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VIEWPOINT

Transporter membrane traffic and function: lessons from a mould

George Diallinas and Olga Martzoukou

Department of Biology, National and Kapodistrian University of Athens, Greece

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Correspondence
G. Diallinas, Department of Biology, National and Kapodistrian University of Athens, Panepistimioupolis 15784, Athens, Greece
Tel: +30(210)7274649
E-mail: diallina@biol.uoa.gr

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Transporters are essential transmembrane proteins that mediate the selective translocation of solutes, ions or drugs across biological membranes. Their function is related to cell nutrition, communication, stress resistance and homeostasis. Consequently, their malfunction is associated with genetic or metabolic diseases and drug sensitivity or resistance. A distinctive characteristic of transporters is their cotranslational translocation and folding in a membrane bilayer, this being the endoplasmic reticulum (ER) in eukaryotes or the cell membrane in prokaryotes. In the former case, transporters exit the ER packed in secretory vesicles and traffic via seemingly unconventional, rather than Golgi-dependent, sorting routes to their final destination, the plasma membrane (PM). Proper folding is a prerequisite for ER exit and further trafficking. Misfolded transporters, either due to mutations, high temperature of chemical agents (e.g. DMSO, DTT) are blocked in the ER. The accumulation of ER-retained transporters, in most cases, elicits endoplasmic reticulum-associated degradation, but also ubiquitination-dependent, chaperone-mediated, selective autophagy. The function of PM transporters is finely regulated at the cellular level, in response to physiological or stress signals that promote, via α-arrestin-assisted ubiquitination, their endocytosis and vacuolar/lysosomal degradation, and in some cases recycling to the PM. Importantly, transporter oligomerization and specific interactions with membrane lipids are emerging as important players in transporter expression, function and turnover. This review discusses how paradigmatic work on transporters of a model mould, *Aspergillus nidulans*, has contributed to novel findings related to transporter functioning in eukaryotes.

Misfolded transporters blocked in the ER are degraded by chaperone-mediated selective autophagy and ERAD

Although not formally shown, it is strongly believed that eukaryotic transporters are cotranslationally translocated, similarly to other transmembrane proteins, from ribosomes to the membrane of endoplasmic reticulum (ER), through the Sec61 translocon protein complex [1,2]. Experimental evidence has shown that an interaction between the first translated transmembrane segment (TMS) of membrane proteins, the signal

Abbreviations
AP, adaptor protein complex; BiFC, bifluorescence complementation assay; CFTR, cystic fibrosis chloride channel; COPII, coat protein complex II; ER, endoplasmic reticulum; ERAD, endoplasmic reticulum-associated degradation; ERES, endoplasmic reticulum exit sites; ERGIC, endoplasmic reticulum-Golgi intermediate compartment; MD, molecular dynamics; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PM, plasma membrane; QC, quality control; TGN, trans-Golgi network; TMS, transmembrane segment; UPR, unfolded protein response.

recognition particle and probably other chaperones, guides the ribosome to the translocon channel [3,4]. Subsequently, other TMSs act as additional signals for correct folding during translocation to the ER [3,5]. The concurrent cotranslational translocation and folding have been shown to provide a primary quality control (QC) point, as several types of misfolded membrane proteins are blocked in the ER membrane and trigger mechanisms leading to an unfolded protein response (UPR) and degradation of the misfolded proteins by the ER-associated degradation (ERAD) [6–8]. However, little is known specifically on the fate of misfolded transporters, except probably the case of the cystic fibrosis chloride channel (CFTR) [9,10] and some yeast transporters (e.g. Gap1, Pca1) [11,12], where misfolded versions are shown to be degraded via ERAD.

In the course of studying loss-of-function mutant versions of UapA, a very well-characterized uric acid–xanthine/H^+ symporter in the filamentous fungus *Aspergillus nidulans* (Fig. 1; [13]), we have detected specific cases showing partial retention in the ER concurrent with increased turnover, which takes place primarily via chaperone-mediated selective autophagy, but also by ERAD [14]. The chaperone involved in the former mechanism, BsdA, is a 4-helix ER transmembrane adaptor that recruits the main HECT/Ned4-type Ub ligase [14], HulA, on ER-resident misfolded versions of UapA. Ubiquitylated ER-resident UapA is then recognized by mature autophagosomes (e.g. Atg8- and Atg9-dependent), which directly promote its sorting into vacuoles. Genetic inactivation of BsdA allows a significant fraction of misfolded UapA molecules to translocate normally in the plasma membrane (PM), even though UapA remains misfolded and transport-inactive. Pharmacological blockage of ERAD seems to have a more moderate effect in allowing misfolded UapA to reach the PM. The case of UapA suggests that a route for degradation of specific misfolded eukaryotic transporters might involve chaperone-mediated autophagy, rather than, or additionally to, ERAD. Interestingly, however, not all ER-retained versions of UapA studied, are recognized by BsdA (unpublished observations). Some evidence suggests that BsdA, a transmembrane protein itself, recognizes misfolded versions due to mutations within TMSs whereas those due to mutations in cytoplasmic loops escape chaperone-mediated autophagy.

