amino acid level, Avt1 and Avt2 do not cluster with any of the other Avt members.

Vba1, Vba2, and Vba3 are MFS proteins that catalyze the transport of basic amino acids into the vacuole (97). Subsequent studies suggested that Vba1 may transport other neutral and acidic amino acids (98). The initial study by Shimazu et al. (97) identified two other potential family members based on sequence similarity. One, Vba4, is a vacuolar membrane protein involved in drug resistance and vacuolar morphology rather than amino acid transport (98). The other, Vba5, is a plasma membrane transporter involved in cellular uptake of arginine and lysine (91). The PM multidrug resistance efflux proteins Sge1 (99) and Azr1 (100) are closely related to the vacuolar basic amino acid transporters (VBAs), enough so that they could be considered part of the same family. It is unknown why some members are localized to the vacuolar membrane and others are localized to the plasma membrane or why their direction of transport differs, e.g., drug export by Azr1 and Sge1 and amino acid import by Vba5.

Atg22, also an MFS protein but distinct from the VBA family, facilitates the efflux of tyrosine, isoleucine, and leucine from the vacuolar lumen (101, 102). Another group of vacuolar amino acid transporters was recently identified based on their similarity to

cystinosin, the mammalian proton-driven lysosomal cystine exporter (103). They belong to a small but distinct group of proteins referred to as the lysosomal cystine transporter (LCT) family or, more broadly, the PQ loop family. Ers1 mediates cystine transport, but activity has been demonstrated only by the accumulation of cystine in whole cells expressing Ers1 with a V5 epitope tag (104). Three other LCT family members, Ypq1, Ypq2, and Ypq3, have been shown to localize to the vacuolar membrane (105), but there is controversy about the directionality of transport. Based on their similarity to cystinosin, they are reported to be exporters (105). In another study, it was concluded that Ypq1, -2, and -3 are importers of lysine/arginine, arginine, and histidine, respectively (106, 107).

There are also questions about the relative contributions of each transport system to the flux of amino acids across the vacuolar membrane. Shimazu et al. (97) found a 70% reduction in vacuolar histidine and lysine import in a Δ*vba1* mutant, while Tone et al. (95) saw an almost complete loss of histidine import in an *avt1*Δ *avt3*Δ *avt4*Δ mutant, and Sekito et al. (106) observed a 90% reduction in lysine import in a Δ*ypq1* mutant. Despite the severe effects of the Avt1 knockout on transport activity in isolated vacuoles, Tone et al. (95) did not find a significant difference in the vacuolar concentrations of basic amino acids. It is possible that knockout mutations in specific Avt genes cause a change in the expression of other proteins. It is also possible that some of these proteins are in fact transceptors; that is, they form a membrane protein-substrate complex that transduces signals and have an indirect effect on the transport and accumulation of amino acids. These questions can be resolved only when *in vitro* transport model systems, e.g., lipid vesicles containing purified proteins (48), are available.

Mitochondrial transporters. The characterized *S. cerevisiae* mitochondrial amino acid transporters all belong to the mitochondrial carrier (MC) family (37) and catalyze solute:solute or solute:proton antiport. Agc1 is an aspartate/glutamate carrier thought to be involved in the delivery of glutamate to the mitochondrial matrix for transamination reactions and amino acid biosynthesis and in glycolysis through exchanging glutamate for aspartate as part of the malate-aspartate NADH shuttle (40). Yeast cells lacking *agc1* are unable to grow with acetate or fatty acids as carbon sources.

Ort1 (ARG11) transports ornithine, arginine, and lysine in both directions by either exchange or proton antiport and is involved in, but not essential for, arginine biosynthesis (41, 108, 109). This is because the enzymes catalyzing the first five steps of the pathway, converting glutamate to ornithine, are located in the mitochondria, while those catalyzing the final three steps, from ornithine to arginine, are located in the cytosol (110). The "leaky" phenotype of *arg11* deletion strains, along with observed residual uptake by isolated mitochondria, indicates that there is another as-yet-unidentified mitochondrial carrier for basic amino acids (108, 109).

Sam5 (Pet8) transports *S*-adenosylmethionine (SAM) from the cytosol into the mitochondrial matrix, where it acts as a methyl group donor for methylation of DNA, RNA, proteins, and sterols as well as a cofactor for the synthesis of biotin and lipoic acid (111). *S*-Adenosylhomocysteine (SAHC), which is produced from SAM in these methylation reactions, is a competitive inhibitor of this transport. It was thus hypothesized that the physiological function of Sam5 is to catalyze the exchange of cytosolic SAM with SAHC in the mitochondrial matrix.

Hem25 transports glycine into the mitochondria, where it is used in both heme biosynthesis and one-carbon metabolism (112, 113). Cells lacking *hem25* exhibit a defect in respiration and decreased stability of specific proteins of the electron transport chain (114, 115). Fernández-Murray et al. recently identified Ymc1 as a potential secondary glycine transporter. However, this is in contradiction with its proposed role in carbon source utilization, which is likely to involve the transport of a TCA cycle intermediate (116, 117). This controversy is addressed in more detail by Lunetti et al. (112), who conclude that Hem25 is a glycine transporter, and so is its mammalian ortholog *SLC25A38*.

Mitochondrial transporters that facilitate the transport of serine or proline have not

been found. However, a recent study in isolated *S. cerevisiae* mitochondria showed that the uptake of proline is dependent on the proton electrochemical gradient, suggesting the presence of a transporter (118).

Other transporters. Uga4, also a member of the APC superfamily, is involved in the transport of γ-aminobutyric acid (GABA). The *uga4* gene was isolated due to its ability to restore growth on GABA as a sole N source in a *gap1 put4 uga4* deletion strain (119), but a subsequent study by Uemura et al. (120) reported that Uga4 is a vacuolar membrane (VM) protein. This is based on indirect immunofluorescence microscopy and the sensitivity of GABA uptake to both azide and bafilomycin A1, which collapse the electrochemical proton gradient across the plasma and vacuolar membranes, respectively. Although other studies on GABA transport and metabolism have suggested that it is accumulated in the vacuole (121, 122), the localization of Uga4 to the vacuolar membrane (VM) is inconsistent with its ability to mediate whole-cell GABA import. It cannot be ruled out that Uga4 is located on the vacuolar membrane and promotes whole-cell uptake via an unidentified regulatory function, so overall, the literature is inconclusive. Unfortunately, large-scale green fluorescent protein (GFP)-based localization studies (www.yeastgenome.org [accessed 29 September 2019]) do not show a clear localization for Uga4. Besides all the characterized amino acid transporters, which function as importers, there is evidence for a possible leucine exporter. Melnykov observed that leucine is excreted from cells after loading with an alanine-leucine dipeptide (123). The known leucine transporters (Table 1) utilize the electrochemical proton gradient (Δp) to accumulate leucine and are not expected to export the solute from the cell, unless the leucine gradient exceeds the Δp through massive uptake and hydrolysis of the dipeptide.

Energy-Coupling Mechanism and Amino Acid Accumulation

The amino acid transporters of yeast are so-called proton-coupled symporters (plasma membrane and vacuole) or antiporters (mitochondria and vacuole). In cells, ATPase-driven proton pumps maintain a neutral to slightly alkaline cytoplasm (thus resulting in a ΔpH) and generate a membrane potential ($\Delta\Psi$); the sum of both is the proton motive force (Δp). In the equation $\Delta p = \Delta\Psi - Z\Delta\text{pH}$ ($Z = 2.3RT/F$, where R is the gas constant, T is the absolute temperature, and F is the Faraday constant, at $T =$ 298 K and $Z = 58$ mV), the components (ΔpH and $\Delta\Psi$) of the proton motive force drive the uphill transport of a solute via proton-coupled symporters and antiporters. The actual driving force depends on the charge of the solute and the number of protons co- or countertransported. Assuming a stoichiometry of 1:1, then neutral amino acids are driven by ΔpH plus $\Delta\Psi$, symport of anionic amino acids is driven by ΔpH only, and symport of basic amino acids is driven by ΔpH plus 2 times $\Delta\Psi$. Thus, at a given magnitude of the proton motive force and at thermodynamic equilibrium, the accumulation of such amino acids is very different. We emphasize that in living cells, the condition of thermodynamic equilibrium is hardly ever (if ever) reached (see reference 124), but here we use this limit to illustrate the impact of solute charge on the apparent accumulation of amino acids.

At equilibrium, the driving force (Δp) for solute-proton symport is equal in magnitude to the solute concentration gradient ($Z \log_{10} [\text{solute}]_{out}/[\text{solute}]_{in}$). If we assume that $\Delta\Psi$ equals −120 mV and $Z\Delta$pH equals 120 mV (i.e., the inside of the cell is 2 pH units higher than the outside), then the overall proton motive force is −240 mV. Under these conditions, at thermodynamic equilibrium, and rounding off Z to 60 mV, neutral, acidic, and basic amino acids would accumulate to values of $[\text{solute}]_{in}/[\text{solute}]_{out}$ of 10^4, 10^2, and 10^6, respectively. Next to being a driving force for transport, the components of the proton motive force can affect specific steps of the translocation cycle and thus have a kinetic effect (125).

As expected for solute symporters, dissipation of Δp in general leads to solute export down the concentration gradient, as has been shown in yeast for most amino acids (25, 126). However, under the same conditions, little or no export of basic amino acids is observed in *S. cerevisiae* (25, 127). Four APC proteins are responsible for the import of

basic amino acids: Lyp1, Can1, Gap1, and Alp1 (13). The deletion of all four genes abolishes the transport of basic amino acids across the plasma membrane (PM) of yeast (128). Mechanistic and bioenergetic studies of amino acid transport *in vivo* are hampered by the sequestration of solutes in the organelles, their metabolism inside the cell, and the difficulty in manipulating the ion gradients. Furthermore, transcriptional or posttranslational regulation of transport and removal of the transporter from the PM influence the measured activity. To measure transport independent of these challenges, Can1 has been studied in hybrid plasma membrane vesicles. In this system, the transport of arginine via Can1 was found to be unidirectional (129), but this conclusion was revisited in a later *in vivo* study when efflux was observed under conditions where cells were growing exponentially (26). In recent work by Bianchi et al. (48), using purified and membrane-reconstituted Lyp1, the unidirectionality was found to be an intrinsic property of the protein and explained by an extreme asymmetry in the Michaelis constant (K_m) for the inward and outward transport of lysine (48). The step(s) in the translocation of the substrate that determines this extreme asymmetry remains elusive, which is hampered by the lack of sufficient structural data.

