Trends in Genetics

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Review

Dissection of Transporter Function: From Genetics to Structure

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Transporters are transmembrane proteins mediating the selective uptake or efflux of solutes, metabolites, drugs, or ions across cellular membranes. Despite their immense biological importance in cell nutrition, communication, signaling, and homeostasis, their study remains technically difficult mostly due to their lipid-embedded nature. The study of eukaryotic transporters presents additional complexity due to multiple subcellular control mechanisms that operate to ensure proper membrane traffic, membrane localization, and turnover. Model fungi present unique genetic tools to study eukaryotic transporter function. This review highlights how fungal transporter genetics combined with new methodologies for assaying their cellular expression and function as well as recent structural approaches have led to the functional dissection of selected transporter paradigms in *Aspergillus nidulans*.

Why Fungal Transporters?

All types of cells need to communicate with their environment. This communication is achieved through the control of uptake and efflux of small molecules such as nutrients, metabolites, and ions. Uptake and efflux of these solutes and ions depends on specialized polytopic transmembrane proteins, classified into two major types: solute transporters and ion channels [1,2]. Solute transporters are considered to act like enzymes [3,4], possessing a major binding site that binds substrates at one side of the membrane; this binding elicits a conformational change that exposes the substrate-occupied binding site on the other side of the membrane where substrates are eventually released [5–8]. By contrast, channels act like selective **gated** pores (Box 1). The biological importance of transporters and channels in cell functioning is associated with nutrition, homeostasis, signaling, defense, and detoxification. Their malfunction is related to several human diseases (e.g., cystic fibrosis, diabetes, neurotransmission defects) [9,10]. In addition, transporters are directly related to drug sensitivity or resistance [11]. The importance of transporters is also reflected in the observation that 8–10% of most known genomes encode transporters [12].

Filamentous fungi are champions of transporters, with at least 10–12% of their genomes encoding such proteins [12]. In a recent *in silico* analysis, aspergilli seem to possess more than 120 different families of transporters (http://fungidb.org/fungidb). Some are *Aspergillus* specific, some fungus specific, some microbe specific, and others ubiquitous in all domains of life (G. Diallinas, unpublished observations). Most metazoan transporters have **homologs** (see Glossary) of fungal proteins and seemingly similar mechanisms for their subcellular expression and turnover regulation. Model fungi such as *Saccharomyces cerevisiae* and ***Aspergillus nidulans*** provide unique novel genetic tools that allow the dissection of structure–function relationships and the regulation of expression or turnover of transporters in living cells. In addition

Trends

Transmembrane transporters are essential for cellular functioning.

Transporter function or malfunction is related to genetic diseases, pathologies, and sensitivity or resistance to drugs.

Transporters function by substrate-binding-induced alterations of their protein conformation exposing the substrate-binding site alternatively to either side of the membrane.

Genetic analysis of fungal transporters predicted the existence of channel-like gating or filtering domains in transporters before crystal structures were available.

Recent crystallographic analyses of transporters reveal mechanistic variations in the outward- to inward-facing alteration of transporters and confirm the presence of gates in transporters.

The genetics and crystal structure of the fungal purine transporter UapA show how different gating elements might function and reveal the functional importance of dimerization.

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Box 1. Transporters versus Channels

Transporters are transmembrane proteins mediating the selective cellular uptake or efflux of solutes, metabolites, ions, and drugs across membranes (Figure I). They possess a major binding site that interacts with substrates at one side of the membrane and this binding elicits a conformational change that exposes the substrate-occupied binding site on the other side of the membrane where substrates are eventually released. The basis for this alternating-access model was established in the 1950s [89,90] and gained rigorous experimental support in more recent years. In its simplest conceptual form the mechanism of the alternating-access model is that of a rocker switch [91,92]. Transporters resemble enzymes with respect to their dependence on specific interactions of substrates with residues of a single binding site and thus their activity is characterized by Michaelis–Menten kinetics [3,4].

Transporters are structurally and functionally distinct from ion channels, which are pore-forming transmembrane proteins controlled by the simultaneous opening and closing of 'gates' rather than by gross alteration of protein conformations. 'Gating' operates in response to various biochemical, biophysical, electric, and mechanical signals. Channel selectivity is controlled by 'molecular filters' restricting the size of the pore that are located close to the outward-facing gate and do not possess a specific substrate (ion)-binding site. When open, channels rapidly translocate ions down their electrochemical gradient without the input of metabolic energy, generating electrical potentials measurable by using patch clamp devices [1].

Transporters can be facilitators (energy-independent transport, similar to channels), primary active transporters requiring ATP hydrolysis for their function, or secondary active transporters coupling the energetically demanding substrate transport with energetically favorable symport or antiport of ions or a second substrate [93].

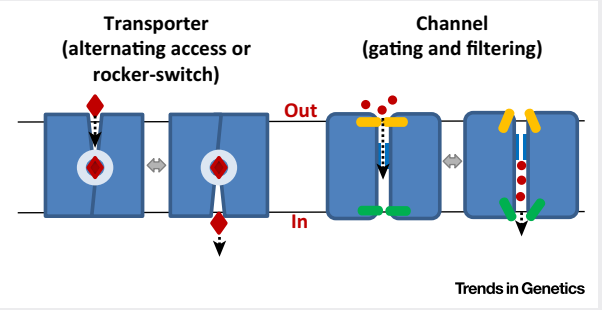

Figure I. Cartoon Highlighting Mechanistic Differences in Transporters and Channels.
Key: red diamonds, solute/substrate; red circles, ions; yellow capsules, outward-gating elements; green capsules, inward-gating elements.

to these tools, physiological, classical genetic, and biochemical studies concerning fungal transporters have a long history, which is proving an extra advantage for studying transporters in fungi [13,14].

A. nidulans has been used since the 1990s as a model system of choice for studying several aspects of transporter biology, such as regulation of expression, membrane traffic, and turnover, structure–function relationships, **substrate specificity**, and molecular evolution [13–17]. This is a rational continuum of the existing genetic knowledge and the uniqueness of combining *Aspergillus* genetics with new specific tools developed in recent years. This review focuses mainly on how our knowledge has evolved from genetic mutations to the structure and mechanism of function of a paradigmatic transporter, namely **UapA**, with references to other well-studied *A. nidulans* and *S. cerevisiae* transporters.