ER exit control: who goes on and who does not

Translocation of transporters into the ER membrane does not seem to require proper folding, as even truncated versions lacking several TMS can be localized in the ER [14–19]. It seems that entry into the ER membrane simply requires a minimum degree of hydrophobicity provided by at least two TMS, as shown in a specific case of a truncated version of UapA (Areti Pantazopoulou and George Diallinas, unpublished). In contrast, exiting of transporters from the ER is more demanding, requiring not only proper folding, but seemingly specific interactions with ER lipids and chaperones (see next paragraph), and a crosstalk with the coat protein complex II (COPII) vesicular machinery ([20–23]; see later). Some evidence also suggests that ER exit is concentrative, requiring proper transporter homo-oligomerization for eliciting membrane curvature and eventual packaging into COPII budding vesicles [15,24]. COPII coat assembly begins with the recruitment of the heterodimeric Sec23/24 on the ER membrane, by Sar1 GTPase. Sec24 is the principle cargo-binding adaptor. Following the formation of a prebudding assembly, heterodimers of Sec13/31 are attracted via the interaction of Sec31 with Sec23. Consequently, Sec13/31 drives membrane curvature aided by the oligomerization of Sec23/Sec24. After vesicle formation, downstream steps lead to uncoating of transport vesicles and recycling of COPII coat components. The process of ER exit often requires specific context-dependent or autonomous sequence motifs in transporters, most commonly located at their cytosolic termini [25]. Some ER exit sequence motifs are known to be essential for specific cargo recognition by Sec24, the COPII adaptor coat protein [26–29]. Most commonly, these are short sequences, such as di-acidic (D/E-X-D/E), hydrophobic and aromatic (FF, YY, LL, FY, ΦXΦXΦ), or other more variable short motifs [30–36]. Interestingly, similar short motifs are also recognized by the adaptor protein (AP) complexes AP-1, AP-2 and AP-3, which regulate clathrin-dependent or clathrin-independent vesicular budding at the trans-Golgi network (TGN), the PM or the endosomes [37,38]. The tripeptide D-I-D, located in the C terminus of the general amino acid permease Gap1 of *Saccharomyces cerevisiae*, just next to the last TMS (TMS12), is probably the first transporter motif shown to be critical for insertion into COPII vesicles [39]. Work with *A. nidulans* transporters has also revealed specific motifs essential for ER exit. In UapA Tyr47, located in the highly conserved motif, G-D-Y-D-Y^{47} found in the cytosolic N-terminal region of fungal members of the NAT family [40], proved critical for ER exit [15]. Tyr47 can be replaced functionally only by Phe, showing that the aromatic ring is critical for its role. Noticeably, Y47 substitutions that lead to ER retention do not affect

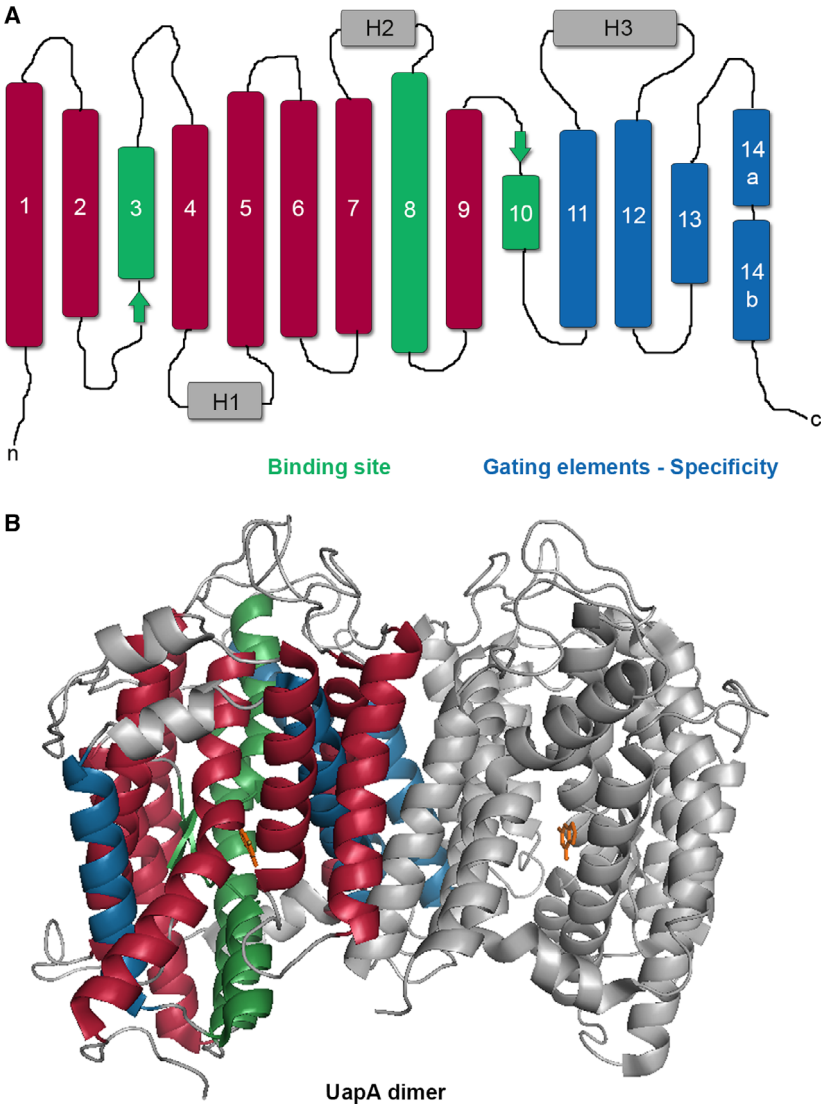

Fig. 1. The UapA transporter as a paradigm for studying membrane traffic and function (A) Topological model of UapA. α-helical TMSs are represented by rectangles and β-strands by arrows. TMSs 3, 8 and 10 host residues involved in substrate binding and are indicated by green colour. TMSs 11, 12, 13, 14 and L1 contain gating elements and residues important for the specificity of the transporter (blue). (B) Crystal structure of the homodimer of UapA. A monomer of UapA is shown in grey, whereas the other monomer is shown with the key regions of the protein coloured as in (A). The position of substrate (xanthine) in the major UapA-binding site is also indicated in range.