The exact mechanism by which a transporter couples a proton to solute accumulation is difficult to deduce, even when crystal structures are available. In general, proton coupling involves the protonation and deprotonation of one or more residues, but strictly speaking, it is difficult to discriminate protonation/deprotonation of amino acid residues from binding and translocation of a hydronium ion (130). The transfer of a proton may happen via a series of protein amino acids, involving carboxylates, hydroxyls, amines, and water (131). Such a proton permeation pathway requires that proton translocation is coupled to solute binding and translocation, as the system would otherwise create a proton leak (124, 132).

It is possible that we can learn about proton coupling from Na^+-coupled transporters, because some systems may also bind hydronium ions (133, 134). For example, the Na^+/Li^+-dependent galactoside transporter MelB from *Escherichia coli* shows proton coupling at low pH, whereas sodium coupling is preferred when sufficient sodium ions are present (135). The structure and functional data for MelB suggest a universal cation-binding site for Na^+, Li^+, and H_3O^+. Whether hydronium permeates the membrane in the same manner as Na^+ or Li^+ or is a transient state of water in the translocation cycle is not known and is difficult to deduce from currently available data (136). For the Na^+-coupled solute-specific transporter BetP (from *Corynebacterium glutamicum* and a member of the APC family), it was shown that a single mutation, Gly153 to Asp, is sufficient to convert the protein into an H^+-coupled solute-specific transporter (137).

Comparison of structures of sodium- and proton-coupled transporters within the LeuT family (from *Aquifex aeolicus* and belonging to the APC superfamily) shows some similarity between the Na^+- and proton-binding sites, but the proteins have distinct pathways for Na^+ and H^+ translocation (137–140). The Na^+ bound in the first sodium-binding site of LeuT and BetP is involved in the coordination of the substrate via a direct interaction with its α-carboxyl. In the H^+-dependent transporters CaiT (from *E. coli*) and ApcT (from *Methanocaldococcus jannaschii*), the substrate is coordinated differently, and a hydronium ion does not fit into the site that would correspond to the first Na^+-binding site of LeuT and BetP. Furthermore, the second sodium-binding site present in BetP is replaced by a lysine residue (Lys158), which is located in transmembrane domain 5 (TM5) of CaiT and ApcT. This is the site that is hypothesized to become protonated prior to substrate binding (138). In ApcT, Lys158 superimposes with the second Na^+ ion of LeuT, Mhp1 (from *Microbacterium liquefaciens*), and BetP (from *C. glutamicum*). The calculated pK_a is 3 to 4 pH units lower than for an unperturbed lysine ($pK_a = 10.5$), and mutagenesis of Lys158 to Ala abolished transport (138). Similar results have been obtained for LysP, a lysine symporter from *Salmonella enterica* serovar Typhimurium (141). In Fur4, the uracil proton symporter from *S. cerevisiae*, the equivalent mutation reduced the affinity for uracil more than 400-fold (142). The fact that the binding affinity is either reduced or abolished when the lysine is mutated to a neutral

residue agrees with the suggestion that binding of a cation activates the substrate-binding site (136, 137).

Next to the above-discussed highly conserved lysine residue in TM5, a conserved glutamate in TM3 plays a role in proton coupling in Can1 and Lyp1. Mutagenesis of glutamate 184 to alanine or glutamine in Can1 destroys translocation, but replacement by aspartate does not. In all these mutants, binding of the substrate was retained (78, 143), showing the direct involvement of an anionic residue in proton-coupled translocation. In conclusion, proton coupling in solute-H^+ symporters has similarities with sodium ion coupling, with the exception that, even with crystal structures at hand, it is difficult to define which residues participate in proton translocation. Furthermore, there is some redundancy in proton-coupling residues. In general, one can state that proton-coupling solute transporters require a chain of residues that are transiently protonated and often include a centrally located protonatable group.

Amino Acid Transporters in Other Fungi

YAT homologs have been characterized in the fission yeast *Schizosaccharomyces pombe* (144–148); the filamentous fungi *Aspergillus nidulans* (149–151), *Penicillium chrysogenum* (152, 153), and *Neurospora crassa* (154); the human pathogens *Candida albicans* (22, 155), *Candida glabrata* (156), *Cryptococcus neoformans* (21, 155), and *Histoplasma capsulatum* (156); the plant symbiotes *Amanita muscaria* (157), *Hebeloma cylindrosporum* (158), and *Glomus mosseae* (159); and the plant pathogens *Fusarium fujikuroi* (160) and *Uromyces fabae* (161).

Several vacuolar amino acid transporters have been characterized in *S. pombe* which are homologous to Atg22 (162), Avt3/4 (163, 164), Avt5/6 (165, 166), and Vba2 (165, 167, 168) of *S. cerevisiae* (Fig. 4). An Avt3 homolog from the plant pathogen *Fusarium oxysporum* has also been characterized (169). In addition, homologs of the mitochondrial carrier Ort1 have been identified in *N. crassa* (170) and *Aspergillus fumigatus* (171).

A note should be made here about Can1 from *S. pombe* (SpCan1), which shares its name with *S. cerevisiae* Can1 on the basis of the isolation of canavanine resistance mutations and not because of sequence similarity (82, 172). Although SpCan1 belongs to the APC superfamily, a BLAST search against the *S. cerevisiae* S288c genome reveals that it is in fact most similar to Vhc1, a vacuolar membrane protein which plays a role in osmoregulation and is thought to be a K^+/Cl^- symporter (173). A recent study in *S. pombe* showed that fluorescently tagged SpCan1 indeed localizes to the vacuolar membrane (174).

For the most part, the transporters characterized in other fungi are functionally analogous to those in *S. cerevisiae*, but there are exceptions. Cyn1 from the pathogenic fungi *C. glabrata*, *C. albicans*, and *H. capsulatum* transports cystine, which is the predominant form of organic sulfur found in blood plasma (156). There are no functional orthologs of Cyn1 in *S. cerevisiae* or *S. pombe*. *C. albicans* has several close homologs of Gap1 with similar or narrower substrate specificities, and some, like Gap1, signal the PKA pathway (175).

Several fungal amino acid transporters are only distantly related to transporters from *S. cerevisiae*. The aromatic amino acid and leucine transporter Mtr has been characterized in *Neurospora crassa* (176), *Penicillium chrysogenum* (177), and *Fusarium proliferatum* (178). A family of fungal oligopeptide transporters (FOTs), identified by soil transcriptomics, contains at least one member that is able to transport cysteine (179). Homologs of this family are absent from common *S. cerevisiae* laboratory strains, but two members are present in the EC1118 wine strain (179). Both the Mtr and FOT permease families are most similar to the AAAPs, with 11 predicted transmembrane segments (TMSs), and are found only in fungi. The exception is the red alga *Galdieria sulphuraria* (180), which has nine separate Mtr homologs.

Additional Functions of Amino Acid Transporters

Some yeast transporters have been shown to possess an additional function as a receptor, and they are therefore termed "transceptors." The best-studied transceptor is

Gap1, which induces the protein kinase A (PKA) pathway (57). The transceptor function of Gap1 has been described in detail (52), and a similar function has been proposed for the phosphate transporter Pho84 and the ammonium transporter Mep2 (181, 182). Gap1 triggers the PKA pathway only in the presence of specific substrates, such as citrulline. Other substrates do not induce this pathway but compete with citrulline and thereby effectively block signaling (58, 59). Specific residues in Gap1 responsible for PKA signaling have not yet been identified. Mutants that affect substrate binding also affect PKA signaling, but a relationship between the transport rate and PKA signaling has not been found (59). The C terminus of Gap1 may play a signaling role, as truncations of the Gap1 C terminus show increased PKA signaling (57).

Structural Features of Protein Families

APC and AAAP families. The amino acid-polyamine-organocation (APC) (TCDB 2.A.3) and amino acid/auxin permease (AAAP) (TCDB 2.A.18) families both belong to the APC superfamily (49, 50, 183). All members of the APC superfamily share the same core fold, referred to as the "5 + 5 inverted repeat," which is a two-repeat unit of 5 TMSs that exhibits pseudo-2-fold symmetry with respect to the membrane plane (Fig. 5). This fold was first identified in the crystal structure of the bacterial Na^+:leucine symporter LeuT (140). LeuT is a homodimer in which TM1 to -10 of each subunit form two antiparallel, intertwined structural repeats, each containing five transmembrane segments. TM11 and TM12 form a V shape that sits at the dimer interface. Similar folds have been determined for related bacterial transporters, including several members of the APC family with similarity to the YATs. These are the arginine:agmatine antiporter AdiC (from *E. coli*) (184, 185), the H^+-dependent amino acid transporter ApcT (138), and the glutamate-GABA antiporter GadC (from *E. coli*) (186). The crystal structures of ApcT and GadC indicate that the proteins do not form dimers, but the antiparallel repeats of five transmembrane segments are conserved (138, 186). AdiC is a homodimer both in the crystal structure and *in vitro*, but each of the subunits transports independently of each other (184, 185).

The availability of crystal structures of several different conformations and binding states of AdiC confirmed the alternating-access model for transport (187). The substrate is bound between two structurally distinct domains, each containing four TMSs. The transition between the inward- and outward-facing conformations is achieved by rigid-body rotations of both domains. A similar model has been described for LeuT, in which one domain remains stationary while the other moves using both rigid-body rotations and hinge-like bending (188). Transport by GadC involves an additional pH-dependent regulatory mechanism mediated by the binding of its C terminus within the inward-open conformation (186).