Developing Tools to Study Transporters in *A. nidulans*

The tools used for studying transporter function in *A. nidulans* and *S. cerevisiae* basically comprise simple and rapid **uptake assays**, the construction of multiple-gene knockout strains that allow the direct assessment of a given transporter in a 'clean' genetic background lacking other similar transporters, and the employment of *in vivo* cell biology approaches to follow

Glossary

***Aspergillus nidulans*:** a filamentous fungus (mold) that is used as a model genetic, cellular, and molecular system to understand basic mechanisms of the eukaryotic cell.
Gating: commonly the mechanism by which channels open and close to control the selective passage of ions across a membrane. For gating, channels have two gates at the entrance and exit of ions that open simultaneously in response to a chemical, electrochemical, biophysical, or mechanical signal. Recent data show that gates also exist in transporters, but in this case the gates at the outward- and inward-facing sites of transporters open and close alternatively, never simultaneously.
Genetic screen: an approach to determine the genetic basis responsible for a phenotype caused by spontaneous or induced genetic mutations.
GFP: a 238-amino-acid protein from the jellyfish *Aequorea victoria* that exhibits bright green fluorescence when exposed to UV light. Genetically modified versions of GFP can be genetically liked to any protein and thus used as a powerful tool to follow *in vivo* the cell fate of any protein of interest.
Homodimerization: the close physical, and most often functional, noncovalent interaction of two molecules of a single protein.
Homologous proteins: proteins originating from a common ancestral protein. Homologous proteins (transporters) conserve a similar basic structure but their functions might have diverged significantly.
Reverse genetics: an approach to discover the function of a gene by analyzing the phenotypic effects of a specifically modified DNA sequence introduced by genetic transformation into an organism/cell of choice. Reverse genetics proceed in the opposite direction to the so-called forward genetic screens of classical genetics.
Substrate affinity: the affinity (strength) by which an enzyme or transporter binds its substrate as expressed with a measured K_m value defined as the substrate concentration that gives half-maximal velocity of an enzymatic/transport reaction. Also called the Michaelis–Menten constant.

transporter subcellular expression and turnover. These simple tools, combined with rational *in vitro* mutagenesis, random **genetic screens**, *in silico* modeling, and, recently, crystallography, have led to the functional characterization of wild-type or mutant versions of transporters and eventually to novel concepts on how transporters work.

Uptake Assays

Transport assays in living cells have been used for decades for unicellular microorganisms, mostly bacteria and yeasts but also protozoa and algae. Uptake assays in multicellular organisms present more technical difficulties. However, in living cells a given transporter is usually studied in the presence of other transporters with overlapping transport functions, so the apparent transport kinetics and substrate specificity profiles observed might represent results from several transporters. Despite these apparent disadvantages, transport assays in living cells are by far the most rigorous and easiest way to characterize the physiological characteristics of a transporter. In *A. nidulans*, problems associated with mycelium development and cellular differentiation were solved by using conidiospores harvested at the isotropic phase of germination before germ tube emergence [18–20]. At this developmental stage, all transporters analyzed so far are highly expressed through *de novo* transcription independent of the physiological conditions imposed.

Studying Transporters in a Genetically Clean Background: The Purine Transporter Paradigm

Given the availability of the genome sequence, the ease of constructing and crossing null transporter mutants, and the employment of simple uptake studies in germinated conidiospores, all seven putative nucleobase/nucleoside transporters of *A. nidulans* have been identified and studied. Additionally, strains that genetically lack any combination of these have been constructed (Table 1). Each of these transporters has been kinetically characterized in the absence of other transporters with overlapping functions [21–38]. Additionally, a strain containing total genetic deletions of all seven genes encoding these transporters has been constructed. This strain (called Δ7), which has no detectable nucleobase/nucleoside **transport capacity**, has proved to be an excellent tool to characterize other minor or cryptic transporters related to purines or pyrimidines [29]. The Δ7 strain permits the direct functional characterization of any putative nucleobase/nucleoside transporter from the several aspergilli sequenced [39] and probably from any other filamentous fungus. Before the construction of the Δ7 strain, a strain lacking simply the three major purine transporters (UapA, UapC, and AzgA) was used to functionally characterize the first plant purine transporter from maize [40]. Thus, in principle the Δ7 strain can be used to functionally express and characterize any nucleobase/nucleoside/allantoin transporter from any eukaryotic organism, if the transporter can be correctly targeted to the plasma membrane.

Following Transporter Expression, Traffic, and Turnover via Protein Fluorescent Tags

Since 1999, **GFP** tagging has become a standard tool to follow *in vivo* the subcellular expression, membrane trafficking, and turnover of wild-type and mutant versions of fungal transporters [41–43]. Transporters are tagged C terminally, as the N-terminal region is critical for proper cotranslational translocation into the endoplasmic reticulum (ER) membrane. Plasma membrane GFP-tagged functional transporters label rather homogeneously the periphery of yeast or hyphal cells. Results from an analysis of 20 different fungal transporters show that there is no *a priori* way to predict whether tagging a transporter will affect its localization or function. For example, all nucleobase ascorbate transporters (NATs) conserve wild-type kinetics and specificity when tagged with GFP, whereas major facilitator superfamily (MFS), amino acid–polyamine–organocation (APC), and nucleobase cation symporter 1 (NCS1) transporters are differentially sensitive to GFP tagging (for transporter families see http://www.tcdb.org/). Addition of an amino acid linker between the transporter and the GFP often solves or minimizes functionality problems [44]. Importantly, fungal systems allow testing the functionality of any GFP-tagged transporter

Substrate specificity: the range of substrates (chemicals) an enzyme or a transporter can recognize, bind, and catalyze their chemical modification or transport, respectively. Enzymes and transporters can show all ranges of specificity.

Transport capacity: the rate or efficiency by which a transporter catalyzes the transport of its substrate as expressed by measured apparent V_m value; that is, the concentration of substrate accumulated within a specific number/mass of cells in a specific period of time. Apparent V_m reflects the amount/number of functional transporter molecules present in a membrane and thus depends on the level of transporter expression and turnover, which in turn depends on the physiological conditions used to grow the cells/organism analyzed.

UapA: a high-affinity, high-capacity xanthine–uric acid/H^+ symporter in *A. nidulans* and one of the eukaryotic transporters most studied at the genetic level.

Uptake assays: method to measure transporter activities using radiolabeled substrates and thus kinetically characterize transporters.

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Table 1. Nucleobase-Related Transporters in *Aspergillus nidulans*

