Evolution of mitochondrial protein import – Lessons from trypanosomes

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Abstract

The evolution of mitochondrial protein import and the systems that mediate it marks the boundary between the endosymbiotic ancestor of mitochondria and a true organelle that is under the control of the nucleus. Protein import has been studied in great detail in *Saccharomyces cerevisiae*. More recently it has also been extensively investigated in the parasitic protozoan *Trypanosoma brucei* making it arguably the second best studied system. Here we provide a comparative analysis of the protein import complexes of yeast and trypanosomes. Together with data from other systems, this allows to reconstruct the ancestral features of import complexes that were present in the last eukaryotic common ancestor (LECA) and to identify which subunits were added later in evolution. We discuss how these data can be translated into plausible scenarios providing insights into the evolution of i) outer membrane protein import receptors, ii) proteins involved in biogenesis of α-helically anchored outer membrane proteins, and iii) of the intermembrane space import and assembly system. Finally, we show that the unusual presequence-associated import motor of trypanosomes suggests a scenario of how the two ancestral inner membrane protein translocases present in LECA evolved into the single bifunctional one found in extant trypanosomes.

Keywords: LECA; membrane translocation; parasite; protein translocases; TIM complex; TOM complex.

Introduction

All eukaryotes have or once had mitochondria which makes them one of the defining features of eukaryotes. Mitochondria provide many important services to the cell, including oxidative phosphorylation, synthesis of FeS-clusters for both mitochondrial and non-mitochondrial proteins, synthesis of certain lipids and many more (Friedman et al., 2014; Nunnari et al., 2012). To achieve all this the organelles have to be under the control of the nucleus and firmly integrated into the physiology of the complex eukaryotic cell. - However, it was not always like that. The origin of mitochondria can be traced back approximately 1.7 billion years ago to a free living α-proteobacterium that was taken up by an archaeal host cell and became an endosymbiont (Archibald, 2015; Dacks et al., 2016; Roger et al., 2017). This on its own is not such an unusual event and we know of many modern examples of such endosymbiotic systems (Lopez-Garcia et al., 2017). However, what happened next was indeed very unusual and - in the case of the mitochondrion - occurred only once in evolution: the endosymbiont converted into an organelle. Which selective forces shaped this process and how exactly this organellogenesis took place is still being debated (Lane, 2014; Poole et al., 2014; Roger et al., 2017). What we do know is that more and more of the genome of the endosymbiont was either lost or transferred to the host cell genome. At some point this became critical and the only way for the endosymbiont to survive was to convert into an organelle. A key event in this process was the evolution of a protein import system that allowed the nascent organelle to make use of proteins whose genes were previously transferred to the host cell genome (Dolezal et al., 2006; Fukasawa et al., 2017; Harsman et al., 2017). Thus, the protein import system defines the boundary between an endosymbiont and a true organelle that is under control of the host cell genome. Today more than 95% of all mitochondrial proteins derive from nuclear genes and need to be

imported from the cytosol. Thus, mitochondrial protein import is a key process for mitochondrial physiology as well as for understanding the organelle's evolutionary history. It is therefore not surprising that mitochondrial protein import has been studied in great detail resulting in a wealth of knowledge about the machineries and the mechanisms that mediate it (Grevel et al., 2019; Hansen et al., 2019; Opalinska et al., 2015; Pfanner et al., 2019; Schulz et al., 2015; Wasilewski et al., 2017). But there is a problem, with the exception of plants, essentially all experimental studies on the mitochondrial protein import have been done in yeast and mammals. Eukaryotes are very diverse and can be divided into at least five major phylogenetic taxons, termed supergroups(Burki, 2014; Burki et al., 2019; Dacks et al., 2008). However, fungi (which includes yeast) and metazoans (which includes mammals) belong to the same eukaryotic supergroup, the opisthokonts.

For a deep understanding of the mitochondrial protein import process and how it evolved we need to know which of its features are similar and which ones are different in unrelated eukaryotes. Moreover, we need to investigate whether the observed similarities are due to common descent or due to the same functional constraints that may have resulted in convergent evolution. In recent years the mitochondrial protein import systems of the parasitic protozoan *Trypanosoma brucei*, a member of the eukaryotic supergroup of the excavates, has been experimentally studied in quite some details making it arguably the best characterized such system outside the opisthokonts (Eckers et al., 2012; Harsman et al., 2017; Hauser et al., 1996; Mani et al., 2016; Schneider, 2018; Schneider et al., 2008). In this review I will introduce the mitochondrial protein import system of *T. brucei*, with an emphasis on the most recent findings, and contrast it to its counterpart in yeast. Furthermore, I will discuss the insights such a comparative analysis can provide into the evolution of the mitochondrial protein import systems.

