Peeping at TOMs—Diverse Entry Gates to Mitochondria Provide Insights into the Evolution of Eukaryotes

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Abstract

Mitochondria are essential for eukaryotic life and more than 95% of their proteins are imported as precursors from the cytosol. The targeting signals for this posttranslational import are conserved in all eukaryotes. However, this conservation does not hold true for the protein translocase of the mitochondrial outer membrane that serves as entry gate for essentially all precursor proteins. Only two of its subunits, Tom40 and Tom22, are conserved and thus likely were present in the last eukaryotic common ancestor. Tom7 is found in representatives of all supergroups except the Excavates. This suggests that it was added to the core of the translocase after the Excavates segregated from all other eukaryotes. A comparative analysis of the biochemically and functionally characterized outer membrane translocases of yeast, plants, and trypanosomes, which represent three eukaryotic supergroups, shows that the receptors that recognize the conserved import signals differ strongly between the different systems. They present a remarkable example of convergent evolution at the molecular level. The structural diversity of the functionally conserved import receptors therefore provides insight into the early evolutionary history of mitochondria.

Key words: **mitochondria, eukaryotes, protein import, TOM complex, trypanosome.**

Protein Import Distinguishes Mitochondria from Its Endosymbiotic Ancestor

The origin of eukaryotic cells arguably is the most important transition in evolution besides the origin of life itself. It is now widely accepted that the development of the nucleus, the name-giving feature of eukaryotes, required a highly efficient energy metabolism that could only be provided by mitochondria (Lane 2014). The acquirement of a bacterial endosymbiont by the archeal ancestor of eukaryotes (Williams et al. 2013; Koonin and Yutin 2014; Spang et al. 2015) that subsequently was converted into the mitochondrion was therefore likely the event that triggered the evolution of eukaryotes. The process of organellogenesis was accompanied by a massive reduction of the endosymbiont's genome. While part of it was lost, some genes were transferred to the host's nucleus, a process designated endosymbiotic gene transfer (EGT) (Thorsness and Weber 1996; Adams and Palmer 2003; Timmis et al. 2004). Today mitochondrial genomes harbor very few genes encoding between 3 and 65 different proteins (Gray et al. 1999; Allen 2003), which stands in sharp contrast to the complexity of the mitochondrial proteomes which even in unicellular organisms amounts to more than 1,000 proteins (Meisinger et al. 2008; Niemann et al. 2013; Huang et al. 2014). This indicates that essentially all mitochondrial proteins, many of which are encoded by genes that had been transferred to the nucleus by EGT, are imported from the cytosol.

Gaining the capability to import proteins is therefore the defining event that marks the transition of the endosymbiont to a genetically integrated organelle that largely is under the control of the nucleus (Dolezal et al. 2006; Lithgow and Schneider 2010; Hewitt et al. 2011; Gray 2012). The question of how mitochondrial protein import evolved is therefore tightly linked to the more general question of the origin of the eukaryotic cell.

Protein Import Has Mainly Been Studied in Yeast

Saccharomyces cerevisiae has been a powerful model to investigate mitochondrial protein import resulting in a wealth of data on the machineries and the mechanism of the process (Chacinska et al. 2009; Schmidt et al. 2010; Schulz et al. 2015). Bioinformatic and experimental evidence shows that protein import and the factors mediating it are highly conserved between yeast and mammals (Dolezal et al. 2006). As these two systems are morphologically very different, it is often assumed that the observed conservation may extend to all eukaryotes. However, this is a misconception: According to the latest molecular phylogenetic tree eukaryotes are divided into a small number of supergroups that diverged very early in evolution (Adl et al. 2005; Burki 2014). Moreover, metazoans including mammals and yeast belong to the same supergroup of the Opisthokonts, indicating that on the large scale they are closely related (fig. 1).

Thus, except for plants which define the supergroup of the Archeaplastida and whose protein import system has been investigated for many years (Murcha et al. 2014), there are few experimental studies on mitochondrial protein import in non-opisthokont organisms. However, since very recently

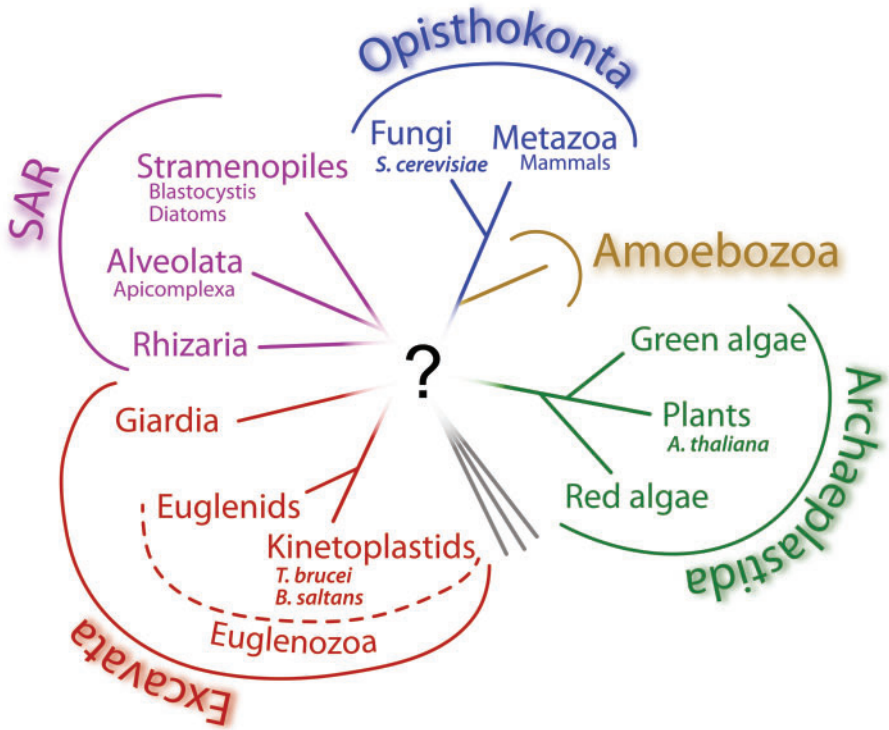

Fig. 1. Eukaryotic phylogeny. Unrooted phylogenetic tree of eukaryotes based on genetic and morphological evidences resolves the five supergroups Opisthokonta, Amoebozoa, Excavata, Archaeplastida, and SAR (Stramenopiles, Alveolates, and Rhizaria), that diverged very early during eukaryotic evolution. A number of organisms could not yet be confidently associated with any of the supergroups. Within the different supergroups the clades are indicated that are discussed in this review regardless of their taxonomic rank. Essentially all popular model organisms (human, mouse, fungi, worms, flies, yeast, etc.) belong to the Opisthokonts. Branch lengths are arbitrary and the branching pattern does not necessarily represent the inferred relationships between the lineages.

there is a new kid on the block. Studies in the parasitic protozoa *Trypanosoma brucei*, a member of the supergroup of the Excavates, identified and characterized the composition and function of the translocase that mediates protein transport across the mitochondrial outer membrane (OM) (Mani et al. 2015).

Thus, for the first time we have sufficient data of the mitochondrial protein import machinery of representatives of three different eukaryotic supergroups that allow us to perform a comparative analysis that is not simply based on sequence similarities but on structural and functional features of import components. We deliberately excluded eukaryotes with mitochondria-related organelles that lack an own genome from our analysis as their protein import systems have been subject to reductive evolution (Heinz and Lithgow 2013).