Transporter	Family[a]	Subfamily[a]	Prokaryotic Homolog[b]	Modeled Structure[c]	Crystal	Specificity	Mechanism	Substrate Binding[d]	Transport Activity[e]	Gating Elements[f]	Refs
UapA	NAT/NCS2 2.A.40	2.A.40.4	**UraA** (uracil) PDB 3QE7 (inward facing)	**14 TMSs 7 + 7 (2 + 5 + 2 + 5)**	**Yes**	Uric acid, xanthine	Two-domain elevator mechanism H^+ symport	**E356** (TMS8), **Q408**, A407 (TMS10) S154 (TMS3)	**Q85**, H86 (TMS1) **D360** (TMS8) **D388** (TMS9) **N409**, G411, T416 (TMS10)	**F528**, **T526** (TMS14) **R481** (TMS13) A441 (TMS11) V463, A469, Q113 (L1)	[15,16, 30,47,55]
UapC	NAT/NCS2 2.A.40	2.A.40.4	**UraA** (uracil) PDB 3QE7	**14 TMSs 7 + 7 (2 + 5 + 2 + 5)**	No	Uric acid, xanthine	H^+ symport	–	–	–	[20]
AzgA	NAT/NCS2 2.A.40	2.A.40.7	**UraA** (uracil) PDB 3QE7	**14 TMSs 7 + 7 (2 + 5 + 2 + 5)**	No	Adenine, guanine, hypoxanthine	H^+ symport	**N131** (TMS3) **N339** (TMS8) **E394** (TMS10)	N342 (TMS8)	Indirect evidence[g]	[36]
FcyB	NCS1 2.A.39	FCY 2.A.39.2	**Mhp1** (benzylhydantoin) PDB 4D1B (occluded)	**12 TMSs 5 + 5(+2)**	No	Cytosine, purines	H + symport	S85 (TMS1), W159, **N163** (TMS3), W259 (TMS6), **N354** (TMS8)	**N350**, **N351**, P353 (TMS8)	Indirect evidence[7]	[31,34,88]
FurA	NCS1 2.A.39	FUR 2.A.39.3	**Mhp1** PDB 4D1B	**12 TMSs 5 + 5(+2)**	No	Allantoin	H^+ symport	–	–	–	[32,37,88]
FurD	NCS1 2.A.39	FUR 2.A.39.3	**Mhp1** PDB 4D1B	**12 TMSs 5 + 5(+2)**	No	Uracil	H^+ symport	N54 (TMS1) **Q134** (TMS3) N249, Y250 (TMS6) **N341** (TMS8)	T57 (TMS1) **W130** (TMS3) L386 (TMS10)	**M389** (TMS10)	[29,37,88]
CntA	CNT 2.A.41	CNT 2.A.41.2	**NupC** (nucleosides) 4PD9 (occluded)	**11–13 TMSs**[h]	No	Nucleosides	H^+ symport	–	–	–	[32,94] (G. Diallinas, unpublished)

[a]According to http://www.tcdb.org/.
[b]Closest prokaryotic homolog with known crystal structure used to build structural models of the *A. nidulans* nucleobase transporters.
[c]5 + 5(+2) and 7 + 7(2 + 5 + 2 + 5) inverted repeat-fold transporters are members of a single superfamily named the APC superfamily. Protein members of the APC superfamily have two inverted intertwined repeat units of five TMSs (5 + 5). Some subfamilies contain two or four extra TMSs, shown here as 5 + 5(+2) or 2 + 5 + 2 + 5 [75].
[d]Based on mutational analysis and substrate docking approaches using modeled structures. Residues altering the K_m and in some cases the specificity of the transporter. Residues in bold interact strongly with substrates via H bonding through their side chains.
[e]Based on mutational analysis and intramolecular interactions in modeled structures. Residues altering the V_m of the transporter without affecting its turnover. Residues in bold are absolutely necessary for transport activity.
[f]Based on mutational analysis, modeled structures, and molecular dynamics. Residues altering significantly the specificity of the transporter or the transport capacity, despite being relatively distant from the major substrate-binding site. Residues in bold have the most prominent effect on specificity.
[g]Based on the observation that mutations in the substrate-binding site do not themselves alter substrate specificity.
[h]Bacterial NupC has ten TMSs [94], but eukaryotic homologs like CntA have one to three putative extra TMSs [93].
Abbreviations: NAT/NCS2, nucleobase ascorbate transporters/nucleobase cations symporters 1; NCS1, nucleobase cations symporters 1; CNT, concentrative nucleoside transporters.

through comparison of **substrate affinity (K_m)** values and specificity profiles of tagged and untagged transporters. GFP labeling also provides a valuable tool to classify transporter mutations into two major classes: those affecting subcellular expression, membrane localization, and stability and those critical for transport activity *per se* (Figure 1A, Key Figure).

Identification of the Putative Substrate-Binding Site of UapA by Reverse Genetics

One of the first eukaryotic transporters to be analyzed systematically by **reverse genetics** at the level of structure–function relationships is the UapA transporter of *A. nidulans*. Through classical genetic and physiological studies, UapA was already known to be specific for uric acid–xanthine uptake [21,45,46]. In a seminal article [23], series of UapA–UapC and UapC–UapA chimeric transporters were constructed via an unbiased *in vivo* recombination approach in *Escherichia coli* and studied by expression in an *A. nidulans* strain genetically lacking the endogenous