Mitochondrial protein import in yeast

Mitochondrial protein import has best been analyzed in the *Saccharomyces cerevisiae* which therefore serves as a golden standard with which all other systems can be compared. There are a number of excellent reviews discussing the various protein import pathways (Hansen et al., 2019; Opalinska et al., 2015; Pfanner et al., 2019; Schulz et al., 2015; Wasilewski et al., 2017). Thus, in the following I will only provide a condensed overview focusing on the protein complexes that mediate import.

Outer membrane

In the mitochondrial outer membrane (OM) we find three complexes that have distinct functions in protein import (Fig. 1) (Dukanovic et al., 2011). The most important one across which essentially all mitochondrial proteins are translocated is the translocase of the OM (TOM) whose structure has recently been solved (Araiso et al., 2019; Bausewein et al., 2017; Tucker et al., 2019). It consists of 7 subunits. The β-barrel protein Tom40 forms the protein import pore. It is associated with the import receptors Tom20, which has a preference for presequence-containing precursor proteins as well as β-barrel proteins, and Tom70, which mainly binds hydrophobic membrane proteins delivered by the cytosolic chaperone heat shock protein 70 (cHsp70). The substrate specificity of the two receptors is not absolute but overlapping explaining why their individual deletion is not lethal. Tom22 acts as a secondary receptor that transfers the substrates to Tom40 and is essential for the stability of the whole complex. The TOM complex furthermore contains Tom5, Tom6 and Tom7 which regulate its stability and assembly (Perry et al., 2008).

The sorting and assembly machinery (SAM) is the most highly conserved import machinery. Its function is to insert β-barrel proteins into the OM after they have been translocated to the intermembrane space (IMS) by the TOM complex. The core component of SAM is the β-barrel protein SAM50, which is a homologue of BamA the β-barrel protein insertase in gram negative bacteria (Ulrich et al., 2015).

Finally, the mitochondrial OM harbors the MIM complex consisting of two small proteins mitochondrial import 1 (Mim1) and Mim2. It facilitates the insertion into the OM and/or possibly other steps in the biogenesis of α-helically anchored proteins (Dimmer et al., 2012; Stefan Dimmer et al., 2010). Some of its substrates are subunits of the TOM complex indicating that the MIM complex is essential for the biogenesis of the main OM translocase. The exact mechanism by which the MIM complex exerts its function is still unknown.

Intermembrane space

There are two protein complexes in the IMS that are required for import of different subsets of proteins (Fig. 1). A collection of small Tim proteins form two distinct hexameric complexes, a soluble one and a membrane-associated one that interacts with the TIM22 complex (see below). They function as chaperones preventing the aggregation of β-barrel or hydrophobic mitochondrial carrier proteins in the IMS and handover their substrates to the corresponding translocation machinery, the SAM or the TIM22 complexes respectively (Koehler et al., 1998; Sirrenberg et al., 1998; Vial et al., 2002).

The mitochondrial IMS import and assembly protein (Mia40), an integral inner membrane (IM) oxidoreductase exposed to the IMS, and the soluble IMS-localized sulfhydryl reductase Erv1 mediate import of small IMS proteins that have typical cysteine motifs. Mia40 and Erv1 form a disulfide relay that transfers disulfide bonds first from Erv1 to Mia40 and then to the

substrate. This stabilizes the substrate and prevents its back translocation to the cytosol. Conversely, the electrons liberated by the oxidation of the substrate are first transferred to Mia40 and subsequently to Erv1 before they are fed into the respiratory chain (Backes et al., 2017; Herrmann et al., 2012; Mordas et al., 2015).