substrate binding and do not elicit UPR, showing that they do not affect the basic folding of UapA. Genetic suppressors of Y47A have been isolated and shown to replace specific residues either in the beginning of TMS7 (V298A) or in the α-helical H3 hinge at the extracellular loop linking TMS11-TMS12 (F437C). Recent findings further showed that a specific structure-stabilizing mutation in TMS10 (T401P) could also restore the trafficking of the Y47A mutant version of UapA to the PM (Anezia Kourkoulou and George Diallinas, unpublished). These findings suggest that Tyr47 has an 'allosteric' effect on transporter structural dynamics necessary for ER exit. Other mutations in UapA leading to total ER retention concern the replacement of several Gly residues in TMS7 [15] or deletion or substitutions of residues in several internal cytoplasmic loops, but these seem to lead to partial misfolding rather than defining specific ER exit motifs (Kohar Chorozian, Sofia Dimou, George Diallinas, unpublished). In FurE, a uracil–allantoin–uric acid transporter, specific mutations in the cytosolic N-tail proximal to TMS1 or in cytoplasmic loop L2 also lead to ER retention [19]. In this case, these mutations replace either positively charged (Lys, Arg) or structural (Pro) residues, which apparently are necessary for proper attachment to the lipid membrane and transporter folding. In conclusion, one might be careful in assigning sorting roles to specific residues as ER exit might be simply blocked nonspecifically due to misfolding [14,41].

The necessity of specific ER-resident chaperones for transporter ER exit has been demonstrated in ascomycetes [11,42–46]. One of the best characterized ER exit chaperones is the *S. cerevisiae* Shr3 protein, which

mediates COPII–cargo interactions essential specifically for the packaging of amino acid transporters in secretory vesicles [42,43]. Shr3 has been shown to assist the folding of amino acid transporters and thus prevent precocious ERAD [11], while a more recent genetic analysis shows that Shr3 acts transiently in a cotranslational manner to prevent translation intermediates from engaging in nonproductive interactions and thus preventing misfolding during transporter biogenesis (Ring A, Myronidi I and Ljungdahl PO, personal communication). True orthologues of Shr3 have also been characterized in *Schizosaccharomyces pombe* (Psh3p; [44]), *Candida albicans* (Csh3; [45]) and *A. nidulans* (ShrA; [46]), and shown to perform similar functions in the cellular expression of amino acid permeases. Other membrane-localized chaperones specific for ER exit of distinct families of transporters have also been identified in yeasts. These concern the ER exit of specific hexose or phosphate transporters [43]. Additionally, members of a distinct family, called Erv14, are hydrophobic chaperones with three transmembrane domains involved in COPII-assisted ER exit of specific transporters [47,48]. However, not all Erv14 members are exclusively transporters, as other transmembrane proteins with variable functions and structures belong to the same family (e.g. lipid flippases or proteins linked to cell polarity or cell wall regulation). Finally, the abundant ER membrane protein complex, present in all eukaryotes, interacts with membrane proteins passing through the ER and thus seems to participate in their folding or assembly by serving as a chaperone (for a recent review, see Ref. [49]).

Conventional or nonconventional transporter sorting to the PM?

In mammalian cells, after ER exit, COPII vesicles move membrane cargo proteins to an ER-Golgi intermediate compartment (ERGIC), a network of membranes that functions as a gate to the Golgi [50]. Whether the ERGIC acts as a stationary compartment or a transient structure formed by fusion of ER-derived vesicles, functioning as a carrier itself, is not known. ER to ERGIC transport is microtubule-dependent, and thus, both anterograde and retrograde trafficking between these compartments are affected in the presence of microtubule polymerization inhibitors [51]. In *S. cerevisiae*, however, a 'hug-and-kiss' mechanism was described for cargo transport from the ER exit sites (ERES) to the *cis*-Golgi which guarantees efficient and targeted transport. Specifically, the *cis*-Golgi approaches and contacts the ERES, triggering the collapse of the COPII coat, and thus enabling the capturing of cargo by the *cis*-Golgi which then leaves the ERES [52]. Traffic of COPII components from ER to Golgi is bidirectional to ensure that proteins required for the formation of vesicles are recycled. After translocation in the *cis*-Golgi, membrane cargoes move to the medial and TGN via Golgi maturation. From the TGN, membrane cargoes are thought to be secreted towards the PM, either indirectly via the endosomal compartment or directly in AP-1/clathrin-coated secretory vesicles, with the involvement of multiple Rab GTPases and the microtubule cytoskeleton [53,54]. Do all transporters follow a Golgi-dependent pathway to reach their final membrane destination?