Key Figure

Residues Critical for Function versus Residues Critical for Specificity in UapA

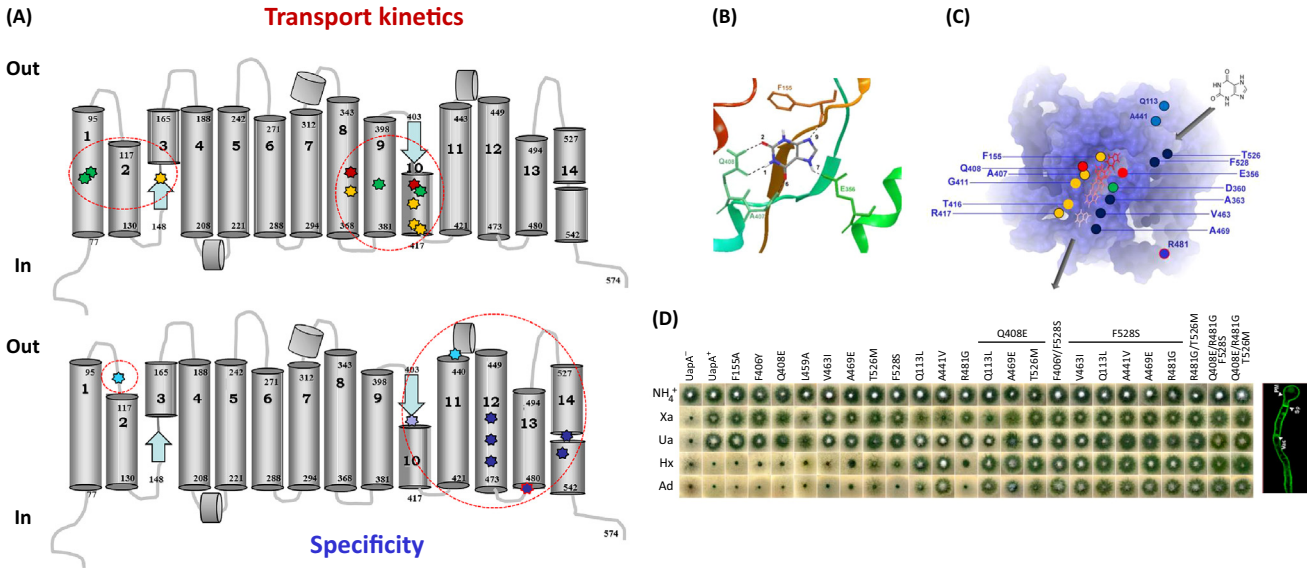

Figure 1. (A) Upper panel: Residues absolutely (red) or significantly (yellow) critical for substrate binding and residues critical for transport rate without affecting substrate-binding affinities (green). All functional mutations are located in TMS1, TMS3, TMS8, TMS9, and TMS10. Lower panel: Residues critical for substrate specificity. Blue coloring denotes gating elements located within transmembrane segments TMS12, TMS13, and TMS14 whereas turquoise represents gating elements in the flexible hinges between TMS1 and TMS2 and TMS11 and TMS12. (B) UapA–substrate interactions modeled via the UraA structure [55] and substrate docking before UapA structure determination, supporting the involvement of specific residues (Glu356 and Gln408) in direct substrate binding. Reproduced from [56]. (C) Modeled substrate translocation trajectory via molecular dynamics revealing that specificity residues (in blue), except Arg481, are located proximal to the substrate translocation trajectory, which could rationalize the effect of these residues on specificity. Coloring of residues is as in (A). (D) Growth tests of *Aspergillus nidulans* strains expressing wild-type or mutant versions of UapA on ammonium ($NH4^+$), xanthine (Xa), uric acid (Ua), hypoxanthine (Hx), or adenine (Ad) as the sole nitrogen source. $UapA^-$ indicates an isogenic strain carrying a total deletion of the *uapA* gene. $UapA^+$ indicates a strain expressing wild-type UapA from its native endogenous locus. All strains shown also carry genetic deletions of two other major purine transporters (*azgA* and *uapC*). Wild-type UapA transport activity confers growth on xanthine or uric acid but not on hypoxanthine or adenine. Notice that among single mutants only those concerning gating residues (e.g., A469E, T526 M, F528S, Q113L, A441 V, R481G) confer growth on novel substrates (e.g., hypoxanthine, adenine). Combinations of gating residue mutations (e.g., Q113L, A469E, T526 M, F528S) with substrate-binding-site mutations (e.g., Q408E, F406Y) increase the ability of the double mutants to grow on hypoxanthine and/or adenine. Further combinations of different mutations in gating elements with substrate-binding-site mutations lead to UapA versions with high-affinity and high-capacity transport of all purines. Adapted from [47]. Notice that all relevant mutations do not affect the localization of GFP-tagged UapA into the plasma membrane, as exemplified in the right-most insert next to the growth tests.

capacity for purine transport (*uapA*$^{-}$ *uapC*$^{-}$ *azgA*$^{-}$). UapC is a paralog of UapA that also transports uric acid–xanthine, but the activities of UapA and UapC can be physiologically and kinetically distinguished [20,22,27]. All chimeras containing a central 70-amino-acid part of either UapA or UapC functioned as UapA or UapC, respectively, as shown by growth tests and direct uptake assays. It was concluded that this central segment, which we now know to contain TMS8, TMS9, and the first part of TMS10 [47], hosts the substrate-binding site of UapA (or UapC). In accordance with the functional importance of this region, the first part of TMS10 contains a characteristic signature motif conserved in all UapA/C-like proteins [26], which at that time already defined a fast growing and ubiquitous protein family, the NATs (also later known as the NCS2 family [15,16]). Today more than 20 NAT members from bacteria, fungi, plants, and mammals have been functionally characterized and the great majority of them are shown to be H^+ (or Na^+ in mammals) symporters of nucleobases. A surprising distinction concerns the NATs of primates, which are Na^+/L-ascorbate symporters totally unable to recognize nucleobases [48].

UapA has subsequently undergone extensive rational mutational analyses concerning mostly conserved or partially conserved residues in TMS8–TMS10, TMS1, and TMS3. More than 100 mutations have been generated, introduced by transformation into a *uapAΔ uapCΔ azgAΔ* [26] mutant background, and analyzed functionally [15–17]. In this analysis, 11 amino acid residues proved critical for function: Gln85 and His86 in TMS1; Ser154 in TMS3; Glu356 and Asp360 in TMS8; Asp388 in TMS9; and Gln408, Asn409, Gly411, Thr416, and Arg417 in TMS10 (Figure 1A, upper panel). Six of these residues, all of polar nature, were essential for wild-type function: Gln85, Glu356, Asp360, Asp388, and Gln408 and Asn409. Importantly, multicopy expression of most mutants, even those that show very low activities, allowed their kinetic characterization by direct uptake assays. Mutations in four of the essential residues (Gln85, Asp360, Asp388, and Asn409) affected substrate transport rates (apparent V_m) while two, Glu356 and Gln408, affected the K_m of UapA for all substrates. Using a GFP-tagging approach, all of the functionally critical mutations, except specific mutations in His86, were shown to have no effect on UapA stability, membrane trafficking, plasma membrane localization, or turnover (Figure 1D).

All functionally interesting mutants were also analyzed for their ability to recognize a large set of purine- and pyrimidine-substituted analogs and compared with wild-type UapA [27,30]. This confirmed that the substrate-binding site of UapA comprises specific residues in TMS3, TMS8, and TMS10 and led to a structure–activity model suggesting that Gln408 and Glu356 directly bind substrates through H-bond interactions with group N1, C2 = 0, N7, or N9 of the purine ring (Figure 1B). Impressively, mutational analysis of the homologous *E. coli* NAT proteins led to nearly identical conclusions regarding the roles of residues involved in substrate binding and transport [49–54].

Importantly, molecular modeling of UapA and substrate docking approaches, which became possible in 2011 when the first bacterial homologous NAT protein (the uracil transporter UraA [55]) was crystallized, fully supported the genetic predictions concerning residues involved in substrate binding and transport and confirmed that residues critical for specificity are localized outside the presumed substrate-binding site (Figure 1C) [56].

Classical Genetics Leads to Novel Concepts: Gating Elements in Transporters

The systematic mutational analysis of UapA has led to the identification of the putative substrate-binding site but left open how the strict specificity of this transporter for uric acid and xanthine is determined, as no mutation analyzed led to a UapA version able to transport other purines. Only a single mutation in one of the presumed substrate-binding residues, Q408E, conferred on