Protein import is mediated by four major heteroligomeric protein complexes in the OM and the inner membrane (IM) (fig. 2). In this review, we focus on the translocase of the OM (TOM) the entry gate for essentially all mitochondrial proteins. It is localized at the interface of the organelle and the cytosol and thus the first machinery with which imported proteins need to engage.

Mitochondrial Import Signals Are Conserved

The mitochondrial proteome of which more than 95% is imported from the cytosol is not only of comparable size in yeast, plants, and trypanosomes but also contains substrates for all the different types of import pathways (Meisinger et al. 2008; Niemann et al. 2013; Huang et al. 2014) (table 1).

The largest class which includes approximately 60% of all mitochondrial proteins is targeted to mitochondria by N-terminal presequences that are rich in basic and hydroxylated amino acids that have the propensity to form amphiphilic α-helices. Presequences are found on most matrix and many IM protein precursors. They mediate import across the TOM and TIM23 (translocase of the mitochondrial inner membrane 23) complexes (fig. 2) (Habib et al. 2007; Chacinska et al. 2009). After import they are generally processed by the heterodimeric mitochondrial processing peptidase (MPP) and in some cases further trimmed by two other proteases, Icp55 and Oct1. Finally, the processed presequences are degraded by Cym1 and Prd1 (Desy et al. 2012; Mossmann et al. 2012; Teixeira and Glaser 2013).

All these proteases as well as the features of the presequences themselves are conserved, although their specific substrates may vary in the different systems (Carrie et al. 2015). The only discernable difference in the presequences is that the plant ones are on average longer and contain more serine residues than the ones in yeast and trypanosomes. This might be due to the fact that in plant mitochondrial targeting signals must be differentiated from the plastid ones (Murcha et al. 2014).

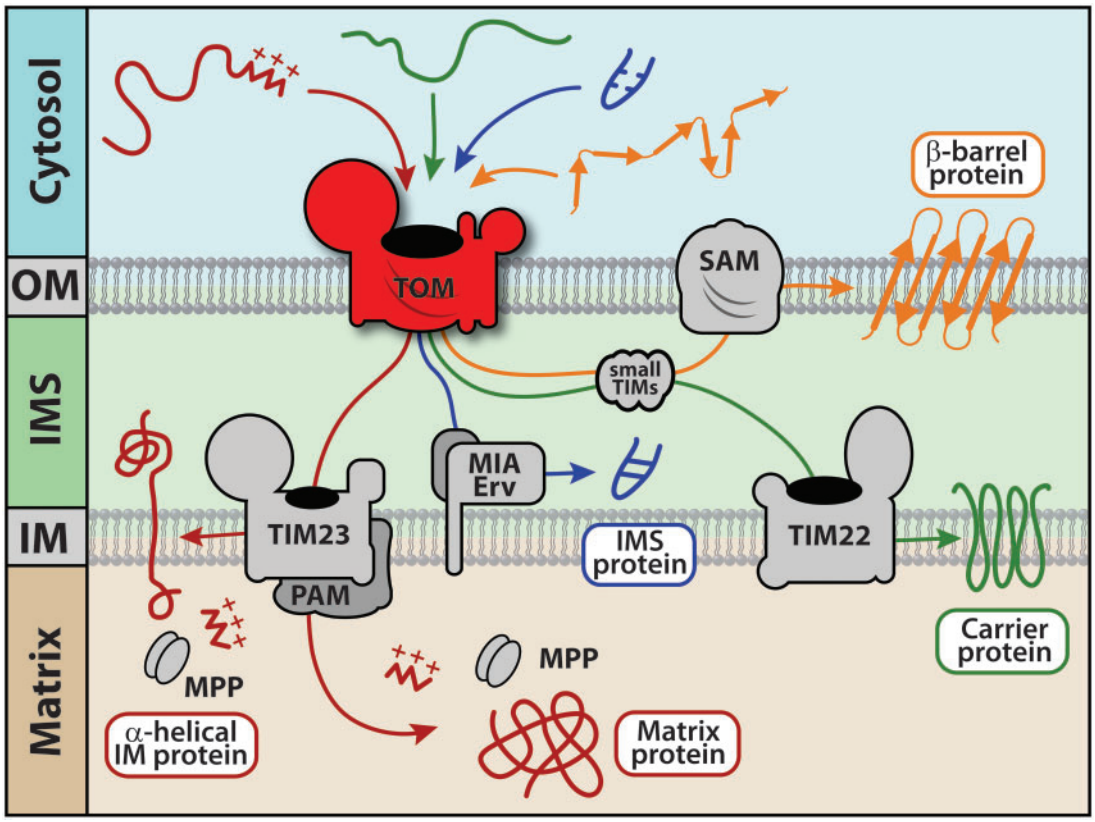

Fig. 2. Mitochondrial protein import pathways in yeast. Essentially all mitochondrial proteins are imported across the heterooligomeric TOM. After OM translocation the import pathways diverge depending on the class of proteins. Presequence-containing proteins are handed over to the TIM23 by which they are either laterally released into to IM, in the case of α-helically anchored IM proteins, or pulled into the matrix by the presequence-associated motor (PAM) module for soluble proteins. In both cases, the presequence gets processed by the heterodimeric MPP. The hydrophobic mitochondrial carrier proteins associate with the small TIM chaperones in the IMS and subsequently are inserted into the IM by TIM22. Small IMS-localized proteins with cysteine-rich signals are retained in the IMS by the formation of disulfide bonds catalyzed by the MIA pathway. The hydrophobic β-barrel proteins interact with the small TIM chaperones in the IMS and are then inserted into the OM by the SAM of the OM.

A few IM proteins facing the intermembrane space (IMS) have in addition to a presequence also a sorting signal that is removed by the IM protease (IMP) or other proteases (Chacinska et al. 2009). IMP is highly conserved, it is found in all eukaryotes and shows homology to the bacterial leader peptidase (Schneider et al. 1991; Teixeira and Glaser 2013).

Presequences are not only highly similar between different species but also functionally conserved. They correctly localize proteins heterologously expressed in different supergroups, both in vivo and in vitro (table 2) although exceptions exist (van Wilpe et al. 1999).

Carrier proteins (MCP) define a conserved mitochondrial protein family that is localized in the IM and whose members have six transmembrane helices. Except for a small subset in plants, MCPs lack presequences and instead have internal as yet poorly defined targeting sequences. MCPs cross the OM using the TOM complex before they engage with the TIM22 complex that inserts them into the IM. Yeast, plants, and trypanosomes have a comparable number of carrier proteins (Palmieri et al. 1996; Colasante et al. 2009; Haferkamp and Schmitz-Esser 2012), all of which must be imported (table 1). Again in vivo and in vitro experiments suggest that carrier proteins are correctly localized when heterologously expressed (table 2).

β-barrel proteins are initially imported into the IMS using the TOM complex and subsequently inserted into the OM by the sorting and assembly machinery (SAM) (Hohr et al. 2015). β-barrel proteins contain a loosely defined signal after the last β-strand that in yeast is recognized by Sam35 of the SAM (Kutik et al. 2007). What features of β-barrel proteins are recognized by TOM is unclear; however, the proteins are generally correctly localized when expressed in heterologous systems. Interestingly, even some bacterial and chloroplast β-barrel proteins can be targeted to mitochondria when expressed in non-plant eukaryotes (table 2).

Finally, there are a handful of IMS-localized small TIM chaperones which have a cysteine-containing internal targeting signal and which are imported by the mitochondrial intermembrane space assembly (MIA) pathway (Herrmann and Riemer 2012). Both the small TIMs as well as their targeting signals appear to be conserved in essentially all eukaryotes.