The crystallographic structures of LeuT and AdiC have been used to generate homology models of *A. nidulans* PrnB and *S. cerevisiae* Bap2, Can1, Gap1, and Tat2 (66, 72, 78, 143, 189, 190). These models, along with mutational studies, have allowed the identification of numerous amino acid residues involved in substrate recognition and transport. The predictive value of the models was demonstrated by modeling lysine in the substrate-binding site. Based on a comparison of Can1 and Lyp1, this resulted in the mutations S176N and T456S, which converted the specificity of Can1 into that of Lyp1 (78). Thus, whereas Can1 transports arginine and lysine and Lyp1 transports lysine, Can1(S176N,T456S) was found to be specific for lysine only. Overall, these experiments indicate that the structural conservation of members of the APC family is relatively high, especially in the substrate-binding site.

AAAP transporters are predicted to contain 11 transmembrane segments, with the N terminus in the cytoplasm. This topology has been confirmed for NAT2/AAP1 and AUX1 from *Arabidopsis thaliana* (191, 192). Recently, the structure of SLC38A9, a lysosomal AAAP transporter from *Danio rerio*, in complex with arginine has been elucidated. The structure shows 11 transmembrane helices and the presence of the 5 + 5 inverted repeat (193). In this model, TM1 to -10 of SNAT2 correspond with TM1 to -10 of LeuT, and TM12 is missing. The current crystal structure of SLC38A9 is devoid

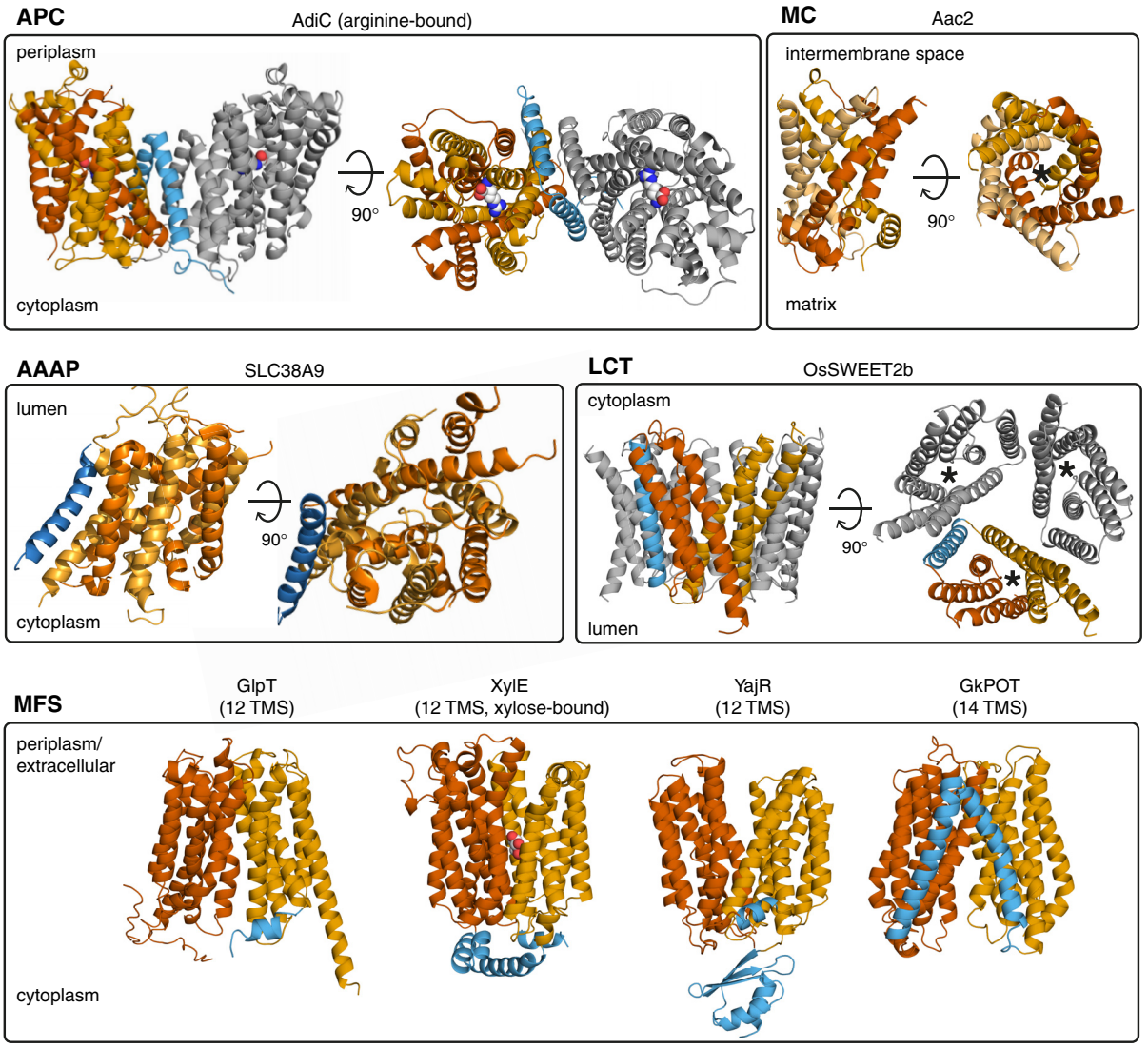

FIG 5 Crystal structures representing the five major families of amino acid transporters. Structures of *E. coli* AdiC (PDB accession number 3L1L) (343); *S. cerevisiae* Aac2 (PDB accession number 4C9H) (198, 199); *Danio rerio* SLC38A9; *Oryza sativa* OsSWEET2b (PDB accession number 5CTG) (204); *E. coli* GlpT (PDB accession number 1PW4) (344), XylE (PDB accession number 4GBY) (219), and YajR (PDB accession number 3WDO) (212); and *Geobacillus kaustophilus* POT (PDB accession number 4IKV) (214) are shown. (Pseudo)symmetrical repeats within the polypeptide chains are shown in orange, gold, and light brown. Additional structural elements, including transmembrane segments, amphipathic helices, and cytosolic domains, are in light blue. Additional protein chains in dimeric AdiC and trimeric OsSWEET2b are in gray. Bound substrates are shown in a sphere-filling representation, with carbon in white, oxygen in red, and nitrogen in dark blue.

of the N-terminal tail, but the core region of the protein is fully functional in transport; it is thus a good model for mechanistic insight into vacuolar AVT members.

Mitochondrial carrier family. Mitochondrial carriers (MCs) (TCDB 2.A.29) contain three sequence-related repeats of roughly 100 amino acids, such that the total carrier domain is around 300 amino acids long (194, 195). Each repeat contains two signature motifs separated by approximately 20 to 30 amino acids: Px[D/E]xx[K/R]x[K/R] and [D/E]Gxxxx[W/Y/F][K/R]G. Crystal structures have been solved for the mitochondrial ATP/ADP carriers from *Bos taurus* (cow) (196, 197), *S. cerevisiae* (198), and *Thermothelomyces thermophila* (199). Each structure shows the same monomeric barrel-like fold formed from six transmembrane helices (Fig. 5). The three sequence-related domains form three pseudosymmetrical structural repeats consisting of two transmembrane α-helices joined by a short matrix α-helix. Experiments both *in vitro* and *in vivo* suggest that the ATP/ADP carriers function as monomers (see references 194 and 195 and

references therein). A unique transport mechanism has been proposed, involving the simultaneous rotation of the three repeat domains around a central translocation pore (194, 195).

Hem25, Ort1, and Sam5 are all approximately 300 amino acids long and are likely to have the same basic fold as the ATP/ADP carriers. Agc1 is 902 amino acids long and contains an ~500-residue N-terminal domain (NTD) and an ~100 residue C-terminal domain (CTD), both of which would be located in the intermembrane space. Residues 508 to 902, containing the mitochondrial carrier domain, are sufficient for transport (40). A similar domain structure is observed for the two isoforms SLC25A12 and -13 of the human mitochondrial aspartate/glutamate carrier (200), although Agc1 is still approximately 125 amino acids longer than either of these proteins. The NTDs of SLC25A12 and SLC25A13 mediate dimerization of the transporters and are responsible for the activation of transport by calcium (200, 201). The structure of an NTD-CTD chimera has been solved in both calcium-bound and calcium-free conformations, and the data confirm that regulation by Ca^{2+} involves both of these domains (200). It was proposed that the regulatory domains dock above the mitochondrial carrier domain and regulate transport by restricting access to the translocation pore. It has been reported that the NTD of Agc1 does not contain the calcium-binding EF hand motifs (202) present in the NTD of SLC25A12/13, which conforms with the notion that yeast mitochondria lack a mitochondrial calcium uniporter. Inspection of the crystal structures confirms that the residues responsible for calcium binding in SLC25A13 are not (completely) conserved in Agc1. However, the NTD of Agc1 shares 20% sequence identity with that of SLC25A13 or SLC25A12, and a search for potential structural homologs using the HHpred server (203) yields several calcium-binding, EF hand structures as significant matches (probability of >99% and E value of $<1e^{-10}$). It would be interesting to determine if the NTD and CTD of Agc1 are regulatory domains and, if so, determine which signal(s) that they respond to.

Lysosomal cystine transporter family. No structures have been solved for members of the lysosomal cystine transporter (LCT) family (TCDB 2.A.43). The closest crystallized homolog is OsSWEET2b, the sugar transporter from rice (*Oryza sativa*) (204). LCT and SWEET transporters both belong to the TOG (transporter–opsin–G-protein-coupled receptor) superfamily and are predicted to have the same 3 + 1+3 topology, which is two repeat units of 3 TMSs that exhibit pseudo-2-fold symmetry with respect to the membrane plane separated by a single TM (205). OsSWEET2b was crystallized as a homotrimer, which corresponds to the *in vivo* oligomeric state (Fig. 5). Each subunit contains seven transmembrane α-helices, with TM1 to -3 and TM5 to -7 forming parallel triple-helix bundles (THBs). The substrate transport path is believed to be at the interface of the two triple-helix bundles (205). This is supported by work on bacterial homologs, the SemiSWEETs, where crystal structures with a bound substrate and/or representing several different parts of the transport cycle are available (206–209). The general consensus is that SemiSWEETs operate via an alternating-access mechanism involving (almost) rigid-body movements of the two THBs.