UapA a capacity for moderate hypoxanthine binding (K_i of 70 μM), but the corresponding mutant still could not transport hypoxanthine [26,30]. Altered specificity mutants were, however, obtained using direct genetic screens. Strains expressing either wild-type or selected mutant versions of UapA but lacking other major purine transporters were mutagenized and colonies that could grow on hypoxanthine or adenine were selected (Figure 1D) [24,30,57] (note that in *A. nidulans*, in contrast to *S. cerevisiae*, all purines are catabolized to ammonium and thus serve as nitrogen sources). These genetic screens led to the identification of several residues that when mutated convert UapA into a transporter capable of transporting all purines. Surprisingly, nearly all specificity mutations obtained were located outside the presumed substrate-binding site (TMS1, TMS3, or TMS8–TMS10) and instead mapped to amino acids within the extracellular loop linking TMS1/2 (Gln113), TMS11 (Ala441), TMS12 (Val463, Ala469), and, most frequently, TMS13 (Arg481) and TMS14 (Thr526 and Phe528) (Figure 1A, lower panel). A single exception was mutation F406Y (Figure 1A, upper panel), which affected a residue within the putative substrate-binding site [49]. Thus, either the substrate-binding site defined through rational mutational analysis is more complex or the specificity mutations define functional elements that have a dynamic effect on substrate binding. The second assumption was strongly supported through the kinetic analysis of specificity mutants [30,57]. All of these mutations showed relatively high transport capacity for, but extremely low-affinity binding of, 'novel' substrates such as hypoxanthine, adenine, or uracil, but conserved nearly wild-type transport characteristics for the physiological substrates uric acid and xanthine. This observation supported the idea that the substrate-binding site of UapA was not affected in the specificity mutants and thus specificity mutations should define other domains acting as 'molecular filters' or 'gating' elements. Given that some of the most frequently obtained specificity mutations concerned residues located towards either the extracellular (e.g., Thr526) or cytoplasmic (e.g., Arg481) side of TMSs, filters or gates should be present on both sides of major substrate-binding sites. Another prediction resulting from this hypothesis is that mutations in the major substrate-binding site and in the filters/gates should be additive. This was indeed the case [30,47,57]. Genetic combinations of specificity mutations (e.g., R481G, F528S, T526 M) with substrate-binding site mutations (F406Y, Q408E), which affect purine binding affinity, led to various UapA versions that transported all purines with both high-affinity and high-capacity characteristics (Figure 1D). Structural evidence for the existence of gating domains in transporters started becoming evident when different transporters were crystallized in outward-facing or cytoplasm-facing conformations in 'occluded' or 'open' states; that is, in states where a gate is closed or open (Box 2) [58–64]. Since then, tens of transporter structures have been crystallized and shown to exist in open or occluded states, helping define the dynamics of alterations of the different conformations (see excellent recent reviews in [65–68]).

The Crystal Structure of UapA Pays Tribute to Genetics and Reveals a Functional Role of Dimerization

Until very recently no eukaryotic solute transporter structure had been determined, in contrast to several available structures for channels. In 2013 the structure of a fungal (*Piriformospora indica*) transporter, PiPT, was published in an inward-facing substrate-occluded state [69]. This structure, however, could not be related to functional approaches and the physiological function of the transporter remains elusive. In 2014–2015, five more eukaryotic transporter structures were published: the *Drosophila* dopamine transporter DAT [70]; the mammalian glucose facilitators GLUT1 (human) [71], GLUT3 (human) [72], and GLUT5 (rat and bovine) [73]; and human anion exchanger 1 (Band3 protein) [74]. In 2016 the structure of UapA became available [47]. The GLUT structures are accompanied by a wealth of genetic data and support the data that members of the MFS superfamily function via an updated rocker-switch mechanism that incorporates the function of gates called the 'clamp-and-switch' alternating-access mechanism [66,68] (Box 2). The *Drosophila* DAT structure conforms to the 5 + 5 LeuT fold [5,67,68], accompanied by extensive functional studies in several prokaryotic homologs.

Box 2. Models of the Mechanism of Transporter Function

All transporters seem to alternate between an outward- and an inward (cytoplasm)-facing conformation (Figure I). Several mechanisms for this conformational alteration have been proposed, such as the gated rocker-switch, the rocking-bundle, the clamp-and-switch, and the elevator mechanisms [64–68]. All mechanistic models consider the existence of various types of 'gating elements' operating alternately at the two sides of transporters. Alternating accessibility can be achieved only through allosteric coupling with extracellular and intracellular gating. Current evidence supports the suggestion that gating closure seems to precede the alteration from outward- to inward-facing conformation.

In the gated rocker-switch, clamp-and-switch, and rocking-bundle models the transporters comprise two domains with the substrate bound at the interface approximately halfway across the membrane. The transitions between outward- and inward-facing states involve movements of the domains relative to each other. Substrate binding is associated with further local rearrangements of gating elements. The gated rocker-switch and clamp-and-switch mechanisms differ from the rocking bundle as the 'moving' domains are structurally symmetric in the former (6 + 6 fold) but asymmetric in rocking bundles, where only one domain seems to move [5 + 5(+2) fold]. Furthermore, there are important differences in their gating events [65–68].

In the elevator alternating-access mechanism (mostly 7 + 7 fold), the substrate-binding site is confined to a single domain that traverses the membrane along a relatively rigid, immobile scaffold or dimerization domain. The scaffold domain provides most of the gating elements and is involved in transporter oligomerization. Transporters using the elevator mechanism are evolutionary very distinct, suggesting that this mechanism has evolved several times independently [65–68]. Another, more complex multimodal mechanism assuming multiple secondary binding sites and encompassing channel-like elements is also proposed [95] but is not described here.

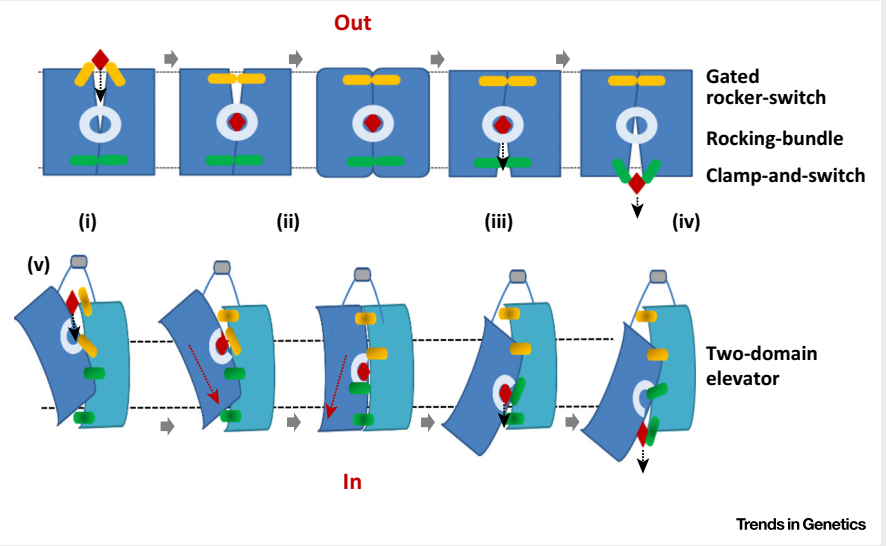

Figure I. Different Mechanistic Models for the Alternation from Outward- to Inward-Facing Conformations in Transporters.
Key: red diamonds, solute/substrate; yellow capsules, outward-gating elements; green capsules, inward-gating elements. I, outward-facing open; II, outward-facing substrate-occluded; III, substrate-bound fully-occluded; IV, inward-facing substrate-occluded; V, inward-facing open.