Certain lines of evidence point towards the theory that *de novo* made transporters, such as the insulin-regulated GLUT4 glucose carrier or the CFTR channel, might not follow conventional Golgi-dependent routes. For example, genetic deletion of regulatory proteins involved in TGN-dependent membrane cargo sorting (e.g. Arfrp1, golgin-160 or AP-1) leads to GLUT4 accumulation in the PM, rather than retention in the Golgi, which indicates the presence of an unknown Golgi-independent route [55]. Additionally, kinesin motor proteins or microtubules, both necessary for the post-Golgi sorting of several proteins to the PM, have a moderate or nonexistent role on GLUT4 accumulation in the PM [56,57]. Most importantly, the CFTR channel has been formally shown to translocate to the PM via direct trafficking from the ER in COPII vesicles [58]. To our knowledge, there is no formal evidence that any *de novo* synthesized transporter traffics to the PM through the Golgi/TGN compartment. It should be noted, however, that in specific cases where transporters were shown to be localized in Golgi and/or endosomes, or to require these organelles for sorting to the PM, the focus of the study was on vesicular recycling from and back to the PM, rather than trafficking of *de novo* made transporters (e.g. GLUT4). Further evidence against a conventional mechanism for transporter sorting to the PM comes from the observation that no mutation or specific condition is known to block transporter trafficking to the Golgi. Our current studies, following the localization of *de novo* made UapA and other nutrient transporters in *A. nidulans*, also favour a mechanism of unconventional transporter trafficking [59]. More specifically, we have found that the localization of several transporters to the PM is Golgi-, endosome- and microtubule-independent, as it does not necessitate key proteins essential for Golgi and post-Golgi function in standard cargo secretion (e.g. SedV, GeaA, HypB, RabC, RabO, RabE, AP-1, AP-3). In line with these findings,

transporters do not colocalize with Golgi molecular markers, at any moment during their secretion. On the other hand, transporter localization seems to depend on a functional COPII coat, as well as on clathrin heavy chain (ClaH) and actin. ClaH possibly operates via its effect on the proper assembly and function of the actin network ([59]; Sofia Dimou, Mariangela Diosysopoulou, Sotiris Amillis, Olga Martzoukou, Vangelis Bouris, George Diallinas, unpublished).

Why would specific transporters follow an unconventional pathway for secretion? It seems that the conventional, Golgi/microtubule-dependent pathway operates mostly for sorting of specific membrane cargoes that need to be targeted in a polar fashion to the actively growing apical region of filamentous fungi or to the budding region of yeast cells, which corresponds to a particular polarized cell membrane or a specific membrane domain of animal and plant cells. In *A. nidulans*, for example, the conventional pathway has been established by using as model cargo proteins that are apically localized in order to perform specific functions related to fungal growth (e.g. chitin synthase or lipid flippases). Similarly, the conventional pathways in yeast or animal cells have also been studied using mostly polar cargoes [60,61]. In contrast to polar cargoes, solute transporters in fungi are homogeneously localized all along the membrane of growing hyphae. Thus, one possible rationalization for the existence of conventional and unconventional trafficking pathways might be that the first operates for polar or site-specific secretion, whereas the latter for nonpolar bulk secretion of housekeeping functions, such as cell nutrition via solute uptake. We believe that the trafficking route discovered through studies on *A. nidulans* that transporters follow, might prove to be a major mechanism by which several nutrient transporters are sorted to the PM not only in fungi, but also in higher eukaryotic cells. In line with this idea, several aspects of specific membrane cargo trafficking in neuronal dendrites, such as Golgi independence and actin dependence, share similarities with our findings in *A. nidulans* [62,63]. Of course, we do not envisage that all transporters, in all cell types, follow an unconventional sorting route to the PM.

Work done, time to go (and come back?)

Fungal transporters are down-regulated in response to physiological or stress signals, or as a result of transport activity in the presence of their substrates [64–68]. This control of protein turnover occurs at the PM and is mediated via transporter endocytosis. Internalized endocytic vesicles are sorted in early endosomes that progressively mature to late endosomes/MVBs and eventually fuse with the vacuole/lysosome, where transporter degradation occurs. In mammals, but also in yeasts, after endocytosis, specific transporters may be recycled to the PM via endosomes or specialized transporter vesicles ([69–72]; see later). In all cases where transporter endocytic degradation or recycling takes place, the ubiquitination of specific Lys residues, present in the cytosolic N or C termini of transporters, is a prerequisite [73–76]. Transporter ubiquitination is catalysed by HECT-type ubiquitin ligases of the Ned-d4/Rsp5-type, recruited to the ubiquitination sites on transporter tails by adaptor proteins, known as α-arrestins [76–78,80,85]. How arrestins recognize specific sequences or structural motifs, and thus recruit ubiquitin ligases to the tails of transporters, is little known. Signals that lead to transporter endocytosis include changes in the carbon and nitrogen sources, the pH of the growth medium or the temperature, and last but not least the presence of drugs and oxidizing solvents that affect the PM or other basic cellular functions (e.g. azoles, amphotericin B, rapamycin, cycloheximide, DMSO or DTT). Such physiological or stress signals lead to activation or/and recruitment of α-arrestin adaptors and thus to increased ubiquitination and endocytic turnover of transporters [68,74,81,82].