In summary, these results strongly suggest that the mitochondrial targeting signals and the machineries that process them were already established in the last common ancestor of

Table 1. Imported Proteins and Their (predicted) Targeting Signals.

	Species (supergroup)			
	Saccharomyces cerevisiae (Opisthokonta)	*Arabidopsis thaliana* (Archaeplastida)	*Trypanosoma brucei* (Excavata)	*Giardia*[a] (Excavata)
No. of mitochondrial proteins	~1,000	~1,000	~1,000	50–100
No. of presequence-containing proteins	~600[b]	~690[b]	~620[c]	Some have presequences, many do not
Average length of presequences (aa)	25–30	~50	25–30 (some are much shorter)[d]	ND
Features of presequences	Amphiphilic helix	Amphiphilic helix (serine rich)	Amphiphilic helix	ND
Presequence processing peptidases	α-MPP/β-MPP	α-MPP/β-MPP[e]	α-MPP/β-MPP	β-MPP
	Icp55	Icp55	Icp55	—
	Oct1	Oct1	Oct1	—
	Imp1/Imp2	Imp1/imp2	Imp1/Imp2	—
	Cym1	Cym1	Cym1	—
	Prd1	Prd1	Prd1	—
No. of carrier proteins	35	58	24	0
Import signals of carrier proteins	Internal sequences	Internal sequences, some have presequences	Internal sequences	—
No. of β-barrel proteins	5	7	5	1
Import signals of β-barrel proteins[f]	β-signal	β-signal	β-signal	ND
No. of small TIM chaperones	4	5	5	ND
Import signals of small TIM chaperones	Internal Cys-containing peptide	Internal Cys-containing peptide	Internal Cys-containing peptide	ND

NOTE.—ND, no data.

[a]*Giardia* is included in this analysis as an example for an organism with mitosomes which underwent extensive reductive evolution. The data listed in this column are from Dolezal et al. (2005), Smíd et al. (2008), and Jedelsky et al. (2011).

[b]Experimentally determined (Vögtle et al. 2009; Lee et al. 2013; Huang et al. 2014).

[c]Bioinformatic prediction.

[d]See Häusler et al. (1997).

[e]The MPP subunits of most plants are identical to the core1 and core 2 subunits of complex III of the respiratory chain (Mossmann et al. 2012).

[f]The β-signal in yeast is recognized by SAM subunit Sam35 (Kutik et al. 2007).

all eukaryotes. This is supported by the observation that a sizable fraction of extant bacterial proteins contains N-terminal extensions predisposed for mitochondrial targeting (Lucattini et al. 2004).

Although, for some substrates, for example, cytochrome c (Babbitt et al. 2015) and c1 (Priest and Hajduk 2003), organism-specific variations in the import pathway exist, the function of the TOM, mediating import across the OM of approximately 1,000 different proteins which contain the same conserved targeting signals, is the same in yeast, plants, and trypanosomes. The situation in plants however is complicated by the fact that they also have plastids (Perry et al. 2008), whose proteins must be excluded from mitochondria. Moreover, there is a large fraction of plant proteins that are dually localized to both organelles (Carrie and Whelan 2013). It has been shown in these cases that in vitro and in vivo import systems do not always faithfully mirror the physiological situation (Hurt et al. 1986; Lister et al. 2001; Fuss et al. 2013).

Thus, as the translocases in yeast, plant, and trypanosomatids were largely shaped by the same functional constraints one might expect their composition and the structures of their subunits to be very similar. Surprisingly, this is not the case and a comparative analysis between the TOM complexes of the three species reveals striking differences (fig. 3).

TOM Complex Architecture

TOM consists of 6–7 subunits which depending on the affinities to each other can be divided into core and peripheral components (Perry et al. 2008). The core of TOM includes a β-barrel protein and 3–4 tightly associated subunits, some of which are very small. The remaining proteins are more loosely associated with the complex. Although in yeast and trypanosomes the TOM subunits are coded for by single copy genes, we often find multiple genes encoding highly similar isoforms of TOM subunits in plants (Lister et al. 2004) (table 3). Except for one β-barrel membrane protein, all subunits contain a single membrane-spanning α-helix. The molecular weight of the whole complex ranges from 220 kDa in plants to 1,000 kDa in *T. brucei*. It is known that all components are present in more than one copy but the exact subunit stoichiometry has not yet been determined for any system. In the next few paragraphs, we discuss the different TOM subunits in order of their degree of conservation.

Table 2. Mitochondrial Proteins Are Correctly Localized in Heterologous Systems.

Species[a]	Import Substrate[b]	Species[c]	Supergroup[d]	Localization[e]	Import Pathway[f]	Experimental Evidence	Reference
Saccharomyces cerevisiae	Sam50	*Leishmania tarentolae*	E	OM	β-barrel	Vitro	Eckers et al. (2012)
	Oep24	*Pisum sativum*	A-Plas	OM	β-barrel	Vitro	Ulrich et al. (2012)
	Oep37	*Pisum sativum*	A-Plas	OM	β-barrel	Vivo/vitro	Ulrich et al. (2012)
	YadA	*Yersinia enterocolitica*	Bact	OM	β-barrel	Vivo/vitro	Ulrich et al. (2014)
	Erv1	*Leishmania tarentolae*	E	IMS	?	Vitro	Eckers et al. (2012)
	Tim1	*Leishmania tarentolae*	E	IMS	?	Vitro	Eckers et al. (2012)
	Tim17	*Trypanosoma brucei*	E	IM	Carrier	Vivo/vitro	Eckers et al. (2012); Weems et al. (2015)
	F1β	*Nicotiana plumbaginifolia*	A	IM	Preseq	Vitro	Chaumont et al. (1990)
	LipDH(1-14)[g]	*Trypanosoma brucei*	E	Matrix	Preseq	Vivo/vitro	Hauser et al. (1996); Häusler et al. (1997)
	Hsp60	*Leishmania tarentolae*	E	Matrix	Preseq	Vitro	Eckers et al. (2012)
	MnSOD	*Nicotiana plumbaginifolia*	A	Matrix	Preseq	Vivo/comp	Bowler et al. (1989)
	AtPreP	*Arabidopsis thaliana*	A	Matrix	Preseq	Vivo/comp	Alikhani et al. (2011)
Trypanosoma brucei	Tom40	*Saccharomyces cerevisiae*	O	OM	β-barrel	Vivo	Chaudhuri and Nargang (2003)
	VDAC	*Neurospora crassa*	O	OM	β-barrel	Vitro	Eckers et al. (2012)
	Tim9	*Saccharomyces cerevisiae*	O	IMS	MIA	Vitro	Eckers et al. (2012)
	ACC	*Saccharomyces cerevisiae*	O	IM	Carrier	Vitro	Eckers et al. (2012)
	Tim17	*Saccharomyces cerevisiae*	O	IM	Carrier	Vivo/comp	Weems et al. (2015)
	Tim23	*Saccharomyces cerevisiae*	O	IM	Carrier	Vivo	Weems et al. (2015)
	Cox4	*Saccharomyces cerevisiae*	O	IM	Preseq	Vivo	Häusler et al. (1997)
	Letm1	*Homo sapiens*	O	IM	Preseq	Vivo/comp	Hashimi et al. (2013)
	Frataxin	*Arabidopsis thaliana*	A	IM	Preseq	Vivo/comp	Long, Vavrova, et al. (2008)
	Frataxin	*Homo sapiens*	O	IM	Preseq	Vivo/comp	Long, Jirku, et al. (2008); Long, Vavrova, et al. (2008)
	Frataxin	*Thalassiosira pseudonana*	S	IM	Preseq	Vivo/comp	Long, Vavrova, et al. (2008)
	Ferredoxin	*Homo sapiens*	O	IM	Preseq	Vivo/comp	Changmai et al. (2013)
	Su9(1-69)[g]	*Saccharomyces cerevisiae*	O	IM	Preseq	Vitro	Eckers et al. (2012)
	Dld1(1-72)[g]	*Saccharomyces cerevisiae*	O	IM	Preseq	Vitro	Eckers et al. (2012)
	Isa1	*Homo sapiens*	O	Matrix	Preseq	Vivo/comp	Long et al. (2011)
	Isa2	*Homo sapiens*	O	Matrix	Preseq	Vivo/comp	Long et al. (2011)
	Adh3	*Saccharomyces cerevisiae*	O	Matrix	Preseq	Vitro	Hauser et al. (1996)
Plants	CytP450	*Homo sapiens*	O	IM	Preseq	Vitro	Luzikov et al. (1994)
	F1β	*Saccharomyces cerevisiae*	O	IM	Preseq	Vitro	Chaumont et al. (1990)
	F1β	*Schizosaccharomyces pombe*	O	IM	Preseq	Vitro	Schmitz and Lonsdale (1989); Chaumont et al. (1990)
	MSW	*Saccharomyces cerevisiae*	O	Matrix	Preseq	Vivo	Schmitz and Lonsdale (1989)