These transporters seem to function via a 'rocking-bundle alternating-access' or 'gated-pore' mechanism, where substrate binding between two structurally distinct domains catalyzes the coupled movement of outside and inside gates around a centrally located substrate-binding site [67,68]. In this mechanism, gating events seem to precede the rocking movement (Box 2). The Band3 [74] and UapA [47] structures conform with a 7 + 7 fold, topologically related to the LeuT 5 + 5 fold [75]. Band3 was captured as an outward-facing open conformer with no substrate, whereas UapA was inward facing with bound xanthine and partially occluded. Both proteins are structurally related to two crystallized bacterial homologs, the uracil transporter of

E. coli [55] belonging, as UapA, to the NAT family, and the proton-coupled putative fumarate symporter SLC26Dg [76] from the poorly tractable bacterium *Deinococcus geothermalis*. Molecular dynamics using the eukaryotic Band3 and UapA transporters and the extensive genetic analysis of UapA strongly support the idea that these transporters function via an 'elevator' mechanism [47,74] first described for the Na^+/H^+ antiporter NhaA in *E. coli* [77]. In this mechanism, the substrate-binding site is confined to a single core domain that traverses the membrane along a relatively rigid, immobile domain (Box 2), which as described below provides most of the gating elements and acts as a scaffold for oligomerization.

The genetic analysis of UapA has played a major role not only in the crystal structure analysis of UapA but also in understanding how this transporter functions and selects its substrates, an issue that could not have been addressed solely through the crystal structure. UapA expression and purification in sufficient amounts was achieved in *S. cerevisiae* strains lacking vacuolar peptidases [78]. Epifluorescence microscopy revealed that UapA tagged with GFP was predominately localized to the plasma membrane and uptake assays confirmed that it was fully active. Successfully purified wild-type UapA was, however, extremely unstable in crystallization attempts. Screening of several UapA mutants, which were thought to be more stable due to reduced transport activity and increased GFP fluorescence in the plasma membrane, led to the purification of mutant G411 V protein, which was extremely stable, especially in the presence of substrates (xanthine) [47,79]. An N-terminally truncated construct, G411VΔ1–11, proved to be excellent for crystallization. The genetically stabilized mutant UapA version was targeted to the plasma membrane and could bind, but not transport, xanthine [47].

The crystal structure showed that UapA comprises two domains, a compact core domain (TMS1–4, TMS8–11) containing the substrate-binding site and a gate domain (TMS5–7 and TMS12–14) hosting most of the residues affecting specificity [47]. Most importantly, UapA forms a dimer in the crystals, with dimer interactions formed exclusively through the gate domain [47]. All assumptions based on genetic, cellular, or biochemical approaches concerning residues involved in substrate binding and/or transport were fully supported by the UapA structure. In addition, the orientation of xanthine within the UapA binding site and its interactions with specific residues were as those proposed via genetic, biochemical, and *in silico* approaches [56]. What was more interesting, however, was the reevaluation of all specificity mutations/residues in light of the crystal structure. The topology of nearly all residues affecting specificity, except Arg481, could rationalize how they might affect substrate selection and supported the two-domain elevator mechanism of transport. These residues could be classified into three types: those located within or in close proximity to the binding site (Phe155 and Phe406 in TMS3 and TMS10, respectively); those found at the interface of the core domain and gate domain, over (Thr526 and Phe528 in TMS14) or under (Val463 and Ala469 in TMS12) the substrate-binding site; and residues located in flexible hinge regions affecting the relative movement of the core and gate domains (Gln113 in TMS1/2 and Ala441 in TMS11) (Figures 1C and 2A). In other words, specificity could be affected by modifying the substrate-binding site itself (type I), by elements acting as specific filters along the substrate translocation trajectory formed between the gate and core domains (type II), or by mutations in 'hinges' that control the sliding of the core domain hosting the substrate-binding site (the so-called 'elevator'; Box 2) against the gate domain. Given that UapA, but also Band3, forms dimers exclusively via the gate domain, it is the core domain that is more flexible to move, rather than the gate domain. This was also strongly supported by molecular dynamics and calculations of flexibility B factors [47].

Arg481, however, seems to be distinct from the abovementioned residues that affect specificity. Its position is distant from the binding site and the trajectory of substrate translocation.