Notably, ubiquitination and endocytic turnover also depend on the conformational changes of transporters themselves. Several *A. nidulans* and *S. cerevisiae* transporters proved more accessible to ubiquitination and endocytosis when they actively transport their substrates, a phenomenon known as substrate-dependent or activity-dependent endocytosis [64,65,68,74]. This observation supported the idea that particular conformations, triggered after substrate binding (e.g. substrate-occluded or inward-facing topologies), increase the accessibility of arrestin adaptors [64,65,74]. It should also be noted that changes in pH, temperature or composition of membrane lipids can affect transporter conformation, leading to partial misfolding or modification of cytoplasmically exposed domains, and thus making the transporter tails more accessible to arrestins and to endocytic turnover [83,84]. In nearly all cases studied, ubiquitination of fungal transporters takes place in one or two specific Lys residues located at either the N- or the C-tail, at a region distal from the most proximal TMS. No universal arrestin-binding domain has been found, but some relevant motifs sharing common elements were recently recognized in a number of transporters [25]. As an example, in the UapA and FurE transporters of *A. nidulans*, a short acidic motif (E/D-X-E-E) located close to the last

TMS and 23–25 residues upstream from the Lys residues modified by ubiquitination at the cytosolic C terminus was shown to be crucial for specific arrestin-mediated endocytosis [19,85]. Acidic sorting signals were also recently described in the N terminus of the methionine or arginine transporters Mup1 and Can1 and in the C terminus of the monocarboxylate transporter Jen1 of *S. cerevisiae* [77,79,86]. Thus, there seems to be a common mechanism for recognition of transporters by α-arrestins in ascomycetes, probably based on interactions of the arrestin C-terminal basic region and the acidic residues present in the N- or C-terminal cytosolic regions of different transporters. This interaction, however, seems insufficient to promote endocytosis as it is usually context-dependent [65]. Interestingly, in some cases, as in the FurE transporter of *A. nidulans* and the Gap1 transporter of *S. cerevisiae*, both the N and C termini contain elements necessary for specific arrestin-dependent ubiquitination and endocytosis [19,25,82].

Genetic evidence from studies with *A. nidulans* nucleobase cation symporter-1 transporters [87], mostly with FurE, further suggests that dynamic interactions of the N and C terminus with each other are critical for endocytic turnover [18,19]. More specifically, short deletions or specific Ala substitutions in triplicates on either terminus of FurE completely block the endocytosis of the transporter. The FurE C terminus contains the Lys residues and the α-arrestin acidic target sequence necessary for ubiquitination, but the N terminus has no sequence predicted to participate in endocytic internalization. The similarity of phenotypes obtained by mutations in the N and C termini in respect to endocytosis was suggestive of an involvement of the two termini in a common mechanism controlling FurE turnover. This was confirmed by intramolecular bifluorescence complementation (BiFC), as the N and C termini of FurE interact dynamically with each other, in a transport activity-dependent manner. In the absence of substrates, FurE is likely in a rather stable outward-facing conformation, which brings the cytoplasmic N and C termini into contact, permitting the reconstitution of a fluorescent signal. In the presence of substrates, the transporter continuously alternates between the outward- and the inward-facing conformation, making the termini more dynamic and the fluorescent complementation signal impossible to detect [18]. Mutations in either of the two termini that perturb the interaction between them seem to reduce or abolish the recruitment of the endocytic machinery. In line with the critical role of dynamic interactions of cytosolic termini in controlling transporter endocytosis in *A. nidulans*, the C terminus of the *S. cerevisiae* Gap1 amino acid transporter is necessary for stress-induced endocytic turnover, via ubiquitination of Lys residues K9 and K16, which are, however, located in the N terminus. It should be noted that the role of tails in endocytosis might also be associated with conformation-dependent membrane partitioning into distinct PM microdomains enriched in specific lipids [66,88,89].

A surprising and rather instructive result that came from studies concerning the mechanism of endocytic turnover of UapA and other *A. nidulans* transporters was the finding that internalization from the PM is clathrin-dependent, but AP-2-independent [38]. This was the first report of clathrin-dependent and clathrin-independent mechanisms of endocytosis in nonmetazoan cells. Furthermore, these findings were the first to show that the AP-2 adaptor can act without its universal partner, clathrin [38]. Thus, in similarity to distinct secretory routes described earlier, there seem to be specific mechanisms for endocytosis of polar membrane cargoes and nonpolar, housekeeping proteins, such as nutrient transporters. The findings *in A. nidulans* likely hold true for all higher fungi, as the σ subunit of the AP-2 complex lacks the entire C-terminal domain containing the putative clathrin-binding box [38].

In sharp contrast to transporter endocytosis, the endocytosis of membrane proteins that are polarly localized in the growing apical region of *Aspergillus* hyphae (e.g. chitin synthase, lipid flippases, syntaxin A) is AP-2 dependent, but clathrin-independent [38]. Overall, these results reveal that in the course of evolution the AP-2 complex of fungi has acquired a specialized clathrin-independent function, necessary for fungal polar growth. Interestingly, clathrin evolved to function in generalized, nonpolar endocytosis, probably by replacing the role of AP-2 with another adaptor, which in the case of transporters might well be the α-arrestins. Thus, it would be interesting to test whether in specialized cells of higher eukaryotic organisms the clathrin and AP-2 roles have been uncoupled to serve the endocytosis of polar and nonpolar cargoes.