NOTE.—The table presents examples for mitochondrial targeting of heterologous substrates. It is not comprehensive. Vivo, protein localizes to mitochondria in vivo; vitro, protein can be imported into isolated mitochondria; comp, imported protein complements function.

[a]System in which the heterologous substrates were imported.

[b]Import substrates tested in the heterologous systems.

[c]Origin of heterologous substrates.

[d]Supergroups to which the system belongs: Opisthokonts (O), Excavata (E), Archaeplastida (A), SAR (S). For *S. cerevisiae*, two heterologous plastid (plas) and one bacterial (bact) β-barrel protein are listed as well.

[e]Localization of substrate in the system of origin.

[f]Import pathway as indicated in figure 2 in system of origin. Preseq, presequence.

[g]Dihydrofolatereductase fusions were tested.

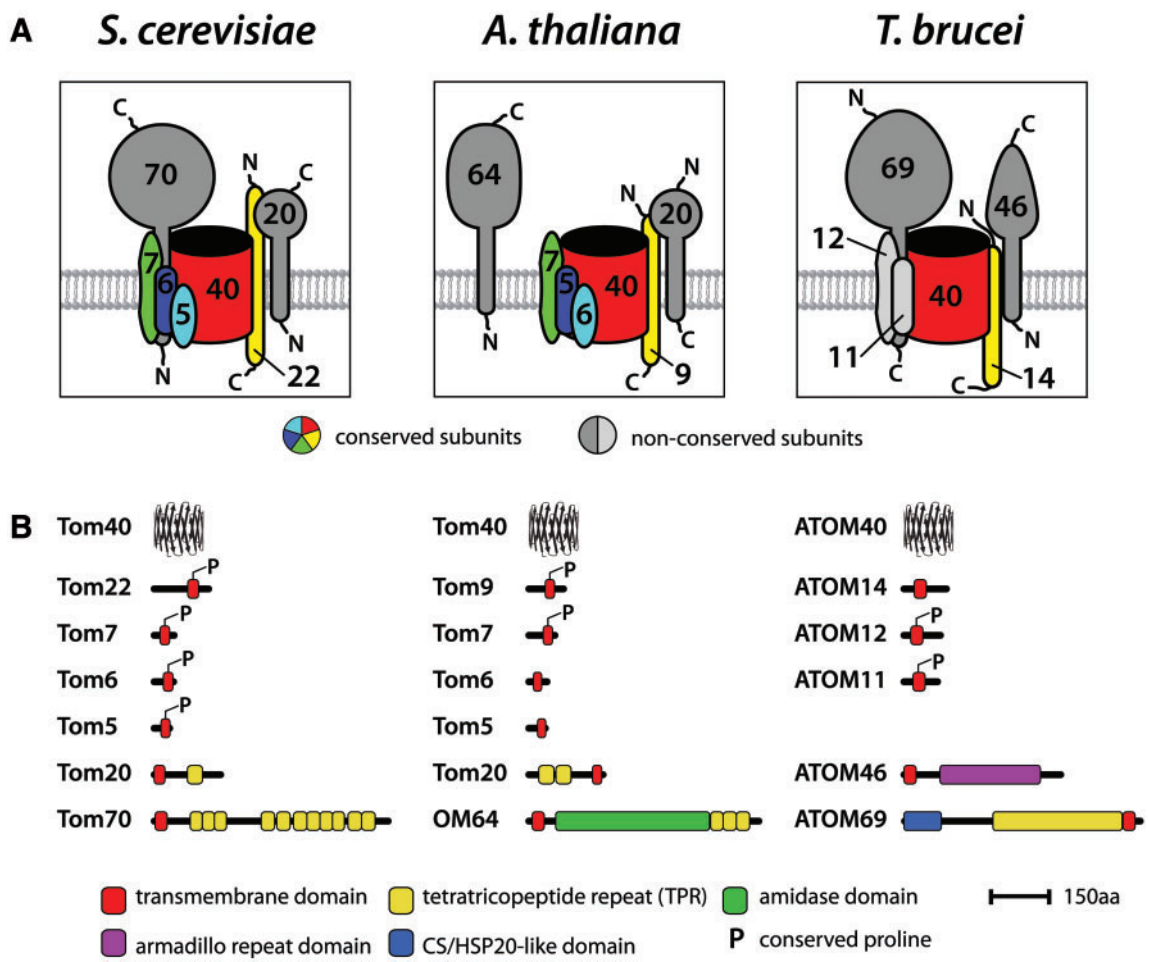

FIG. 3. TOM and its subunits in yeast, plants, and trypanosomes. (*A*) Model of TOM in the indicated systems. Conserved subunits are shown in the same color. Organism-specific components are depicted in gray. The topology of the Tom22-orthologs (Tom22/Tom9/ATOM14) and the two peripheral receptor subunits (dark gray) is indicated. Plant OM64 is not stably associated with TOM but likely has a receptor function. (*B*) To scale representation of the domain structure of the TOM subunits in the indicated systems. Membrane anchors, functional domains, and conserved prolines in the transmembrane domains are indicated.

The β-Barrel Pore

The protein-conducting pore of TOM is formed by a β-barrel protein of approximately 40 kDa that has been identified in the isolated TOM complexes of fungi, plants, and trypanosomes (Moczko et al. 1992; Sollner et al. 1992; Jansch et al. 1998; Werhahn et al. 2001; Mani et al. 2015). An ortholog of this protein, termed Tom40, can be found in all eukaryotes (table 4 and supplementary table S1, Supplementary Material online) and was shown to be essential for life in all species where it has been tested (Maćasev et al. 2004; Dolezal et al. 2006; Perry et al. 2008; Pusnik et al. 2009; Hewitt et al. 2011).