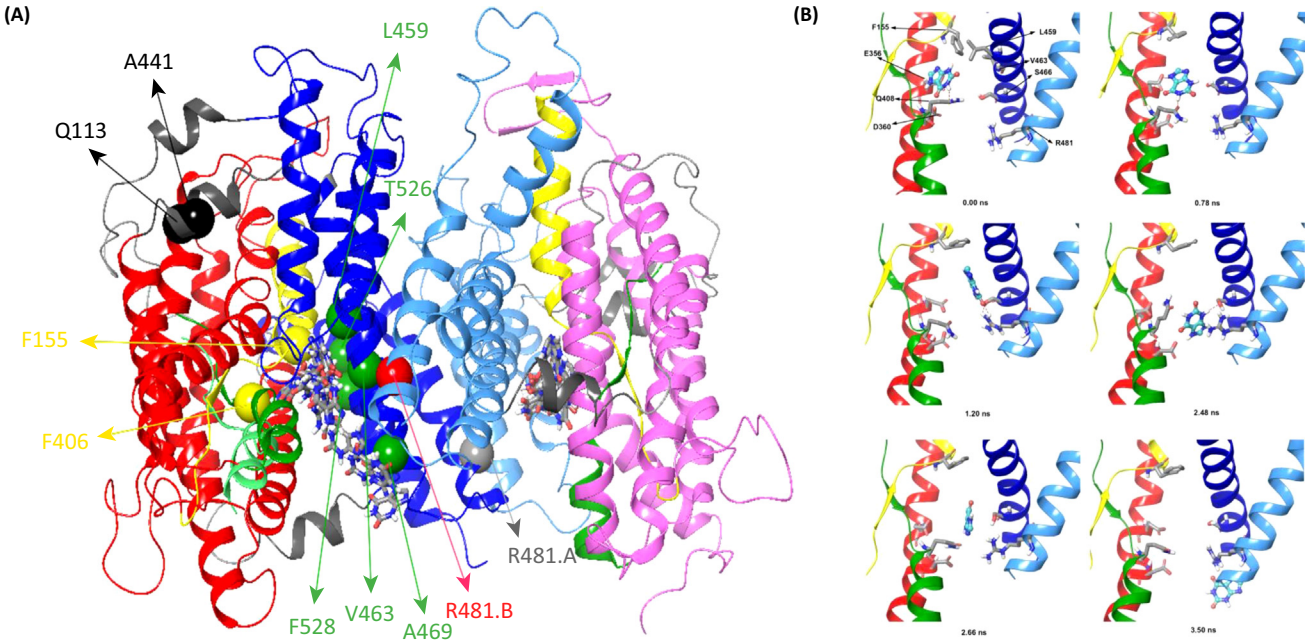

Figure 2. Functional Role of Arg481 in the UapA Dimer. (A) Structure of the inward-facing UapA dimer and molecular dynamics of xanthine translocation from the major substrate-binding site towards the cytoplasm [47]. The core domain (TMS1–4 and TMS8–11) hosting the substrate-binding site is depicted in red (monomer on the left) or purple (monomer on the right) ribbons. In the core domain, the broken half-helices of TMS3 and TMS10 'holding' the substrate are colored yellow and green, respectively. The scaffold or dimerization domain (TMS5–7 and TMS12–14) hosting most of the gating residues is shown in blue (monomer on the left) or turquoise (monomer on the right). Critical specificity residues are depicted by spheres in the left monomer. Yellow (F406 and F155) signifies substrate-binding-site residues affecting specificity (type I gating elements). Green (L459, V463, A469, T526, and F528) signifies specificity residues lying along the substrate translocation path at the interphase of the core and dimerization domains (type II gating elements). Black signifies specificity residues (Q113 and A441) located in flexible hinges affecting the sliding of the core (i.e., the so-called 'elevator') along the dimerization domain (type III gating elements). Stepwise xanthine translocation from the major substrate-binding site to the cytoplasm, as obtained by molecular dynamics, is shown in stick model form [47]. Notice the close proximity of the side chain of Arg481 (shown as a red sphere) from the right monomer (R481.B) to the binding cavity of the left monomer. (B) Selected snapshots of the translocation of xanthine in the UapA inward-facing structure, extracted from molecular dynamics simulations. Xanthine, shown as a cyan-colored stick model, interacts with residues characterized as crucial for transporter activity, forming hydrophobic, π–π, or H-bond interactions (red dashed lines) in the binding cavity. Movement of the substrate is closely associated with reorientation of the side chain of Arg481 from the opposite monomer (R481.B), which protrudes into the binding cavity and mediates the sliding movement of the substrate towards the cytoplasmic side of the transporter. Note also the outward movement of TMS10 (green helix) away from the translocation channel as the substrate moves through. Adapted from [47].

How could Arg481 replacements with small aliphatic amino acids allow the transport of all purines, enlarging UapA specificity? The functional role of Arg481 became evident when the dimeric structure of UapA was considered. In the dimer, the side chain of Arg481 of monomer B extends towards the substrate-binding site of monomer A and vice versa (Figure 2A). Furthermore, molecular dynamics show that Arg481 of monomer B is further approaching the central binding cavity of monomer A, where xanthine remains bound by Asp356 and Gln408. This movement of Arg481 allows a direct interaction via both H bonding and π–π stacking with xanthine, which subsequently is pulled intracellularly due to a characteristic conformational change of the Arg481 side chain and the Arg481–Gln408 interaction. In this translocation trajectory, xanthine interacts with Leu459, Val463, Val469, and Ser466, all residues genetically shown to affect specificity. Thus, dimerization explains the rather mysterious role of Arg481 in UapA function and specificity (Figure 2B) [47].

The functional importance of dimerization was further supported experimentally through the kinetic analysis of strains coexpressing wild-type and mutant versions of UapA [47]. In brief, the strains analyzed expressed the endogenous wild-type UapA, with each one of four

kinetically distinct UapA mutant versions exhibiting undetectable (G411 V, Q408P, N409D) or significantly reduced (Q408E) transport activities. Importantly, Q408E has also moderate-affinity binding for hypoxanthine, which, however, cannot be transported. All mutants when coexpressed with wild-type UapA had a dominant-negative effect for growth on xanthine and reduced xanthine uptake but had little effect on the K_m value for xanthine, supporting the idea that 'heterodimeric' complexes are functionally defective, so that the measured K_m and transport rates reflect the wild-type UapA dimers. Most importantly, in the Q408E/wild-type UapA strain, xanthine transport could be significantly inhibited by hypoxanthine, unlike what is observed in the wild-type control. The chimeric transport characteristics of Q408E/wild-type UapA can be explained only if hypoxanthine binds to the mutant monomer and thus inhibits the transport activity of the opposite wild-type UapA monomer. Functional cooperativity between individual monomers has been suggested for other transporters, but these functional assays have not been supported by structural data in this respect.

Structure–Function Relationships in Other Fungal Transporters: Emergence of Common Themes

Several other *A. nidulans* [34,36,37,80–83] and *S. cerevisiae* [81,84–87] transporters have also been analyzed by mutational analyses and *in silico* modeling, most following the UapA paradigm (Tables 1 and 2). Importantly, these carriers belong to structurally, functionally, and evolutionarily distinct protein families. Models of all of these proteins have been built and analyzed based on crystal structures of their closest structural homologs and related to genetic data. Functionally important residues have been identified and in the modeled structures these are located in putative substrate-binding-site cavities. Docking approaches further supported the possible direct implications of some of these residues in substrate binding. Most interestingly, however, mutations modifying the specificity of some of these transporters are located both within and outside the major substrate-binding site, the latter not significantly affecting affinities for physiological substrates, presumably concerning residues acting as gating elements, as in UapA. For example, in the FurD uracil transporter (Table 1) a rationally designed specificity mutation (M389A) lies in the TMS10 domain, outside the presumed substrate-binding site. Flexible tilting of TMS10 has been proposed to function as an outward gate in the bacterial Mhp1 homolog [88] on which the FurD model was built. Thus, a mutation in an outward-facing gate affects substrate specificity, converting FurD to a carrier capable of transporting uracil, uric acid, xanthine, allantoin, and hypoxanthine with variable binding affinities. Furthermore, combinations of M389A with mutations in the presumed FurD substrate-binding site (N249A, Y250A, and Y250F) show additive effects, leading to modified versions of FurD with still different substrate specificity. Similarly, genetic analysis of PrnB has shown that specificity is determined by residues of the major substrate-binding site (e.g., Lys245 in TMS6) but also by gating elements located peripherally or distant to the binding site (Ser130 in TMS3, Phe252 and Ser253 in TMS6, Trp351 in TMS8, and Thr414 in TMS10) (Table 2) [80,81]. Importantly, yeast amino acid transporter (YAT) homologs of PrnB are specifically conserved in these residues, in accordance with their specificity differences. The genetic results obtained with PrnB are corroborated by similar data obtained from the analysis of structurally modeled YAT homologs such as Put4 and Can1 [81,84,85].

The above observations constitute strong evidence that the function and specificity of members of distinct transporter families are determined by synergistic interactions of genetically distinguishable substrate-binding sites and gating elements. Further evidence for the importance of gating elements in transporter specificity comes from the fact that there are cases where two transporters have practically identical substrate-binding-site residues but transport different substrates. This is particularly evident within the Fur family of fungal NCS1 transporters [37].