Specific *S. cerevisiae* transporters such as the Mup1 methionine transporter, the Tat2 transporter and the Jen1 monocarboxylate transporter [90,91] have been shown to recycle back to the PM, rather than being degraded in the vacuole, after endocytosis. Although in yeast cells endocytic recycling generally requires the Golgi apparatus, a more direct pathway bypassing the TGN, requiring the F-Box protein Rcy1p and Sec1, but not Sec7, is also known to exist [92,93]. The latter pathway has been visualized with the use of FM4-64

and more recently with a synthetic reporter, namely Ste3-GFP-Dub, and is most probably the endocytic recycling pathway followed by some transporters [90,92]. In the indirect pathway [94,95], cargoes are targeted to the Golgi through a VFT/Golgi-associated retrograde protein complex - and Ypt6-dependent pathway and rerouted to the PM in a Sec7-dependent manner. The Jen1 transporter seems to follow this TGN-dependent pathway for recycling, after endocytosis, in response to glucose deprivation, which promotes its deubiquitylation and escape from degradation in the vacuole [70]. Interestingly, a more recent study suggested that the *S. cerevisiae* recycling system consists basically of the TGN, which serves as an early and as a recycling endosome simultaneously [96]. Very little is known regarding transporter recycling in filamentous fungi. In mammalian cells, membrane protein recycling back to the surface can also occur via different pathways. These can be either rapid and direct, or slower and indirect, involving the maturation of postendocytic early endosomes to Rab11-positive recycling endosomes and retrograde sorting to the TGN [93,97,98]. Internalized recycling proteins, such as the transferrin receptor, follow mostly the direct pathway and very rarely transit to the TGN [93]. Recycling of transporters is best studied for the insulin-sensitive GLUT4 glucose transporter, but this seems to constitute a rather particular case. Following endocytosis and prior to recycling, GLUT4 is temporarily sorted to either perinuclear storage compartments or GLUT4 storage vesicles. Insulin-triggered recycling to the PM occurs through a specialized Rab10-dependent secretory pathway, whereas in the basal state a slow constitutive general pathway operates with the involvement of endosomal recycling intermediate compartments and Rab11 [93,99,100].

Transporter cytosolic tails regulate gating and substrate specificity

An interesting novel concept concerning transporter function comes from genetic studies addressing the role of the cytosolic termini of the *A. nidulans* Fur transporters. In particular, it has been shown that the cytosolic tails of the uracil–allantoin–uric acid FurE transporter, through their interactions with other internal loops, regulate not only endocytosis (described above), but also the mechanism of fine gating, and thus substrate specificity (Fig. 2). In the course of systematically analysing the roles of segments of the cytosolic C and N termini of the FurE transporter, it became apparent that specific terminal elements are critical for substrate specificity [18,19]. The specificity changes obtained via certain mutations were of two types. The first extended the number of substrates transported by FurE to include xanthine, in addition to uracil–allantoin–uric acid, while the second type increased the specificity, by limiting the substrates down to uracil and allantoin. Mutations leading to increased specificity were Ala substitutions in the N-tail motif N-X-D-Φ-D-P (residues 24–29), while the ones restricting specificity were deletions of the most distal part of the N-tail (residues 1–21) or Ala substitutions of N-terminal residues 15–17 or of C-terminal residues 504–512. In all cases, specificity modifications did not result in significant changes in substrate-binding affinities or apparent transport rates (K_m and V values, respectively). These findings revealed that cytoplasmic termini of FurE affect substrate specificity from a distance. The roles of the N and C termini are not essential for transport activity *per se*, as FurE versions truncated in either one or both termini can still function, at least for uracil transport. In other words, the interaction of the tails is not an essential part of the mechanism that promotes the alteration from the outward- to the inward-facing conformation but is critical for selective translocation of substrates. Genetic and biophysical evidence [i.e. molecular dynamics (MD)] further suggested that the interactions of the FurE termini are critical for the opening and occlusion of the substrate translocation trajectory and the gating process. Genetic suppressors restoring loss of uric acid transport by N- or C-terminal FurE mutants were located at the external gate, along the substrate translocation path, or in flexible loops that act as dynamic hinges during transport. Moreover, MD and relative rational mutational analysis showed that specific polar residues in the N terminus (Asn24, Asp26 and Asp28) interact dynamically with residues of several internal cytosolic loops and thus control the opening and closing of outer and inner gates [19]. Given the fact that positioning of the N terminus depends on its interaction with the C terminus, both FurE tails control the dynamic molecular crosstalk with its internal loops. What was also notable in the case of FurE is that the substrate specificity of this transporter is pH-dependent, and this seemed to be due to an effect of the pH on the interactions of the N and C termini with the internal cytosolic loops. At pH 5.5, FurE is little functional, transporting only uracil very poorly, while at neutral pH FurE transports well uracil, allantoin and uric acid, and at pH 8.0 it additionally transports xanthine. pH-dependent changes in the specificity of wild-type FurE are somehow mimicked by specific mutations in the critical polar residues of N terminus (Asn24, Asp26 and Asp28). It

seems that loosening of the crosstalk between termini and cytosolic loops, promoted at higher pHs or by mutations at neutral pH, leads to relaxed gating and thus higher promiscuity of the transporter [19].