Using bioinformatics it was initially not possible to identify a Tom40 ortholog in trypanosomes (Schneider et al. 2008; Pusnik et al. 2009) and the β-barrel protein forming the OM import pore was finally discovered by a biochemical approach and termed ATOM40 for archaic TOM of 40 kDa. BLAST (Basic Local Alignment Search Tool) analyses revealed a limited sequence similarity of ATOM40 to a subgroup of the bacterial Omp85-like proteins (Pusnik et al. 2011). HHPred analyses (Soding et al. 2005) on the other hand suggested that ATOM40 might be a highly diverged voltage-dependent anion channel (VDAC)-like protein (Zarsky et al. 2012). The main members of this protein family are the protein import pore Tom40 and the metabolite transporter VDAC (Pusnik et al. 2009), which are found in all eukaryotes. However, although ATOM40 could be grouped into the VDAC-like protein family, it is too diverged to be categorized into a specific subfamily in (Schnarwiler et al. 2014).

Recombinant yeast Tom40 and trypanosomal ATOM40 have been analyzed by electrophysiology using the planar lipid bilayer technique that allows single channel measurements (Hill et al. 1998; Künkele et al. 1998; Harsman et al. 2012). Consistent with their function as protein-conducting pores both proteins form a wide hydrophilic channel that shows selectivity for cations and that can be blocked by addition of a synthetic presequence. However, a more in depth analysis performed in parallel for yeast Tom40 and ATOM40 revealed that recombinant Tom40 inserted as a monomer whose gating behavior is dominated by fast flickering, whereas recombinant ATOM40 was active as a trimer that showed low frequency gating only (Harsman et al. 2012). Thus, in this respect ATOM40 behaves more similar to chloroplast and

Table 3. Composition of TOM and Function of Its Subunits in Yeast, Plants, and Trypanosomes.

Saccharomyces cerevisiae TOM (450 kDa)[a]			*Arabidopsis thaliana* TOM (230 kDa)[b]			*Trypanosoma brucei* ATOM (450–1,000 kDa)[c]		
Subunit	Function	Essential	Subunit	Function	Essential	Subunit	Function	Essential
Core complex								
Tom40	Translocation channel	Yes	Tom40-1 Tom40-2	Translocation channel	Yes	ATOM40	Translocation channel	Yes
Tom22	Secondary receptor TOM organizer	No	Tom9-1 Tom9-2	ND	ND ND	ATOM14	Stabilizes ATOM complex	Yes
Small subunits								
Tom7	Destabilizes TOM complex	No	Tom7-1 Tom7-2	ND	ND	ATOM12	Destabilizes the association of core with ATOM46/69	Yes
Tom6	Stabilizes the TOM complex	No	Tom6	ND	ND	ATOM11	Promotes assembly of core with ATOM46/69	Yes
Tom5	Promotes TOM complex assembly Substrate transfer for Tom22 to Tom40	No	Tom5	ND	ND			
Peripheral subunits								
Tom20	Receptor for precursors with a presequence	No	Tom20-1 Tom20-2 Tom20-3	Receptor for precursors with a presequence	No No No	ATOM69	Receptor for all precursor proteins	Yes
Tom70	Receptor for hydrophobic precursor proteins	No	OM64	Putative receptor for a subset of proteins	No	ATOM46	Receptor for all precursors (preference for hydrophobic proteins)	No
Tom71	Low abundance Tom70 ortholog	No					Mediates interaction of core complex with ATOM69	

NOTE.—ND, no data.
[a]Data from Chacinska et al. (2009).
[b]Data from Murcha et al. (2014).
[c]Data from Mani et al. (2015).

bacterial versions of Omp85-like protein import and export channels, rather than to Tom40 of yeast.

The β-barrel nature of Tom40 and ATOM40 indicate their bacterial origin. The fact that both can be grouped into the VDAC-like protein family points to a single evolutionary origin of the protein. However, with which—if any—specific β-barrel protein in extant bacteria it shares common ancestry is unclear at present.

Tom22-Like Proteins

All TOM complexes isolated so far have a subunit with a cytosolically exposed N-terminus, whose molecular weights range from 10 kDa in plants to 18 kDa in yeast. They share homology within and around their single transmembrane domains, suggesting that they derive from a single common ancestor (Maćasev et al. 2004) (supplementary fig. S1, Supplementary Material online).

The yeast subunit, termed Tom22, functions as a secondary receptor. It is tightly bound to Tom40 and interacts with the primary receptors Tom20 and Tom70 (see below). In addition to its transmembrane region Tom22 consists of an N-terminal cytosolic and a C-terminal IMS domain, which both contain clusters of acidic amino acids (Kiebler et al. 1993; Mayer et al. 1995). Most importantly, independent of the receptor function of Tom22, its transmembrane domain is required for TOM assembly into a 450 kDa complex (Wilpe et al. 1999). However, despite its dual function yeast lacking Tom22 can grow, albeit very slowly.

The plant ortholog of Tom22 was first identified in isolated TOM from *Arabidopsis thaliana* and *S. tuberosum* (Jansch et al. 1998; Werhahn et al. 2001). Surprisingly the protein, termed Tom9, has a much shorter cytosolic domain, which is basic. The IMS domain of Tom9, on the other hand, retained an excess of acidic residues (Maćasev et al. 2004). Studies investigating the specific function of plant Tom9 have not been published and it is not known whether the protein is essential.

The purified ATOM complex of *T. brucei* contains a 14 kDa protein whose transmembrane domain including flanking regions shows similarity to Tom22 and Tom9 when analyzed by HHPred (Soding et al. 2005) (supplementary fig. S1, Supplementary Material online), although it lacks the conserved proline residue in the transmembrane domain (fig. 3). The protein was termed ATOM14 and is highly conserved in all trypanosomatids. An alignment with yeast Tom22 and plant Tom9 suggests that the cytosolic domain of ATOM14 essentially lacks acidic amino acids and is even shorter than in

Table 4. TOM Subunits in Representatives of All Eukaryotic Supergroups.

			Tom40	Tom22		Tom5	Tom6	Tom7	Tom70	Tom20	Tom20	OM64	ATOM11	ATOM12	ATOM69	ATOM46
				Yeast -Type	Plant -Type					Yeast -Type	Plant -Type					
Supergroup																
Ophistokonta	Fungi	*Saccharomyces cerevisiae*	+	+	–	+	+	+	+	+	–	–	–	–	–	–
	Metazoa	*Homo sapiens*	+	+	–	+	+	+	+	+	–	–	–	–	–	–
	Choanoflagellates	*Meloe brevicollis*	+	–	+	+	–	–	+	+	–	–	–	–	–	–
Amoebozoa	Dictyostelia	*Dictyostelium discoideum*	+	+[a]	–	–	–	+	+	+[a]	–	–	–	–	–	–
	Discosea	*Acanthamoeba castellanii*	+	+[a]	–	–	–	+	+[a]	+[a]	–	–	–	–	–	–
Archaeplastida	Plants	*Arabidopsis thaliana*	+	–	+	+[b]	+[c]	+	–	–	+	+	–	–	–	–
		Oryza sativa	+	–	+	+[b]	+[c]	+	–	–	+	+	–	–	–	–
		Physcomitrella patens	+	–	+	+[b]	+[c]	+	–	–	+	+	–	–	–	–
	Red algae	*Cyanidioschyzon merolae*	+	–	+	–	–	–	–	–	–	–	–	–	–	–
	Green algae	*Chlorella variabilis*	+	–	+	–	–	+	–	–	+	–	–	–	–	–
		Chlamydomonas reinhardtii	+	–	–	+[b]	–	+	–	–	+	–	–	–	–	–
Excavata	Kinetoplastids	*Trypanosoma brucei*	+	–	+[d]	–	–	–	–	–	–	–	+	+	+	+
		Bodo saltans	+	–	–	–	–	–	–	–	–	–	+	+	+	+
	Heterolobosea	*Naegleria gruberi*	+	–	–	–	–	–	–	–	–	–	–	–	–	–
SAR	Stramenopiles	*Ectocarpus siliculosus*	+	–	+	–	–	+	+	+[e]	–		–	–	–	–
		Blastocystis hominis[f]	+	–	+	–	–	+	+	–	–	–	–	–	–	–
		Phaeodactylum tricornutum	+	–	+	–	–	+[g]	+	–	–	–	–	–	–	–
	Alveolata	*Plasmodium falciparum*	+	–	+	–	–	–	+	–	–	–	–	–	–	–
		Tetrahymena thermophila	+	–	+	–	–	+	–	–	–	–	–	–	–	–
	Incertae sedis	*Guillardia theta*	+	–	+	–	–	+	–	–	+	–	–	–	–	–

Note.—For accession numbers and other details, see supplementary table S1, Supplementary Material online.