Homology modeling using OsSWEET2b may be a solid basis for future studies of the *S. cerevisiae* LCT family amino acid permeases. However, OsSWEET2b is significantly shorter than these permeases (230 amino acids versus 260 amino acids for Ers1, 308 for Ypq1, 317 for Ypq2, and 296 for Ypq3), and thus, its crystal structure does not account for the entire sequence of these other permeases. Analysis using the TOPCONS server (210) indicates that Ypq1, -2, and -3 have an extended loop between the third and fourth transmembrane segments, which would be located in the cytoplasm.

Major facilitator superfamily. The major facilitator superfamily (MFS) (TCDB 2.A.1) is the largest family of secondary carriers and is found in all branches of life, mediating numerous physiological processes (88, 89, 211). All MFS proteins share a characteristic core fold consisting of two 6-TMS domains with a 2-fold pseudosymmetry axis perpendicular to the membrane (Fig. 5). The binding site lies between these two domains and is formed by residues from both domains. The most basic model for transport (termed the rocker-switch mechanism) involves pseudosymmetrical rigid-body rotation

of the N and C domains (compare, for example, YajR and GkPOT in Fig. 5). More details on proposed transport mechanisms can be found in recent reviews (89, 211).

Many MFS transporters contain membrane-associated or -embedded α-helices in addition to the 12-TMS core. A short amphipathic α-helix is often found in the interdomain linker (see GlpT and YajR in Fig. 5). This helix has been suggested to play a role in the transition from an outward- to an inward-facing conformation by acting as a sensor for the position of the TMS core relative to the membrane (212, 213). A similar role has been proposed for the interdomain TM pair found in mammalian and bacterial proton-dependent oligopeptide transporters, which sit outside the core fold in a V shape (214). In *E. coli* YbgH, the rigidity of these two helices has been shown to be important for transport (215). Members of the drug:proton antiporter 2 (DHA2) family (TCDB 2.A.1.3) are also predicted to have 14 TMSs, with the extra TM pair located between the N and C domains in the primary sequence (88). No structures have been solved for members of this subfamily. Interestingly, removal of TMVII and -VIII from the *Bacillus subtilis* antibiotic resistance protein Tet(L) abolishes tetracycline efflux but not cation transport (216).

Several other cytosolic accessory domains have been identified in MFS transporters. YajR from *E. coli* has a 67-residue C-terminal cytosolic domain whose secondary structure is similar to those of metal-binding domains from ATPases responsible for heavy metal efflux in *Arabidopsis thaliana* (212, 217). The domain is predicted to play a regulatory role, but this has not yet been confirmed because the YajR transport substrate is unknown. Members of the sugar porter subfamily, including the human glucose transporter GLUT1 (218) and the *E. coli* xylose transporter XylE (219), contain a conserved intracellular four-helix domain involved in gating (Fig. 5).

Possible cytosolic domains. Four *S. cerevisiae* amino acid permeases are predicted to have extended N-terminal domains (longer than 200 amino acids). Avt1, Avt3, Avt4, and Vba4 have all been demonstrated or predicted to be located in the vacuolar membrane, with their N terminus in the cytoplasm. Analysis using the TOPCONS Web server (210) indicates that Avt1 has 212 amino acids before the first transmembrane domain (TMD), Avt3 and Avt4 each have 301, and Vba4 has 250. These N-terminal sequences are less conserved than the rest of the protein. A search of the KEGG database (http://www.genome.jp/kegg/) (220) returns 652 hits (E value of $<1e^{-25}$) for full-length Avt1 but only 22 hits (E value of $<1e^{-10}$) for the N terminus alone. Similar results are obtained for Avt3 (447 versus 26 hits) and Avt4 (590 versus 24 hits). Hits against the full-length proteins are found in fungi, protists, and plants (Avt1, -3, and -4) and also in animals (Avt3 and -4), while hits against the N termini are found only in fungi. Full-length Vba4 returns only 13 hits, all from other fungi, and there are no matches for its N-terminal region. The scarce conservation of the N-terminal parts may suggest a different, perhaps regulatory, function specific only to fungi, protists, and plants. No predictions on the structure or function of these domains have been made so far, and no matches have been found when these N-terminal sequences were searched against either the Protein Data Bank (PDB) (using the HHpred server [203]) or the NCBI Conserved Domains Database (CDD) (221). Analysis of the sequences using Jpred4 (222) suggests that they have some (limited) secondary structure: four β-sheets and one α-helix are predicted for Avt1, four α-helices are predicted for Avt3 and Avt4, and one α-helix is predicted for Vba4.

The homologs of Avt3 and Avt4 that have been characterized in other fungi also have extended N termini, which share some conserved sequence elements with those from *S. cerevisiae* (165, 168, 223). Experiments in whole cells suggest that the N terminus of *S. pombe* Avt3 is not required for the localization of the protein to the vacuolar membrane or for amino acid transport but has a small positive effect on the sporulation efficiency (165).

REGULATION OF AMINO ACID TRANSPORT

Yeasts contain many different pathways for the regulation of cellular metabolism, each of which is triggered by sensors that monitor changes in the internal or external

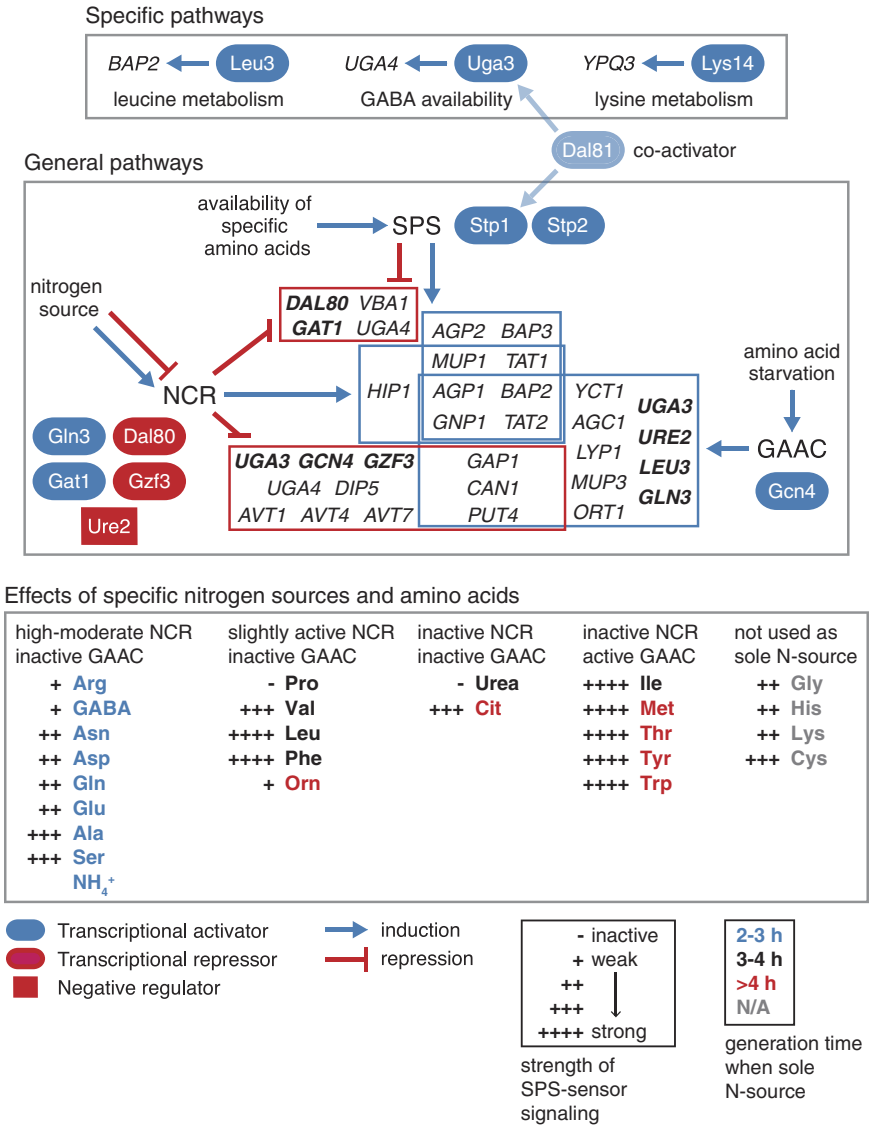

FIG 6 Pathways regulating the expression of *S. cerevisiae* amino acid transporters. The bottom panel indicates the effects of various amino acids and nitrogen sources (N sources) on the activity of each pathway (14). Activity of the SPS pathway was measured using urea as the N source. Compounds are colored according to the generation time when used as the sole N source (14). GABA, γ-aminobutyrate; NCR, nitrogen catabolite repression; GAAC, general amino acid control; N/A, not applicable.

environment (13, 224, 225). Amino acid transporters are regulated by pathways that respond to both the general nutritional status of the cell and the availability of specific amino acid substrates. This regulation occurs both through the activation or repression of gene transcription and through the trafficking of proteins to and from specific membranes. Although these processes have been studied mostly in *S. cerevisiae*, evidence suggests that the core mechanisms are conserved in other fungi.

Regulation via Gene Expression

Figure 6 outlines the specific and general pathways that regulate the expression of genes encoding *S. cerevisiae* amino acid transporters. Each of these pathways also regulates the expression of genes involved in amino acid biosynthesis and other cellular processes. Although the regulatory pathways operate via distinct mechanisms, they can overlap both directly, e.g., through interactions between several transcriptional factors at the same promoter region, and indirectly, e.g., through changes

resulting from the activity of one pathway triggering another pathway. This makes it difficult to define specific targets of transcription factors and also to predict the expression levels of any particular transporter under a given condition. It is important to be aware of strain-specific differences, such as the fact that ammonia is a preferred nitrogen source for strains derived from *S. cerevisiae* Σ1278b but not for those derived from S288c (226).