Oligomerization and lipids as emerging players in transporter functioning

Nearly 10 years ago, we noticed a rather surprising phenomenon with respect to endocytosis of the UapA purine transporter. The wild-type protein undergoes endocytosis in response to the presence of primary N sources (i.e. ammonium or glutamine are preferred N sources compared to purines), but also in the presence of continuous supply of its physiological substrates (xanthine or uric acid) [64,85]. In the latter case, however, only transport-active alleles of UapA undergo endocytosis, which was interpreted as evidence that specific conformational intermediates associated with transport activity are more efficient in recruiting the ubiquitination and endocytic machineries [64,85]. This idea of substrate-elicited, activity-dependent turnover gained strong evidence by subsequent work with transporters of *S. cerevisiae* [65,74]. What, however, has come as a surprise is that PM-translocated UapA mutants that cannot undergo endocytosis, because either they lack activity or the specific Lys for ubiquitination, can be internalized when co-expressed with wild-type UapA. The simplest scenario of the apparent in *trans*-endocytosis of mutant UapA molecules was to consider that UapA dimerizes and that 'restoring' endocytosis was due to homodimerization (or oligomerization). At this time, there was no or little evidence supporting that solute transporters might oligomerize and no evidence on the possible biochemical or physiological role of transporter oligomerization. Since then, a multitude of approaches, such as analysis of dominant-negative mutations, light scattering, pull-downs, *in vivo* BiFC assays and crystallization, confirmed that UapA dimerization occurs *in vivo* and is required for the function and specificity of the transporter [15,101]. In the past few years, it is becoming well established that many transporters form oligomers crucial for effective trafficking to the membrane, function and turnover [102,103]. In fact, all studied structural homologues of UapA, namely the prokaryotic uracil UraA [104] and fumarate/sulfate SulP [105,106] transporters, the plant borate efflux transporter Bor1 [107], the Band3 anion exchanger in human erythrocytes [108] and the human sodium bicarbonate cotransporter NBCe1 [109], have been shown to dimerize. As with UapA [102], UraA mutants trapped in a monomeric state exhibited no transport activity although they bind substrate with normal affinity [104]. Additionally, transporter of the

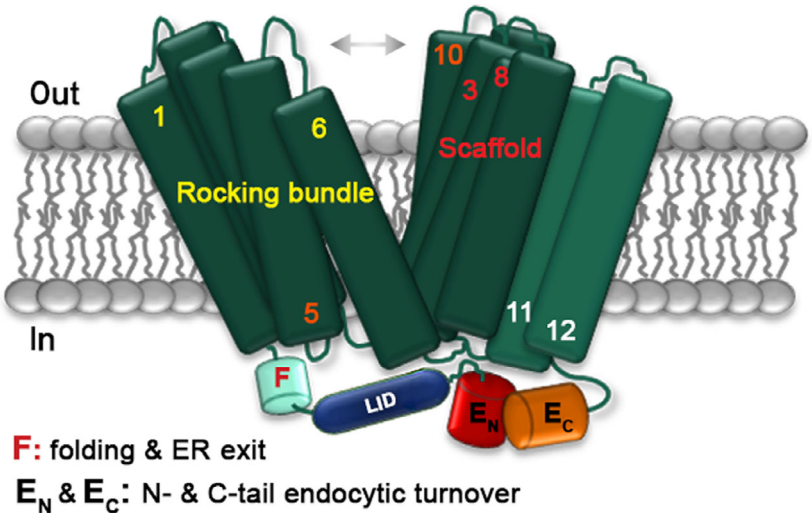

Fig. 2. Simplified model showing the multiple roles of FurE cytosolic termini in folding, ER exit, endocytosis and substrate specificity. Details are described in the text and in [17]. LID stands for a central segment of the N-tail that is specifically involved in interactions with several other cytosolic internal loops and thus regulates the gating and substrate specificity from a distance. E_N and E_C are distal parts of the tails that interact dynamically with each other during transport. Their interaction seems essential for recruiting the ubiquitination machinery and for promoting endocytosis, but is also critical for the topology of the LID and thus for substrate specificity. F is the part very proximal to TMS1 that is critical for correct folding, packaging in COPII vesicles and ER exit. The interactions described above strongly depend on the pH of the medium and the membrane lipid composition.