[a]Wojtkowska et al. (2015). We find no bioinformatic support for these proteins to be orthologs of Tom22, Tom70, or Tom20, respectively.

[b]HHPred and hmmscan analysis suggests that annotated plant Tom5 proteins are in fact orthologs of yeast Tom6.

[c]HHPred and hmmscan analysis suggests that annotated plant Tom6 proteins are in fact orthologs of yeast Tom5.

[d]Mani et al. (2015). The Tom22 ortholog in trypanosomes is termed ATOM14.

[e]Annotated as Tom20 in UniProt. We find no bioinformatic support for this protein to be a Tom20 ortholog.

[f]With the exception of *B. hominis*, which harbors mitochondrion-related organelles, our analysis was restricted to organisms with bona fide mitochondria, capable to perform oxidative phosphorylation.

[g]Maćasev et al. (2004).

plant Tom9 (supplementary fig. S1, Supplementary Material online). In contrast, ATOM14 has an IMS domain that is twice as long as yeast Tom22 or plant Tom9. ATOM14 is tightly associated with ATOM40. The protein is essential under all conditions and, as Tom22 in yeast, plays an important role in (A)TOM assembly. In its absence, much less of the ATOM complex was formed and ATOM46 and ATOM69 and to some extend ATOM11 became unstable (Mani et al. 2015).

In summary, there are two types of Tom22-like proteins. The ones typified by the yeast protein which have a cluster of acidic residues in the cytosolically exposed N-terminal domain and the ones exemplified by the plant Tom9 that lack this domain and thus generally are shorter. In yeast the acidic cytosolic domain has been implicated in presequence binding, whereas in plant Tom9 the corresponding much shorter, nonacidic domain cannot bind presequences (Rimmer et al. 2011). However, even in yeast Tom22 the cytosolic acidic residues can be replaced without significantly affecting protein import or cell growth (Nargang et al. 1998). This indicates that in yeast presequences may preferentially be bound by hydrophobic interactions or in the case of plants that another protein may compensate for inability of plant Tom9 to bind presequences. It has been shown that also the IMS domain of yeast Tom22 can bind presequences (Kiebler et al. 1993; Mayer et al. 1995; Komiya et al. 1998). The same likely applies for the IMS domain of plant Tom9 as it is able to functionally replace the corresponding domain of the yeast protein (Maćasev et al. 2004).

Yeast-type Tom22 orthologs with a cytosolic acidic cluster are largely restricted to Opisthokonts (Maćasev et al. 2004) (table 4). Moreover, the yeast *Saccharomyces castellii* has a plant-type Tom22 ortholog with a short cytosolic domain lacking an acidic cluster. This lack appears to be compensated for by the gain of an acidic cluster in the cytosolic domain of the primary receptor Tom20 (Hulett et al. 2007).

The most widespread form of Tom22 is of the plant-type. It is not only found in most Archeaplastida but also in Excavates, for example, ATOM14 in trypanosomatids, and in at least a few representatives of the Stramenopiles and the Alveolates (Maćasev et al. 2004) (table 4).

Tom7

The TOM complex of yeast and plants contains a small protein each consisting of 60 and 75 amino acids, respectively. These proteins, termed Tom7, show only low sequence similarity but have a conserved sequence motif in their single atypical membrane-spanning domains (Maćasev et al. 2004). This suggests that yeast and plant Tom7 derive from the same common ancestor. One of the functions of yeast Tom7 is to destabilize TOM possibly to allow the incorporation of new subunits (Hönlinger et al. 1996; Becker et al. 2011). This role is antagonistic to yeast Tom6 described below. The function of plant Tom7 has not been investigated yet (table 3).

Bioinformatic searches identified putative Tom7 orthologs in representatives of all eukaryotic supergroups except the Excavates which include the trypanosomatids (Maćasev et al. 2004) (table 4).

Tom5 and Tom6 in Yeast and Plants

Purified TOM of yeast and plants each contain two proteins, of approximately 50 and 60 amino acids in length, termed Tom5 and Tom6, which have a single transmembrane domain each.

Yeast Tom5 is tightly associated with Tom40 and has its N-terminus exposed to the cytosol. It helps to transfer precursor proteins from the receptors to Tom40 and supports TOM biogenesis. Tom6 stabilizes the large TOM complex and thus has an antagonistic function to Tom7 which promotes its disassembly (Dietmeier et al. 1997; Model et al. 2001). Neither Tom5 nor Tom6 is essential for yeast. In plants, the specific functions of Tom5 and Tom6 have not been investigated.

Interestingly, yeast Tom5 appears to be similar to plant Tom6 and yeast Tom5 to plant Tom6 (supplementary fig. S2, Supplementary Material online). This suggests that the two proteins in yeast and plants share a common evolutionary origin. However, a bioinformatic analysis did not reveal any candidates for orthologs of Tom5 and Tom6 in other supergroups (table 4). It should be considered though that very small open-reading frames are often missed when genomes are annotated.

Small ATOM Subunits in Trypanosomatids

ATOM from *T. brucei* contains two small subunits with a single membrane spanning domain, termed ATOM11 (100 amino acids) and ATOM12 (105 amino acids), which are conserved but specific for Kinetoplastids (Mani et al. 2015) (table 4). Both proteins are essential and RNAi-mediated ablation shows that ATOM11 mediates the interaction of the ATOM core complex with the two peripheral subunits ATOM46 and ATOM69 (see below), whereas ATOM12 has an antagonistic function and prevents this association (Mani et al. 2015). Thus, despite the lack of sequence similarity with any Tom subunit of yeast and plants the function of trypanosomal ATOM11 and ATOM12 seem at least in part analogous to yeast Tom6 and Tom7, respectively.

TOM Receptor Subunits

The TOM core complex dynamically interacts with more loosely associated subunits that function as primary import receptors. The best studied ones are yeast and mammalian Tom20 and Tom70 (Endo and Kohda 2002). Tom20 is an N-terminally anchored membrane protein whose cytosolically exposed domain contains a single tetratricopeptide repeat (TPR) domain. TPR folds provide a protein–protein interaction platform, the specificity of which is determined by the variable residues in the conserved TPR motif (Abe et al. 2000). Tom20 preferentially recognizes soluble precursor proteins carrying N-terminal targeting signals. It binds to the hydrophobic surface of presequences and subsequently transfers the precursors to Tom22 (Söllner et al. 1989; Ramage et al. 1993; Saitoh et al. 2007).