Table 2. Fungal Transporters Analyzed Genetically and by Modeling with Respect to Structure–Function Relationships

Transporter	Family[a]	Subfamily[a]	Prokaryotic Homolog[b]	Modeled Structure[c]	Specificity	Substrate Binding[d]	Transport Activity[e]	Gating Elements[f]	Refs
UreA *Aspegillus nidulans*	**SSS** 2.A.21	SSS 2.A.21	**vSGLT** (inward facing, PDB 3DH4) **Mhp1** (outwardfacing, PDB 2JLN)	**14 TMSs** (1 + 5 + 5 + 3)	Urea	W82 (TMS2) N275, D286 (TMS7) Y388 (TMS9)	W82 (TMS2) Y106, A110 (TMS3) T133 (L3) N275, D286 (TMS7) Y388 (TMS9) Y437 (TMS11)	**Y106**, A110 (TMS3) **T133** (L3) **Y437** (TMS11)	[82]
NrtA *A. nidulans*	**MFS** 2.A.1	NNP 2.A.1.8	**GlpT** (inward facing, PDB 1PW4) **FucP** (outward facing, PDB 3O7Q)	**12 TMSs** 6 + 6	Nitrate	**R87** (TMS2) **R368** (TMS8)	N168, G171 (TMS5) N459, G461, G462 (TMS11)	–	[83]
PrnB *A. nidulans*	**APC** 2.A.3	YAT 2.A.3.10	**AdiC** (occluded) PDB 3L1L	**12 TMSs** 5 + 5(+2)	L-proline	**G56**, **T57** (TMS1) **E138** (TMS3) K245, **F248** (TMS6)	G58 (TMS1) F250, **E255** (TMS6)	**S130** (TMS3), **F252**, **S253** (TMS6) W351 (TMS8) **T414** (TMS10)	[80,81,85]
Can1 *S. cerevisiae*	**APC** 2.A.3	YAT 2.A.3.10	**AdiC** (occluded) PDB 3L1L	**12 TMSs** 5 + 5(+2)	L-arginine	**S176**, **T180** (TMS3)	**E184** (TMS3)	**Y173** (TMS3) **W451**, **T456**, **F461** (TMS10)	[84,85]
Put4 *S. cerevisiae*	**APC** 2.A.3	YAT 2.A.3.10	**AdiC** (occluded) PDB 3L1L	**12 TMSs** 5 + 5(+2)	L-proline, alanine, glycine, γ-aminobutyric acid	G125, **T126** (TMS1) **E211** (TMS3) **K318**, **F321** (TMS6)	–	C203 (TMS3) L325, G326 (TMS6) F424 (TMS8) S487 (TMS10)	[81,85]
Jen1 *S. cerevisiae*	**MFS** 2.A.1	SHS 2.A.1.12	**GlpT** (inward facing) PDB 1PW4 f	**12 TMSs** 6 + 6	Pyruvate, lactate, acetate	**Q386**, T391 (TMS7)	**R188** (TMS2) **N379**, **H383**, **D387** (TMS7) **F270** (TMS5) **N501** (TMS11)	R188 (TMS2) **F270**, S271 (TMS5) **Q498**, N501 (TMS11) H383, D387 (TMS7)	[86,87]

[a]According to http://www.tcdb.org/.
[b]Closest prokaryotic homolog with known crystal structure used to model relevant fungal transporters. PDB structural codes are given (http://www.rcsb.org/pdb/home/home.do).
[c]5 + 5(+2) and 7 + 7(2 + 5 + 2 + 5) are inverted intertwined repeat-fold transporters of the APC superfamily. 6 + 6 transporters are members of the MFS superfamily, which is structurally, functionally, and evolutionarily distinct from APC [75]. The two six-helix domains of MFS are connected by an extended cytoplasmic loop.
[d]Based on mutational analysis and substrate docking approaches using modeled structures. Residues altering the K_m and in some cases the specificity of the transporter. Residues in bold interact strongly with substrates via H bonding through their side chains.
[e]Based on mutational analysis and intramolecular interactions in modeled structures. Residues altering the V_m of the transporter without affecting its turnover. Residues in bold are absolutely necessary for transport activity.
[f]Based on mutational analysis, modeled structures, and molecular dynamics. Residues altering significantly the specificity of the transporter or the transport capacity, despite being relatively distant from the major substrate-binding site. Residues in bold have the most prominent effect on specificity. Underlined TMSs highlight gating elements distal from the presumed binding site. All transporters shown seem to function as H^+ symporters.
Abbreviations: MFS, major facilitator superfamily; APC, the amino acid-polyamine-organocation family; SSS, the solute: sodium symporter family; NNP, the nitrate/nitrite porter (NNP) family; YAT, the yeast amino acid transporter family; SHS, the sialate: H^+ symporter family.

Concluding Remarks

Fungal genetics, as exemplified by the analysis of UapA, has predicted the existence of gating elements that determine the substrate specificity of a transporter. These gates or filters work in concert with a major substrate-binding site, also identified genetically, to catalyze the selective translocation of a specific solute and exclude other, chemically similar compounds. Unlike substrate-binding-site residues, which are rather easily identifiable by the primary amino acid sequence similarities of transporters and targeted reverse genetics, gating elements and their role in specificity could have not been predicted *a priori* as these elements are not well conserved [66–68]. It is classical genetics that led to the novel concept of gating in transporters. It is also through genetics and *in vivo* cell biology that fungal transporter **homodimerization** was shown to be functionally critical [38], before this was further confirmed by crystallography and additional functional assays. Last but not least, a genetically stabilized fungal transporter (UapA) led to the first crystal structure of one of the most studied, at the functional level, eukaryotic transporter. The crystal structure of UapA signifies the beginning of a new era where genetic, functional, and structural approaches, uniquely combined for studying model fungal transporters, can be used to fully dissect the transport mechanism of eukaryotic transporters. Several outstanding questions are thus awaiting an answer (see Outstanding Questions).

Outstanding Questions

How do gating elements function – are they substrate selective? If gates also function as selective filters, how is this achieved? Do gates function as transient substrate-binding sites? Is the closing of the outward-facing gate the key conformational step that promotes transition to the inward-facing conformation?

Are gates and filters universally found in all transporters? Are the gates substrate selective in other transporters?

Is dimerization and oligomerization the rule for solute transporters? What is the role of oligomerization in function and localization? Are there allosteric effects on transport activities related to specific substrate analogs or drugs?

How is H^+/Na^+ symport coupled to substrate transport? What is the role of gates in H^+/Na^+ transport?

Can we express and study eukaryotic transporters from higher organisms in appropriate genetic backgrounds in model fungal systems?

How do transporter specificities evolve? Given that present-day transporters within the same family might have dramatically different specificities, what might have been the function of evolutionarily 'intermediate' steps? What is the function and specificity of thousands of orphan transporters? Are all transporter-like homologs of unknown function real transporters or could some of them be pseudo-transporters?

Can we design drugs specific for specific transporters?

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