Only three *S. cerevisiae* amino acid transporters are known to be regulated via specific pathways: Bap2, Uga4, and Ypq3. Full expression of Bap2 is dependent on Leu3, a transcription factor that acts as either a repressor or an activator depending on the concentration of α-isopropylmalate, a precursor of leucine biosynthesis (227). Uga4 expression is specifically induced by its substrate GABA via the transcriptional activator Uga3 (228, 229). The vacuolar transporter Ypq3 is under the positive control of the Lys14 transcriptional activator and is thus repressed by the lysine precursor α-aminoadipate semialdehyde (105). In *A. nidulans*, transcription of the proline utilization gene cluster, including the permease PrnB, is upregulated by the transcriptional activator PrnA in response to proline availability (230).

SPS signaling (reviewed in reference 231) is named after three proteins (Ssy1-Ptr3-Ssy5), which together form an amino acid sensor. Ssy1 shares a high level of sequence similarity with members of the YAT family but has no measurable transport activity. It contains an extended cytosolic N-terminal domain that is necessary for sensing (232, 233). SPS signaling is substrate specific, with hydrophobic amino acids inducing the strongest response (234, 235). Binding of amino acids by the SPS complex leads to proteolysis of the transcription factors Stp1 and Stp2, which otherwise would enter the nucleus and induce the transcription of specific genes (231), leading to the expression of *AGP1*, *AGP2*, *BAP2*, *BAP3*, *GNP1*, *MUP1*, *TAT1*, and *TAT2* (234, 236, 237). Several genomewide transcriptional studies have shown that SPS signaling affects the expression levels of a range of genes other than amino acid transporters, although in some cases, this may be due to secondary effects such as changes in the uptake of amino acids and other nitrogen sources (238). There is uncertainty about the location of Ssy1 in the cell and how it senses amino acid availability. Several groups have demonstrated that SPS signaling responds to changes in the extracellular amino acid concentration; however, Ssy1 was more recently shown to localize to the endoplasmic reticulum (ER) at the cell periphery rather than the plasma membrane (239). This contradicts proposed models for amino acid sensing (240), as it does not allow for direct binding of extracellular amino acids by Ssy1 (67, 232, 241). Ssy1, however, is essential for the sensing cascade, suggesting that the signal may be from a protein partner in the plasma membrane and transduced to Ssy1 in the cortical ER.

An SPS sensor system has also been identified in the opportunistic pathogen *C. albicans*, where it is involved in nutrient sensing, morphology, and evasion of the host immune response (242, 243). The *C. albicans* system appears to consist of the same three basic components (Ssy1-Ptr3-Ssy5) that activate the transcription factors *Ca*Stp1 and *Ca*Stp2 (244). To allow for growth on proteins as the sole nitrogen source, the transcription factors upregulate amino acid transporters and extracellular proteases. The latter is not known to be the case in *S. cerevisiae* (245). The *C. albicans* system also seems to respond to different amino acids, which may be caused by Csy1, an ortholog of Ssy1 that is highly divergent in the extended cytosolic N-terminal sensing domain. This divergence may have an effect on the substrate specificity of Csy1 compared to Ssy1 (242).

NCR, or nitrogen catabolite repression, refers to the general regulation of gene expression in response to nitrogen availability (13, 226). Effectively, cells use preferred nitrogen sources when available (14). The core effectors of NCR are the transcriptional activators Gln3 and Gat1 (Nil1), the transcriptional repressors Dal80 (Uga43) and Gzf3 (Deh1 or Nil2), and the negative regulator Ure2. NCR activity is strongly linked to the ability of yeast to utilize a specific nitrogen source. Gap1 is subject to NCR, especially when a rich nitrogen source, such as glutamine, glutamate, or ammonium ions, is used

(14, 246). For Agp1, which is also subject to NCR, the effect of the nitrogen source is less pronounced, presumably because the system is also under SPS control.

GAAC, or general amino acid control, is a pathway which responds to starvation for one or more amino acids by global inhibition of translation initiation and accumulation of the activator protein Gcn4 (reviewed in references 247 and 248). Transcriptional profiling under GAAC induction has shown that Gcn4 is responsible for the derepression and repression of at least 539 and 1,000 genes, respectively (249). Gcn4 mainly controls biosynthesis and regulatory pathways but also derepresses a wide range of transporters, including Agp1, Bap2, Can1, Gap1, Gnp1, Put4, and Tat2 (249). Gcn4, and its targets, may also be induced by various other stresses that inhibit translation, e.g., heat and oxidative stress (250).

There are several known examples of cross talk between these regulatory pathways. For instance, the transcriptional coactivator Dal81 (Uga35) is required for both Uga3-induced expression of Uga4 and Stp1-induced expression of Agp1 (234, 251, 252) (Fig. 6). In addition, leucine prevents the GABA-induced upregulation of Uga4 expression by recruiting the limited amounts of Dal81 molecules to promoter regions controlled by the SPS pathway (251, 253). This regulatory hierarchy mediated by Dal81 also extends to metabolic enzymes and thereby allows for the sequential use of three poor nitrogen sources (GABA, leucine, and allantoin) (254). Both the NCR and GAAC pathways solely regulate the expression of effectors of their own and alternate between pathways, and there is substantial overlap between their target genes. For example, Gcn4 negatively regulates Gat1-dependent induction of Gap1 and Put4 (255), while a genomewide analysis found that binding sites for Gln3 and Gcn4 are found within the same promoter region more often than would be expected by chance (256).

Regulation via Trafficking

Once synthesized, transporters are regulated by processes either controlling their location within the cell or triggering their degradation (reviewed in references 33, 257, and 258). This typically involves (de)ubiquitination of the proteins. Ubiquitin (Ub) is a 76-amino-acid protein that can be conjugated to substrate proteins by the E3 ubiquitin ligase Rsp5 via the formation of an isopeptide bond between the C-terminal glycine of Ub and a lysine side chain on the target protein. Ub contains seven lysine residues that can be modified additionally, allowing the assembly of polyubiquitin chains. This increases the diversity of possible signals and allows quality control at the ER, Golgi complex, and PM (Fig. 7). Some of these processes, such as the tagging of misfolded proteins in the endoplasmic reticulum for degradation, are general quality control mechanisms, while others are triggered in response to more specific signals.

ERAD. The general quality control mechanism termed ER-associated degradation (ERAD) is triggered when proteins fail to fold or assemble correctly in the endoplasmic reticulum (259, 260). Correct assembly of *S. cerevisiae* YAT family transporters requires the packaging chaperone Shr3, an ER-resident integral membrane protein with 4 transmembrane segments (261, 262). Shr3 holds the first 5 TMDs in a stable conformation so that the remaining 7 TMDs can fold properly as well. Without Shr3, Gap1 and other YAT proteins (e.g., Agp1 and Gnp1) aggregate into high-molecular-weight complexes that are degraded by ERAD. This process involves multimeric protein complexes that contain the E3 ubiquitin ligases Doa10 and Hrd1, the extraction of the aggregate from the ER by the CDC48 ATPase complex, and the subsequent breakdown of the proteins by the cytoplasmic proteasome (263).

Other "Shr3-like" substrate-specific ER-resident chaperones have been identified in *S. cerevisiae*. Gsf2 is required for the ER exit of the hexose transporters Hxt1 and Gal2, Pho86 is required for that of the phosphate transporter Pho84, and Chs7 is required for that of chitin synthase III (Chs3) (261). Likewise, packaging chaperones specific for YATs have been identified in *A. nidulans* (264), *C. albicans* (23), and *S. pombe* (265).

ARTs as adaptors for ubiquitination of YATs. Ubiquitination of plasma membrane proteins is carried out by the E3 ubiquitin ligase Rsp5, a homolog of mammalian Nedd4 (266). Rsp5 contains three WW domains that enable the recognition of target proteins