Tom70 is the primary receptor for mitochondrial carrier proteins that have internal targeting sequences and can also bind hydrophobic precursor proteins that carry presequences

(Hines et al. 1990; Steger et al. 1990; Hines and Schatz 1993). It is N-terminally anchored in the membrane and contains a large cytosolically exposed segment consisting of 11 TPR motifs. The eight TPR motifs distal to the membrane directly recognize substrate proteins (Chan et al. 2006; Wu and Sha 2006). The three TPR motifs proximal to the membrane form the clamp domain that interacts with the cytosolic chaperone Hsp70 in yeast as well as Hsp90 in mammals from which Tom70 can receive precursors proteins (Hachiya et al. 1995; Young et al. 2003).

Although Tom20 and Tom70 have a preference for hydrophilic and hydrophobic substrates, respectively, they have in part redundant functions. Yeast can grow and respire in the absence of Tom70. Loss of Tom20 abolished respiration but was not lethal and respiration could be restored by overexpression of Tom70. Finally, deletion of both receptors caused a severe growth phenotype but did not kill the cells provided that the secondary receptor Tom22 was still present (Ramage et al. 1993; Harkness et al. 1994; Lithgow et al. 1994; Moczko et al. 1994; Yamamoto et al. 2009).

Tom20 and Tom70 are found in all Opisthokonts. Although Tom20 is restricted to this supergroup, putative orthologs of Tom70 were recently discovered in the Stramenopiles of the SAR (Stramenopiles, Alveolates, and Rhizaria) supergroup. In *Blastocystis*, the protein localizes to the mitochondria-related organelle and its cytosolic domain was functional in the context of the yeast protein (Tsaousis et al. 2011). However, in the Excavates and the Archeaplastidae no Tom70 orthologs could be found (Chan et al. 2006) (table 4).

The single protein import receptor associated with plant TOM is also termed Tom20 (Heins and Schmitz 1996). Superficially plant and opisthokont Tom20 are very similar, both have a single transmembrane helix and a cytosolic domain containing TPR motifs—the yeast Tom20 has one and the plant protein two—which recognize presequence-containing proteins. Furthermore, both proteins show the same domain organization and share conserved residues in their transmembrane regions, but only if their sequences are aligned in an antiparallel way. In other words, yeast Tom20 is signal-anchored, whereas plant Tom20 is anchored to the membrane through its C-terminus (tail-anchored). It is difficult to imagine genetic mechanisms that during evolution could lead to the sequence reversal that is observed between the two proteins. Therefore plant and yeast Tom20, while being functional analogs, most likely have different evolutionary origins (Lister and Whelan 2006; Perry et al. 2006).

Besides Tom20 plants have another protein, termed OM64, that likely acts as a receptor for protein import (Chew et al. 2004). OM64 is N-terminally anchored in the mitochondrial OM but not associated with isolated TOM. Its large cytosolic segment includes an amidase domain flanked by three C-terminal TPR domains, that similar to Tom70 of yeast can bind the cytosolic chaperones Hsp70 and Hsp90 (Panigrahi et al. 2014). OM64 plays a role in import of at least some mitochondrial proteins in vivo and was shown to interact with a number of precursor proteins in vitro (Lister et al. 2007). It is a paralog of Toc64, a protein associated with the OM protein translocase of plastids, that likely functions as a receptor for plastid protein import.

Inactivation of all three active Tom20 genes in *A. thaliana* results in a moderate reduction in growth but is not lethal. OM64 mutants showed only mild phenotypic abnormalities (Lister et al. 2007). However, if all three Tom20 isoforms and OM64 are knocked out in the same plant an embryo-lethal phenotype is obtained (Duncan et al. 2013). C-terminally anchored Tom20 is found in the Archeaplastida and within this supergroup appears to be absent in red algae, which also lack a classical Tom20. The phylogenetic distribution of OM64 is more restricted, it is present in most vascular plants but absent in green and red algae, as well as in other lower plant lineages (Carrie et al. 2010). Thus, it is likely that other as yet undiscovered receptors are present in these clades.

ATOM of trypanosomatids contains two receptors, termed ATOM46 and ATOM69, that have large domains exposed to the cytosol (Mani et al. 2015). ATOM69 is superficially similar to Tom70. Both have the same molecular weight and multiple TPR-like motifs. However, ATOM69 in addition has an N-terminal CS/Hsp20-like domain, which in other proteins was shown to bind Hsp90. Moreover, analogous to yeast and plant Tom20, ATOM69 is tail-anchored whereas Tom70 has an N-terminal membrane anchor. ATOM46 also has an N-terminal membrane anchor and an armadillo (ARM) repeat domain. The ARM motif functions as a protein–protein interaction module, it is specific for eukaryotes and is found in a number of unrelated proteins including soluble nuclear transport receptors (Tewari et al. 2010). Thus, except for the TPR domain in ATOM69, the two trypanosomal import receptors do not share any similarity to TOM subunits of other species which illustrates their independent evolutionary history. The cytosolic domains of ATOM69 and ATOM46 were shown to bind a number of different precursor proteins. In these assays, ATOM69 showed a preference for presequence-containing substrates and ATOM46 most efficiently bound the hydrophobic MCPs (Mani et al. 2015).

Ablation of ATOM46 did not cause any growth or import phenotype under standard conditions. Inducible RNAi of ATOM69, however, caused an accumulation of cytosolic precursor proteins that was accompanied by a growth arrest. If both proteins are ablated simultaneously these phenotypes are strongly exacerbated and occur much earlier, suggesting that ATOM69 and ATOM46 are to some extent redundant mitochondrial protein import receptors with distinct but partially overlapping substrate specificities (Mani et al. 2015).

ATOM69 and ATOM46 have been found in all kinetoplastids including the free-living relative of trypanosomatids *Bodo saltans*, illustrating that the unique features of ATOM are not an adaptation to the parasitic life style of *T. brucei* (table 4).

Implications for Mitochondrial Evolution

The comparative analysis of yeast, plants, and trypanosomes supports a two-step model for the evolution of TOM. It posits that a simple version of TOM evolved in the mitochondrial ancestor (Cavalier-Smith 2006; Dolezal et al. 2006; Perry et al. 2006). It consisted of the β-barrel import pore that was commandeered from the endosymbiont, and a tightly associated