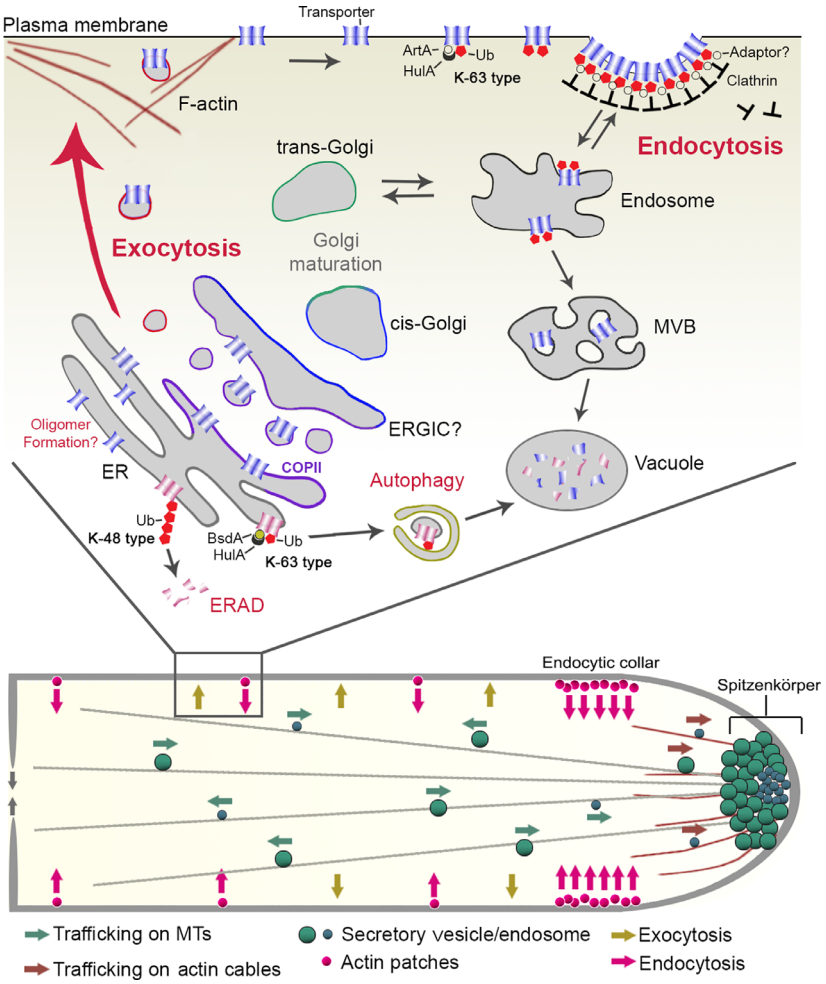

Fig. 3. Schema showing highlights of novel findings concerning the trafficking of solute transporters of *A. nidulans*. For details, see the text. In brief, transporters are cotranslationally inserted into the ER membrane and, if properly folded, exit the ER embedded in COPII vesicles. They are subsequently sorted to the PM via a nonpolar route that bypasses the Golgi and the need for microtubules and endosomes. This route requires a functional actin network and the ClaH, being distinct from the conventional traffic route used for polar secretion of apical markers related to fungal growth (depicted in the lower panel in the figure; for details, see [49]). Transporter endocytic turnover, which comes as a response to specific or general physiological or stress signals, operates via specific HECT-type ubiquitination (e.g. HulA Ubiquitin ligase), assisted by α-arrestin adaptors (e.g. ArtA), followed by clathrin-dependent, but AP-2-independent, internalization. This endocytic mechanism is different from polar cargo apical endocytosis (e.g. lipid flippases), which is AP-2-dependent and clathrin-independent. Specific ER-retained misfolded transporters are degraded by chaperone-mediated (e.g. BsdA) ubiquitination (e.g. HulA), which recruits the machinery of selective autophagy (e.g. Atg8 and Atg9), but also by ERAD. The schema is based principally on studies with the UapA purine transporter, but also with several other *A. nidulans* transporters. Notice that the *cis*- and *trans*-Golgi in *A. nidulans* are distinguishable, dynamic organelles. The Spitzenkörper is a dynamic structure present at the tips of hyphal cells of ascomycetes, crucial for polar cargo secretion, polar growth and cell morphogenesis.

LeuT or 5 + 5 fold also seems to form oligomers necessary for proper trafficking to the membrane and function. In several cases, methodologies such as fluorescence resonance energy transfer [110], cross-linking [111], pull-downs [112], co-immunoprecipitation [113] or size-exclusion chromatography–multiangle light scattering [114], have been employed to confirm oligomerization [102,103]. Also, members of the NSS transporter family can form dimers, depending on their localization [115–119]. Finally, other transporters that oligomerize are members of the SWEET transporter family, the ammonium transporter/MEP/Rh transporter family and the major facilitator superfamily ([102] and references therein).

The role of membrane lipids in the formation of oligomers is emerging as a critical issue. Studies with

UapA contributed in showing the importance of membrane lipids in transporter function. More specifically, it has been shown that phosphatidylinositol (PI) and phosphatidylethanolamine (PE) are crucial in stabilizing a functional UapA dimer, whereas delipidation during purification causes dissociation of the dimer into monomers, but subsequent addition of PI or PE re-establishes the functional UapA dimer. MD simulations predicted the possible lipid-binding sites near the UapA dimer interface, and mutational studies confirmed that Arg287, Arg478 and Arg479 act as the lipid-binding residues involved in the formation of UapA dimers and are imperative for transport activity [120]. Interestingly, while the loss of transporter–lipid interaction led to total lack of function, a fraction of UapA could still dimerize in the R287A/R478A/R489A mutant, suggesting that specific interactions with lipids might also interfere with the mechanism of transport, and not only with dimer formation. MD simulations further predicted that lipids could also bind to the outermost, membrane-facing regions of the core domains of the UapA dimer [120]. Thus, current investigation is employed in fully understanding the role of membrane lipids in UapA folding, subcellular traffic and transport function [121]. In this direction, the characterization of genetic suppressors restoring defects in UapA–lipid interactions allowed the identification of residues that have a role in UapA folding, in the specific environment of fungal membranes.

Conclusions

Work with *A. nidulans* transporters has led to a number of original new concepts that extend beyond fungal biology and specific transporter families. These are as follows: AP-2-independent, but clathrin-dependent endocytosis, Golgi- and microtubule- independent sorting of transporters to the PM by an apparent post-ER/COPII unconventional route, turnover of misfolded transporters by specific ubiquitination and chaperone-mediated selective autophagy, a critical role of cytoplasmic termini of transporters in selective gating and substrate specificity, and an emerging role of transporter membrane lipid interactions in oligomerization, traffic and function. Figure 3 depicts the main points of this review with respect to transporter subcellular traffic and turnover.

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Conflict of interest

The authors declare no conflict of interest.

Author contributions

GD and OM wrote the manuscript.

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