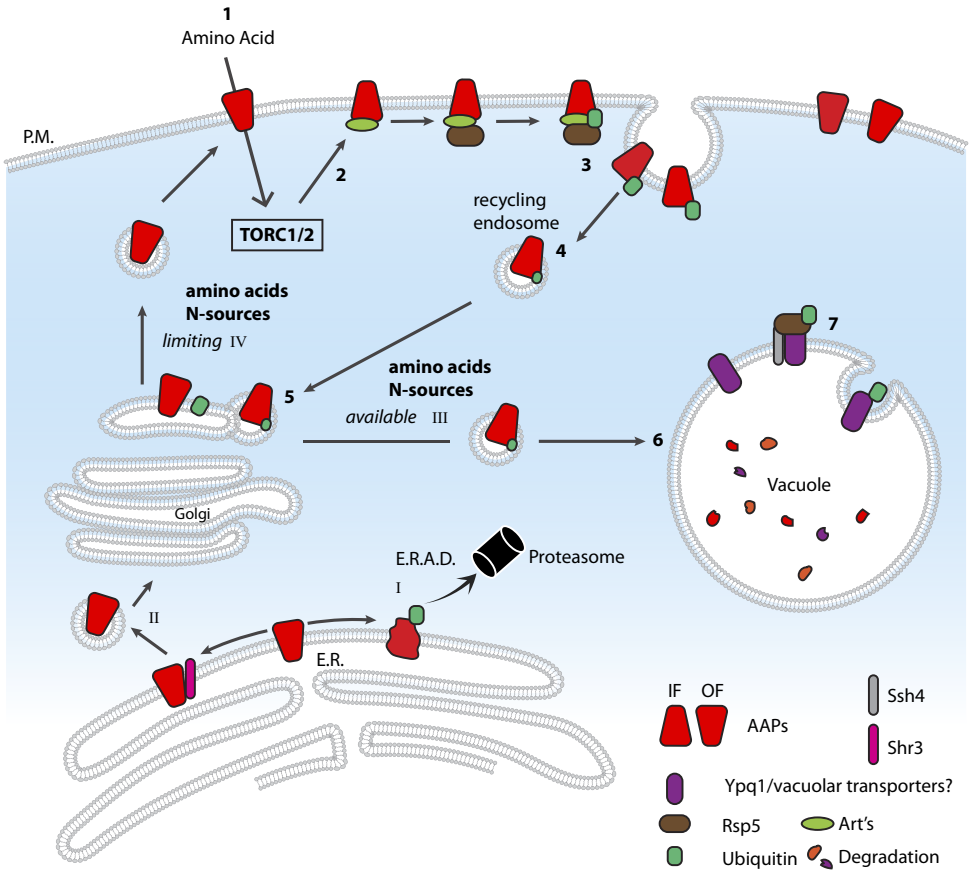

FIG 7 Biogenesis, quality control, and ubiquitin-mediated membrane protein trafficking of YATs in yeast. In the ER, misfolded YATs are degraded via ERAD (I). The Shr3 chaperone stabilizes YATs in the ER and sorts proteins into vesicles for trafficking to the Golgi complex (II). When nitrogen sources are available, YATs travel from the TGN to endosomal vesicles (III), while under nitrogen-limiting conditions, YATs are sorted into exocytic vesicles that fuse with the PM (IV). Import of amino acids (1) signals TORC1/2 for upregulating ARTs that recognize the N-terminal tail of inward-facing YATs (2) and recruits Rsp5 to ubiquitinate lysine residues (3), which are most often located in the N-terminal tail. This initiates endocytosis by the endosomal sorting complex required for transport (ESCRT) and results in the formation of endosomes (4) that first fuse with the TGN (5) and from there (when nitrogen sources are limited) are sorted into exocytic vesicles that fuse with the PM (IV). Under nitrogen-available conditions, YATs are sorted to endosomal vesicles (III), which, after fusion (6), eventually leads to degradation of the proteins. For vacuolar amino acid transporters, the depletion of specific amino acids leads to the binding of chaperones, e.g., Ssh4, which also recruit Rsp5 for ubiquitination (7). After this, intraluminal vesicles are formed by ESCRT components directly on the vacuolar membrane, and subsequent proteolysis occurs. IF and OF refer to the inward- and outward-facing conformations of a YAT.

with PY motifs (PPxY/F). However, many plasma membrane proteins do not contain PY motifs, and recognition therefore requires a specific set of adaptor proteins called ARTs (arrestin-related trafficking adaptors). *S. cerevisiae* has 14 α-arrestin family proteins (Art1 to -10, Bul1 to -3, and Spo23) containing PY motifs that act at the plasma membrane and many more adaptors for other subcellular compartments, e.g., Bsd2 and the Ear1-Ssh4 pair for vacuolar sorting (267) of Smf1 and Fur4 (268–271).

Functions for 4 (Art1, -2, -4, and -8) of the 14 α-arrestin family proteins are known to be correlated with YATs. Monitoring external substrate- and stress-induced endocytosis of a subset of transporters in a 9-arrestin-deletion strain revealed external substrate-induced functions for Art1 (Fur4, Tat2, Mup1, Can1, and Lyp1), Art2 (Smf1, Tat2, and Fur4), Art4 (Hxt6), Art5, (Ltr1), and Art8 (Smf1) and on top of that stress-induced functions for Art1 (Fur4), Art2 (Tat2), Fur4 (Lyp1), and Art8 (Hxt6). Furthermore, weak external substrate- and stress-dependent functioning was observed for Art8 (Tat2 and Fur4) (269).

From the data described above, it follows that ARTs show redundancy, e.g., Tat2

external substrate-induced endocytosis is accommodated by Art1 and Art2 and is unaffected when the gene for either one of the proteins is deleted. This phenotype also implies that the weak functioning of Art8 is sufficient for endocytosis. Additional screening for ART complementation of the 9-arrestin-deletion strain showed high redundancy among ARTs but more importantly also revealed the requirement for downstream endocytic sorting of the endosomes. For example, Art2 and -8 mediate Fur4 endocytosis from the plasma membrane, but the adaptor pair Ear1-Ssh4 is needed to complete the passage through endosomal structures; without the Ear1-Ssh4 pair, Fur4 accumulates in MVBs (multivesicular bodies) (269, 272, 273). Also, various ARTs can recognize the same target protein, but the downstream effect may be different. A clear example of this is Can1, where endocytosis mediated by Art1 leads to vacuolar degradation, while Bul1/2 leads to recycling of Can1 (274).

Cues for ubiquitination. Rsp5-mediated ubiquitination can occur in response to various signals. Tat1 and Tat2 show Rsp5-dependent endocytosis in response to high pressure or low temperature (275–277). Also, heat-induced misfolded Lyp1 is increasingly localized to the PM when the gene encoding Art1 is deleted. This shows a direct correlation between the ubiquitination of Lyp1 by Art1 and temperature stress (278).

Ubiquitination also occurs in response to the availability of nitrogen sources in the cell (271, 279–281). When amino acids and/or nitrogen sources are widely available, Gap1 is removed from both the PM and the *trans*-Golgi network (TGN) and transferred to endosomes. When the same nutrients are limiting, Gap1 is recycled from the endosome back to the TGN or PM, which requires different adaptor proteins for each step.

Gap1 is a broad-specificity permease, but its main function may be that of a transceptor to signal the nitrogen status of the environment. Homologs of Gap1 with a narrower specificity appear to be regulated by the availability of the substrates that they transport. In these cases, not only does intracellular nitrogen availability act as a signal, but endocytosis is also increased by specific amino acids: Can1 (arginine), Dip5 (glutamate), Lyp1 (lysine), Mup1 (methionine), and Tat2 (tryptophan) (143, 269, 282–284).

The PKA signaling of Gap1 shows analogy with substrate-mediated endocytosis (see "Regulation via Trafficking," below). However, the downstream events depend on the type of substrate transported, and thus, translocation *per se* is not enough to induce signaling. While for substrate-mediated endocytosis, binding is sufficient, this has not yet been established for the transceptor function. Gap1 selectively binds the α-carboxyl group of amino acids and accommodates a large variety of side chains. The broad substrate specificity of Gap1 together with the fact that different substrates activate distinct downstream processes led to the conclusion that the amino acid side chain triggers diverge conformations in the protein.

Can1 is in the outward-facing conformation when arginine is absent or limiting, and under these conditions, the protein is stably present in the PM. When arginine is present in excess, the conformational state changes from outward to predominantly inward facing, and the protein is then rapidly endocytosed (143). For both Can1 and Gap1, it has been shown by mutant analysis that transport is not required for endocytosis; the binding of the substrate is sufficient (143). This substrate-specific downregulation may be a mechanism to avoid toxicity by excessive accumulation of amino acids. For instance, *S. cerevisiae* cells that cannot endocytose Gap1, Can1, or Lyp1 experience severe growth defects in the presence of their substrate(s) (53, 274, 278). A similar regulation- and substrate-induced growth defect has also been reported for the di/tripeptide transporter Ptr2 (123).

In summary, the concentration of amino acid permeases in the PM is regulated via ubiquitin-mediated endocytosis, which requires ARTs. The binding of ARTs to YATs requires a specific conformation of the transporter, which can be elicited by stress conditions or binding of substrates. Polyubiquitination is a requirement but not necessarily sufficient to induce endocytosis (Fig. 7). ARTs themselves are regulated via signaling cascades: the activity of Art1, for example, is negatively regulated by a

phosphorylation cascade that involves TORC1 (target of rapamycin complex 1) and Npr1 kinase (257).

TORC1 and -2. *S. cerevisiae* contains the evolutionarily conserved TORC1 and TORC2. The complexes share certain components but have specific factors also. TORC1 contains Lst8p, Tco8p, Kog1p, and either Tor1p or Tor2p, while TORC2 contains Lst8p, Avo1p, Avo2p, Avo3p, Bit61p, and Tor2p. Interestingly, only TORC1 is sensitive to rapamycin, a feature that can be acquired for TORC2 by deleting the C-terminal part of Avo3 that normally masks the rapamycin-binding site in TORC2 (285). Because of this natural insensitivity of TORC2 to rapamycin, which makes it difficult to regulate TORC2 activity, TORC1 is the best studied of the two. TORC1 and -2 act as central regulators of cell growth and proliferation in response to various environmental and intracellular cues (286–288). They are involved in many processes, with TORC2 being involved in membrane tension homeostasis, actin polymerization, and sphingolipid synthesis (289) and TORC1, among others, being involved in protein turnover, autophagy, translation, and the transcriptional regulation of genes induced by nutrient availability (290–293). The latter includes nitrogen homeostasis, in which amino acids play an essential role.

Since this review focuses on YATs, we restrict our discussion to what is known about the role of either TORC1 or -2 in the regulation of these transporters. Part of this regulation involves a kinase cascade reaction of TORC1-Npr1-Art1. Art1 is phosphorylated on the N terminus by the Npr1 kinase, preventing association with a target protein (e.g., Can1). Upon TORC1 activation, the Npr1 kinase is inhibited, which yields unphosphorylated Art1 and the subsequent association of Art1 with the target protein (e.g., Can1) (294). A similar mechanism has been shown for Gap1, but it involves the arrestin-like Bul1 and Bul2 adaptors instead of Art1 (281). In addition, Npr1-dependent phosphorylation has been shown for Art2 and Art3, suggesting that the mechanism might extend to all ARTs (268, 283, 294). TORC1 activation has been proposed to occur as a result of fast proton influx coupled to metabolite import by YATs rather than by the intracellular amino acid levels themselves (295). However, the activation by proton influx is transient. Although the proposed mechanism is appealing, the expected drop in intracellular pH is not observed, which leaves questions about the actual signal that is sensed. Other candidates for sensing internal levels of amino acids and sustained TORC1 activation are the GTPase-activating proteins Lst4 and -7 via Gtr2 and the leucine-tRNA transferase via Gtr1(296). Another and recently identified candidate is Pib2, which acts as a sensor for internal glutamine levels (297, 298). Thus, TORC1 activation is complex, and more work is needed to resolve the precise role of YATs.