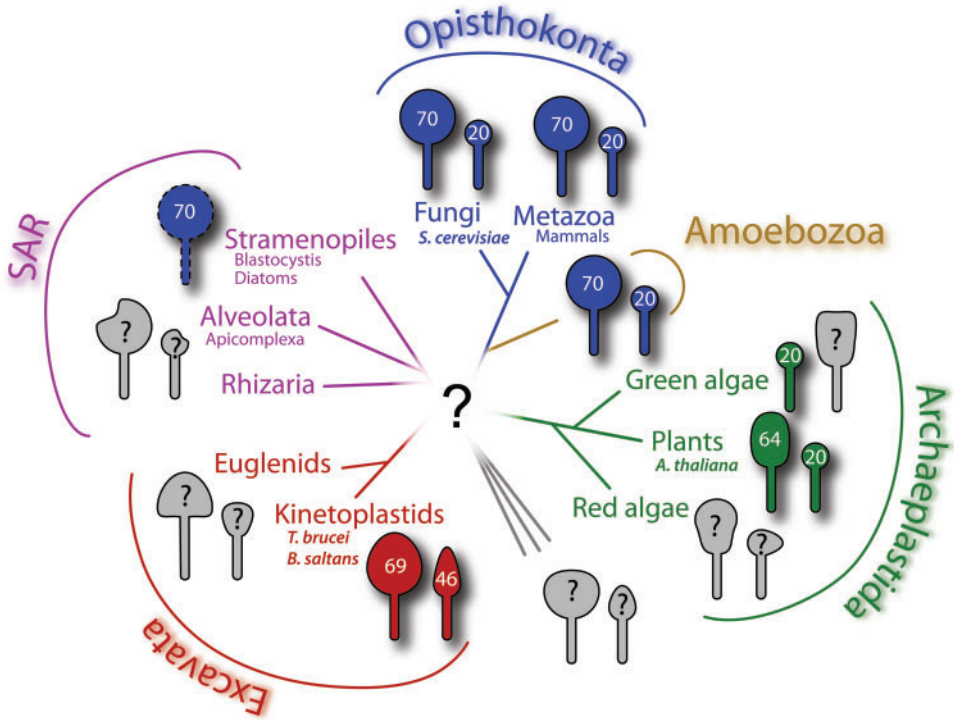

Fig. 4. Diversity of mitochondrial protein import receptors mapped on a schematic eukaryotic phylogenetic tree. Only clades that are discussed in this review are indicated regardless of their taxonomic rank. Opisthopkont Tom20 and Tom70 are shown in blue. A number of Stramenopiles appear to have a Tom70 ortholog (indicated in blue and by broken lines), although convergent evolution cannot be excluded (see text for discussion). Plant Tom20 and OM64 are indicated in green. At least a few green algae have Tom20 but lack OM64. Kinetoplastid ATOM46 and ATOM69 are indicated in red. Expected novel receptor and/or receptor pairs that have not been identified yet are indicated in gray with a question mark.

accessory protein of the Tom22/Tom9-type, that acted as primordial receptor recognizing preexisting targeting signals on the imported substrates. This is a plausible scenario since the function of yeast Tom22 as a secondary receptor is well established and yeast lacking both primary receptors are viable provided that Tom22 is still present (Lithgow et al. 1994). Alternatively, one or more of the present or as yet to be discovered receptors may have been present in the ancestor of all eukaryotes and later been replaced in at least two probably more of the basic eukaryotic lineages.

All TOMs contain a suite of small proteins whose main function is to regulate the assembly and disassembly of the complex. Tom7 is found in a wide range of eukaryotes but not in the Excavates (Maćasev et al. 2004), whereas Tom5 and Tom6 appear to be present in Opisthokonts and plants only (table 4) (supplementary fig. S2, Supplementary Material online). The Excavates lack any of these proteins and the ATOM complex of trypanosomatids instead contains the two unrelated small proteins ATOM11 and ATOM12 that are functionally analogous to Tom6 and Tom7, respectively. In summary this suggests that Tom5, 6, and 7 evolved after the ancestor of the Excavates diverged from all the other eukaryotes, supporting models that place the root of the eukaryotic evolutionary tree at this position (He et al. 2014).

All three systems have two primary receptors on the surface of the OM (fig. 4). These receptor pairs are functionally equivalent but evolutionary distinct, which is surprising since the signals they recognize are conserved (table 2). They therefore arose by convergent evolution after the fundamental eukaryotes lineages were already established. The occurrence of the distinct receptors overlaps but is not congruent with the eukaryotic supergroups.

Yeast-type Tom20 as well as Tom70 are present in all Opisthokonts and in at least some members of its sistergroup the Amoebozoans. However, orthologs of Tom70 also appear to occur in the Stramenopiles (Tsaousis et al. 2011) (table 4). This is difficult to explain and it is possible that the stramenopile Tom70 might have been acquired by horizontal gene transfer from an Opisthokont. Alternatively it might not be a true Tom70 ortholog. Indeed the observed sequence identity between the stramenopile and the opisthokont Tom70s is only weak, which makes it difficult to exclude that it arose by convergent evolution. Not all Archeaplastidae have both Tom20 and OM64, and the red algae lack both proteins (Carrie et al. 2010) (table 4). ATOM46 and ATOM69 are specific for Kinetoplastids (table 4). This suggests that they are a deep branching clade within the Excavates or, as has been proposed before, that this supergroup has a polyphyletic origin (Cavalier-Smith 2010). In any case, we expect that additional as yet unidentified import receptors are present in this group (fig. 4).

Protein import was already operational in the last common ancestor of eukaryotes. However, it appears that only after the establishment of the major eukaryotic lineages the number of imported proteins became so large, probably driven by ongoing EGT, that it required an increase in the

specificity and efficiency of the process, that could only be achieved by a pair of dedicated receptor proteins. Mitochondrial protein import is one of the first—if not the first—mitochondria-specific character. The variations that are seen in functionally identical but evolutionary distinct modules of TOM, such as the receptor subunits, therefore likely mirror the early diversification of eukaryotes. Uncovering the diversity of protein import receptors of the mitochondrial OM might therefore help to reveal the early branches of the eukaryotic evolutionary tree.

Biochemical Constraints on Receptor Function

The comparative analysis presented in this review shows that only the β-barrel import pore and the Tom22/Tom9 component of TOM are universally conserved (table 4). A Tom7 ortholog is present in all but one and Tom5 and Tom6 in two supergroups. The small ATOM subunits, on the other hand, evolved independently. This suggests that small proteins that regulate the assembly state of the complex are a basic requirement for a functional TOM.

All three TOMs dynamically interact with protein import receptors which evolved independently. These receptors represent different solutions to the same biological problem, namely the efficient and specific import of 1,000 or more different mitochondrial proteins (table 1).

All systems appear to need a pair of receptors that have distinct substrate preferences and some degree of redundancy. Removal of the receptor, which binds the broader range of substrates including presequence-containing proteins, causes stronger effects on protein import and fitness than if the other receptor with a preference for hydrophobic substrates is ablated. Moreover, in all cases ablation of both receptors causes a stronger effect than their individual removal might suggest.

Based on the three known receptor pairs we can identify the overarching structural features of the individual import receptors. Both require a single transmembrane region and an exposed cytosolic domain, however, whether the protein is anchored in a $N_{in} - C_{out}$ or $N_{out} - C_{in}$ orientation is not important. The cytosolic domain of at least one receptor subunit must have a substrate binding domain consisting of multiple TPR motifs and a binding site for cytosolic chaperones such as Hsp70 or Hsp90 that might be based on specialized TPR motifs or possibly on a CS/Hsp20 domain. However, in which order these modules are arranged is not important. The soluble domain of the second receptor also requires a protein–protein interaction domain, which may include a TPR motif or an ARM domain.

There are still many clades, especially in the Excavates and the SAR supergroup, where mitochondrial protein import receptors have not been identified yet (fig. 4). The general features of import receptors defined above indicate that TPR motif-containing proteins with a predicted N- or C-terminal transmembrane domain are excellent candidates for such proteins. However, the TPR domain is widespread and also found in other receptor-like proteins such as Pex5 and Sec72 (Schlegel et al. 2007). Confident identification of novel import receptors therefore requires an experimental approach. Although the genomes of many eukaryotes of interest have been sequenced, they are often not easily accessible to biochemical investigations. However, even in such systems it should in many cases be possible: 1) To determine the mitochondrial localization of the candidate receptors as well as 2) to test whether they can bind import signals, as such an experiment requires recombinant proteins only.

Thus, we believe that characterizing novel import receptors across the eukaryotic phylogeny is both feasible and rewarding. We expect that the study of the pattern of receptor variation will shed light on the basic eukaryotic lineages, whereas identifying the shared traits between them will allow to define the fundamental biochemical features mandatory for their function.

Supplementary Material

Supplementary figures S1 and S2 and table S1 are available at *Molecular Biology and Evolution* online (http://www.mbe.oxfordjournals.org/).

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