TORC2 has not been a major component in the study of YAT endocytosis. A recent study using a TORC2 mutant sensitive to rapamycin shows that TORC1 is essential for the exocytosis of Mup1 to the PM, and TORC2 is needed for the endocytosis of Mup1 from the PM (299). It is noteworthy that this finding was made under conditions where endocytosis was induced by an external substrate. Also, by combining observations made in several studies (143, 268, 294, 299, 300) that tracked the endocytosis of multiple YATs (e.g., Lyp1, Fur4, Can1, and Mup1) under external substrate- and stress-induced conditions, we conclude that external substrate-induced endocytosis is not dependent on TORC1 but most likely depends on TORC2. This conclusion is based mainly on the natural rapamycin insensitivity of TORC2 (i.e., TORC2 can be active in the presence of rapamycin) and the fact that rapamycin (inactivation of TORC1) inhibits stress-induced (e.g., internal nitrogen availability) endocytosis but not external substrate-induced endocytosis.

ART target protein recognition. The search for the recognition site of ARTs yielded the N-terminal cytosolic tail as a target. The first proposal for a recognition mechanism involving the binding of ARTs to the N-terminal tail of the target protein came from Keener and Babst, who showed that truncations of the N terminus of Fur4 stabilized the protein at the cell surface (301). Using a homology model of Fur4 based on the bacterial sodium benzyl-hydantoin symporter Mhp1, a "lid region" was proposed, which would interact with internal loops of Fur4 when the protein is in the outward-open state. Destabilization of the lid region (e.g., upon substrate binding or temperature/peroxide

stress) led to increased susceptibility to ubiquitination and, hence, removal of Fur4 from the PM.

Those authors proposed that the lid region works as a stress and substrate sensor that controls the accessibility of the N-terminal lysine residues for ubiquitination (301). Although the amino acid sequence comprising the lid region is not conserved in YATs, similar observations were subsequently made for Mup1, Can1, and Lyp1 (274, 302). Accordingly, Lin et al. constructed chimeras of Can1 and Lyp1 by swopping their N termini, thereby making Can1 prone to cues for Lyp1 degradation and vice versa (268). Moreover, they showed that lysine residues prone to ubiquitination are found mostly in the N-terminal tail of plasma membrane YATs (274, 278, 301–303). A note should be made here about the high-affinity monocarboxylate transporter Jen1, where lysine residues in the cytosolic loop region affect glucose-induced polyubiquitination, while the N-terminal lysine residues have no effect (304). Thus, the tail or loop regions that mediate endocytosis may vary for different transporter families.

The molecular interaction of Art1 with the N terminus has only recently been specified: Guiney et al. showed that an acidic patch in the N-terminal tail interacts electrostatically with a basic region in the C terminus of Art1 (302). It is noteworthy that endocytosis occurs only if the acidic N-terminal patch is proximal to the TM region, suggesting that additional parts of the protein or other partners, which are yet to be identified, are required for endocytosis.

Other regulatory signals on YATs. Specific examples of ubiquitin-mediated endocytosis of plasma membrane amino acid transporters have been identified in several other fungi. Orthologs of Rsp5 (Pub1) and the ARTs (Arn1/Any1) have been identified in *S. pombe* and are involved in the regulation of the amino acid transporters Aat1 and Cat1 (305, 306). In *A. nidulans*, the arrestin-like protein ArtA is required for HulA-dependent ubiquitination and turnover of the proline transporter PrnB (190).

Much less is known about the degradation pathways for vacuolar membrane proteins. The vacuolar lysine importer Ypq1 is selectively degraded in response to lysine depletion (307), which requires a PY-motif-containing vacuolar membrane protein, Ssh4, that recruits Rsp5 in a fashion similar to that of ARTs (307). The authors of that study initially proposed that ubiquitinated Ypq1 was selectively sorted into endosomal vesicles before delivery to the vacuolar lumen, but they later concluded that intraluminal vesicles are formed directly from the vacuolar membrane (308). Overexpression of Tul1, a newly identified vacuolar RING domain E3 ligase, was found to increase the degradation of Vba4. However, it is unknown what triggers the ubiquitination of Vba4 (309). Tul1 is also responsible for the ubiquitination and subsequent degradation of the vacuolar zinc transporters Cot1 and Zrt3 in response to low and high zinc levels, respectively (309).

Although most regulatory signals with respect to YAT regulation are found on the N terminus, the C-terminal tail of Gap1 contains a predicted 19-amino-acid-long amphipathic α-helix (310) and harbors sequence motifs that play a role in the processing of the protein in the secretory pathway, nitrogen-dependent gene regulation, membrane anchoring, endocytosis, or PKA signaling (310–312). The hydrophobic-diacidic motif (M/I/V)D(L/I/V)D is required for exiting the ER and transport to the Golgi complex (313, 314). The multiple regulatory roles of the C-terminal tail are best documented for Gap1. Mutants of Gap1 that are resistant to ammonium-induced endocytosis have been mapped in the C terminus, while the protein is still ubiquitinated to an extent similar to that of wild-type Gap1. Furthermore, substitution of two leucine residues preceding the diacidic motif in the predicted α-helix or deletion of the last 11 amino acids yielded proteins that are less efficiently ubiquitinated and partially protected against endocytosis. A lysine-to-alanine substitution within the predicted α-helix protects Gap1 against ammonia-triggered degradation, but the protein is still normally ubiquitinated (310, 315). Overall, these data suggest that the C-terminal α-helix has a role in the downstream steps of endocytosis but is not ubiquitinated itself. A similar phenotype was obtained when a deletion was made in the C terminus of Bap2, whereby resistance to cycloheximide-induced endocytosis was obtained (316).

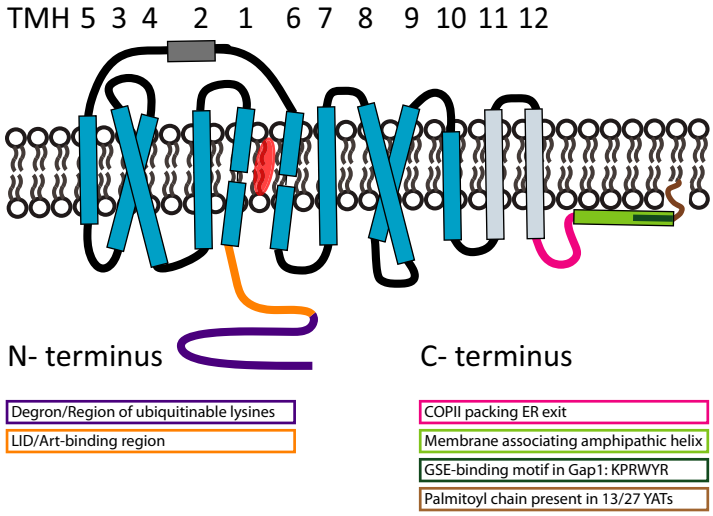

FIG 8 Topology model of YATs and known regulatory regions. The typical 5 + 5 inverted repeat is represented by TM segments 1 to 5 and 6 to 10; the quasi-2-fold axis in the plane of the membrane is indicated by the red oval. In the model, 2 additional TM segments are depicted (light gray), which are typically found in PM-localized YATs. Cytosolic regions known to play a role in regulation and posttranslational modification are shown in colors. From the N to C termini are the regions with lysine residues targeted for ubiquitination (red), Art binding (orange) (see "ART target protein recognition"), COPII for ER exit (magenta), the membrane-associating amphipathic helix (light green), the GSE-binding motif (dark green), and the palmitoyl chain (brown) (see "Other regulatory signals on YATs").

The KPRWYR motif within the last 11 residues of Gap1 is responsible for the association of the protein with a GTPase-containing complex for Gap1p sorting in the endosomes (GSE complex) (317). The GSE complex is involved in the trafficking of Gap1 from the TGN to the PM, and mutations (i.e., tyrosine to alanine) in the motif lead to sequestration of Gap1 in endosomal membranes. Deletion of the last 11 amino acids abolishes the association of Gap1 with the GSE complex and bypasses this regulatory step (317). A detailed summary of regulatory signals established for Gap1 was reported by Kriel et al. (318). An overview of most signals and regulatory domains known for YATs is projected onto a topology model (Fig. 8).

For the core cluster of YATs, as defined by Ljungdahl and Daignan-Fornier (13), the C termini have been subdivided into two classes: those that end with a palmitoylation motif, "FWC," and those that lack the motif and have a shortened C-terminal α-helix. Many of the C termini of the YATs have been shown to interact with membranes even in the absence of the palmitoyl modification (312). This could mean that the C-terminal tail has a defined secondary structure that could play a role in protein trafficking. In Mup1, the C-terminal region has phosphorylation and ubiquitination sites and is involved in endocytic turnover. A conserved region in the C-terminal tail of MUP1 (FWRV [positions 534 to 537]) has been shown to overlap a loop region in the crystal structure of the homologous protein GadC (186). This loop region is referred to as the "C plug" and is positioned close to the core of the TMD segments in GadC. Removal of the conserved region in Mup1 abolishes the substrate-dependent endocytosis and localization of the protein to the MCC (microcompartment of Can1) domain of the PM. Possibly, the C plug of Mup1 is involved in a conformational switch upon substrate transport that allows the endocytic machinery to access ubiquitination sites (299).

In conclusion, the C-terminal tail might have the ability to interact with the membrane and seems to be involved in downstream sorting after ubiquitination. However, our understanding of the role of the C terminus is far from complete and might differ from YAT to YAT. The N-terminal lysine residues seem to be the main sites for ubiquitination and are recognized by ARTs upon various cues. Subsequent recruitment of Rsp5 by ARTs results in endocytosis. On top of regulation via trafficking and gene regulation, a new type of regulation mechanism has emerged in recent years. This type

of regulation involves domain partitioning, in which the ubiquitination machinery can access only target proteins (i.e., Lyp1, Can1, Mup1, Tat2, and Fur4) excluded from PM domains such as MCC/eisosomes (see below) and thereby functions as a protective compartment.

Regulation by Domain Partitioning

The yeast plasma membrane is divided into multiple domains ranging from dynamic networks to static patches. One of these domains is called the MCC (microcompartment of Can1) domain after the YAT Can1, which was the first protein detected to partition into this compartment (319, 320). Subsequently, it was shown that the permeases Fur4, Tat2, Mup1, and Lyp1 can also reside in MCC domains. Also, Sur7 and related tetraspanner proteins (Ygr131, Ynl194, Ydl222, Ylr414, and Nce102) are associated with this domain (299, 319, 321–324). The MCCs make furrow-like invaginations in the PM that are supported by subcortical structures called "eisosomes," which are composed of so-called BAR (Bin, amphiphysin, Rvs) domain proteins, of which Pil1 is the most abundant (325, 326). Eisosomes are reported to be enriched in ergosterol relative to the rest of the PM, but this proposal is so far based only on filipin staining. Pma1, the most abundant protein in the plasma membrane, is excluded from the MCC domains. Pma1 instead forms its own meshwork compartment, which has been named MCP (microcompartment of Pma1) (324, 325, 327). There are also proteins that do not show a strong apparent domain localization and distribute homogeneously over the PM, like the general amino acid permease Gap1 and the hexose transporter Hxt1p (320, 328).

The partitioning of YATs in the eisosome is well studied (299, 300, 323, 324, 327, 329–332), but the exact function of the eisosome remains elusive. A number of studies point toward an environment that is protective against endocytosis. For instance, deletion of the essential MCC/eisosome constituents Nce102 and Pil1 resulted in a more rapid endocytosis of Can1 and Fur4 (330). This finding was later rebutted by a study showing that the rates of endocytosis of Can1 and Tat2 did not significantly change in a Pil1 deletion strain (331). Additionally, a claim was made that the MCC area is essential for the functionality of Can1 by showing that a Pil1 deletion strain is less sensitive to canavanine, a toxic analog of arginine that is transported by Can1 (324). Also, this observation was subsequently rebutted by a study showing that the rate of transport is the same inside and outside the MCC/eisosome structure (300). However, MCC/eisosome-entrapped Can1 and Mup1 are less sensitive to endocytosis, suggesting that this PM domain provides a protective environment for at least some YATs (299). It has been speculated that storage in MCCs/eisosomes is a mechanism induced by nutrient starvation, during which sphingolipid biosynthesis is enhanced. This storage of proteins in MCCs/eisosomes allows for fast influx of lysine and arginine when these amino acids become available in the medium (300). Recent studies have shown that Can1, Lyp1, and Mup1 partition mainly at the edges of the MCC/eisosome domain, and the addition of a substrate induces a conformational change in the proteins, which lowers their affinity for the MCC/eisosome (299, 300, 323). For Can1 and Mup1, release is proposed to be associated with a reduced affinity for sphingolipids when the proteins change conformation from an outward- to an inward-facing state (300). Bianchi et al. (323) proposed that, once released from the MCCs/eisosomes, the transporters redistribute to a site where endocytosis may occur (Fig. 9). This is supported by a study by Busto et al. (299), who found a so-called "network domain'' upon Mup1 release from the MCC that strongly colocalizes with the late endocytic marker Abp1. Interestingly, in this study, Nce102 exited from the MCC together with Mup1; however, this has not (yet) been shown for other YATs. The redistribution of Can1, Fur4, and Mup1 from the MCCs/eisosomes (327, 332) has been observed upon dissipation of the pH gradient while keeping the eisosomal marker Sur7 in place. This suggests a connection between the energy status of the cell (or a crucial role for the internal pH) and residence of YATs in the MCC/eisosome.

PMF dissipation can have different effects on YATs, and in some cases, this leads to downhill excretion of the substrate, e.g., of ammonia by Fur4 but not of arginine or

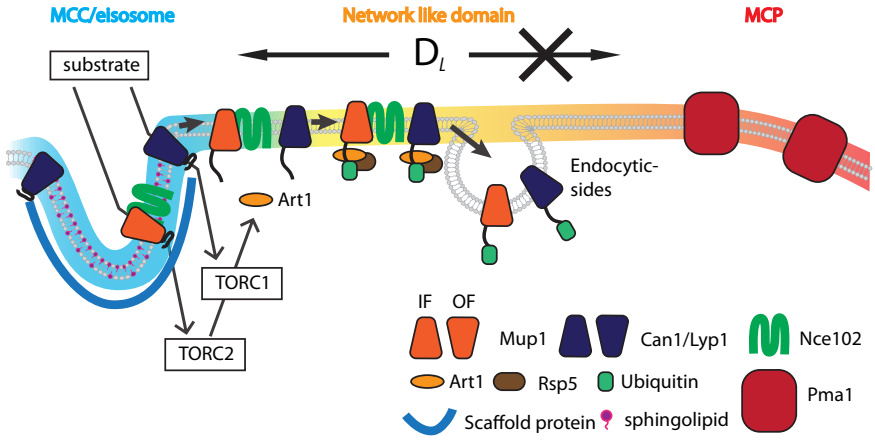

FIG 9 Protection of transport proteins by domain partitioning and regulation of endocytosis of YATs in the yeast PM. The MCC is structurally stabilized by the scaffold proteins Pil1 and Lsp1, which were first identified as the eisosome structure. Mup1, Can1, and Lyp1 are entrapped in the MCC/eisosome, possibly by interacting with sphingolipids or (structural) protein components of the MCC/eisosome. Nce102 may interact with Mup1 specifically. Substrate transport by Can1 and Mup1 activates ARTs via TORC2. The substrate-induced conformational change of the YATs from outward to inward facing may break the interaction with specific lipids or proteins, and as a result, Can1, Lyp1, and Mup1 leave the MCC/eisosome. Endocytosis occurs outside the MCCs/eisosomes. D_L, lateral diffusion; IF, inward facing; OF, outward facing.

lysine by Can1 and Lyp1 (25, 127, 129), leaving the question open of whether the redistribution of the proteins over the PM is dependent on a conformational change from an outward to an inward state or a change in the affinity for a protein or lipid partner. In summary, the MCCs/eisosomes may have a regulatory function in lipid homeostasis, but the majority of evidence points toward a role as a protective storage compartment for APC family transporters and protection of the proteins from endocytosis under nutrient-starved conditions.

Regulatory Mechanisms in Mammalian Cells

Many of the regulatory processes described for yeast that influence the expression of amino acid transporters, and, hence, amino acid homeostasis, are also present in mammalian cells, e.g., mTorc1 (Torc1 in yeast), GAAC, and unfolded protein response (UPR). In both mammalian cells and yeast, total cytoplasmic amino acid levels are sensed, but there are important differences in mTorc1 signaling. In mammalian cells, arginine and leucine are sensed by CASTOR1 and SESN2, respectively, which leads to mTorc1 activation (296, 333, 334). In yeast, a specific methionine-sensing pathway is present, which uses protein phosphatase 2 (PP2A) (335). Also, the G-protein-coupled receptor T1R1/T1R3, a sensor for extracellular amino acids, is not present in yeast and affects mTorc1 signaling in mammalian cells (336). Other mechanisms, like the SPS or NCR pathway, are exclusive to yeast and not reported in mammalian cells.

The human genome consists of ~50 amino acid transporters, the majority of which are required only in specific cell types, and they can be uniporters, Na^+-coupled symporters, and antiporters (337). Contrary to yeast cells having mainly proton-coupled symporters and antiporters, mammalian cells have many antiporters that facilitate the direct exchange of amino acids between the cytoplasm and mitochondria but also the extracellular environment. For instance, the cytoplasmic pool of glycine, alanine, and glutamine is exchanged against extracellular serine and cysteine by ASCT1 and ASCT2 (338, 339). Interestingly some mammalian transporters are not localized exclusively to a single organellar membrane but can function in both the plasma and lysosomal membranes (340, 341), which is not known for yeast. Much less is known in mammalian cells about the cues that lead to the degradation of the individual amino acid transporters. Finally, mammalian cells differentiate to function in different organs, and cells can have a basal side and an apical side of the plasma membrane, which requires an additional level of regulation that is not present in yeast (337).

CONCLUDING REMARKS

YATs have been studied for more than 50 years. The first studies reporting the transport of amino acids in yeast date back to 1966. From there, it took roughly 30 years to discover the identities of the proteins responsible for transport (Table 1) and to determine the mechanisms of energy coupling and the role of the transporters in amino acid homeostasis. Research on the regulatory mechanisms seriously came to life in the 1990s, which led to the discovery of multiple systems that act at either the gene (SPS, GAAC, and NCR) or protein (ERAD and ART/Rsp5) level. Many findings have been translated to studies in higher-eukaryote systems (e.g., endocytosis and trafficking); other mechanisms, such as the necessity of ARTs for the recruitment of the ubiquitin ligase Rsp5 and subsequent endocytosis (see "ARTs as adaptors for ubiquitination of YATs," above), are found strictly in yeast. Also, in the 1990s, lipids were proposed to play a role in the regulation of YATs, not only in trafficking but also in the activity of the proteins. The exact role(s) of lipids in the mechanism and regulation of transport has remained elusive, as solid structural data are lacking. Owing to technical developments in the field of fluorescence microscopy, research on YATs has more recently become focused on the role of partitioning of proteins in specific membrane domains. This work was initiated in the mid-2000s by the discovery of the MCC/eisosome structure. Despite the progress in recent decades, there are still many outstanding questions that remain to be answered.

Outstanding Questions

- What is the exact role of the YAT tail and loop regions in the regulation of amino acid transport?
- What is the sorting signal for Golgi-to-PM trafficking?
- What discriminates sorting to the plasma membrane from sorting to the vacuolar membrane?
- What determines the kinetic offset between inward and outward transport of YATs?
- What is the molecular basis for sensing by TORC1?
- What is the definitive role of the MCC/eisosome in yeast?
- What role do lipids have in YAT functioning and domain partitioning?
- What is the functional role of palmitoylation in YATs?
- What makes MCC/eisosome partitioning specific to given YATs, and what is the retention mechanism?

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Continued next page

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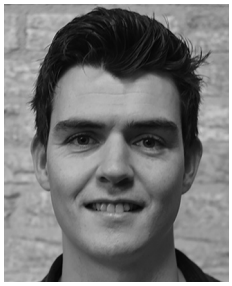

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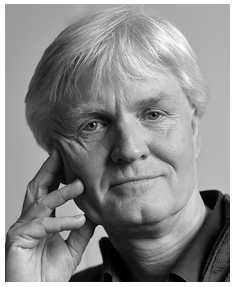

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