Inner membrane

The IM contains two main protein translocases with distinct substrate specificities termed translocase of the IM 22 and 23 (TIM22 and TIM23 complex), respectively (Fig. 1). The TIM22 complex consists of four subunits, of which Tim22 forms the protein conducting pore, and is associated with the IMS-localized small TIM chaperone complex. It mediates the insertion of integral IM proteins with multiple transmembrane domains, such as mitochondrial carrier proteins. The TIM23 complex consists of five subunits, of which Tim23 and Tim17 form the protein-conducting pore and Tim50 acts as a presequence receptor (Dudek et al., 2013; Rehling et al., 2004; Schulz et al., 2015). While the TIM23 and TIM22 complexes do not share any subunits Tim23, Tim22 and Tim17 belong to the same protein family. The TIM23 complex translocates or inserts presequence-containing precursor proteins, which make up to 70% of all mitochondrial proteins, across or into the IM. To that end it is associated, on the matrix side, with the presequence translocase-associated motor (PAM), that consists of mitochondrial heat shock protein 70 (mHsp70), Mge1, the two essential J-domain-containing chaperones Pam16 and Pam18, as well as Tim44 that links the PAM module to the TIM23 complex (Craig, 2018; Marom et al., 2011; Schulz et al., 2015). The TIM23 complex together with the PAM allows ATP-dependent translocation of precursors proteins across the IM. After translocation the presequence is processed by the

heterodimeric matrix protease and the imported protein is refolded by the mHsp70 and Hsp60 chaperones.

Mitochondrial protein import in trypanosomes

It has been assumed that the mitochondrial protein import systems would be highly conserved in all eukaryotes. However, studies of mitochondrial protein import in the experimentally highly accessible parasitic protozoan *Trypanosoma brucei* have begun to show that this is not necessarily the case (Harsman et al., 2017; Mani et al., 2016; Schneider, 2018). In the following chapters I will introduce the protein complexes that mediate mitochondrial protein import in trypanosomes focusing on the differences they show to the ones in yeast (Fig. 1). Moreover, I will discuss what these differences may tell us about the evolution of mitochondrial protein import in general.

Outer membrane: ATOM complex and protein import

The trypanosomal analogue of the TOM complex has been termed atypical translocase of the OM (ATOM). As its yeast counterpart it consists of seven subunits, all of which are essential for mitochondrial protein import (Desy et al., 2016; Mani et al., 2015; Pusnik et al., 2011). ATOM40 is the protein conducting pore and a remote orthologue of Tom40 (Harsman et al., 2012; Zarsky et al., 2012). ATOM14 is a highly diverged orthologue of Tom22. It has a very short functionally dispensable cytosolic domain lacking the acidic clusters found in yeast Tom22. Its IMS domain in contrast is twice as long as the one in yeast Tom22. It is essential for function of ATOM14 and was shown to bind precursor proteins in vitro (Mani et al., 2016).

The other five subunits ATOM69, ATOM46, ATOM19, ATOM12 and ATOM11 are specific for kinetoplastids. The cytosolic domains of ATOM69 and ATOM46 were shown to bind a number of different precursor proteins, with quantitative rather than qualitative differences. This suggests that these two ATOM subunits, similar to yeast Tom70 and Tom20, function as import receptors which have distinct but overlapping substrate preferences. ATOM69 is superficially similar to Tom70. Both share 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, in contrast to yeast Tom70, ATOM69 is tail-anchored. ATOM46 has an N-terminal membrane anchor and armadillo (ARM) repeat domains that likely function as a protein–protein interaction module. Thus, except for the TPR domains in ATOM69, the two trypanosomal import receptors do not share any sequence similarity to the two receptors of yeast indicating that they evolved independently (Mani et al., 2015).

Interestingly, the import receptors in plant mitochondria are different to both the yeast and the trypanosomal receptors. One of them, plant Tom20, looks very similar to yeast Tom20, both have a single transmembrane domain and a TPR repeat in their cytosolic domains. However, the tail-anchored plant Tom20 is coded in reverse when compared to its yeast counterpart which is a signal-anchored protein (Lister et al., 2006; Perry et al., 2006). Some plants furthermore have a second import receptor, termed OM64, that is not tightly associated with the TOM complex but required for import of at least a few mitochondrial proteins. The cytosolic segment of OM64 includes an amidase domain that is flanked by three C-terminal TPR domains (Chew et al., 2004; Lister et al., 2007). Thus, the two plant receptors Tom20 and Om64 appear to be functional analogues but are neither orthologous to yeast Tom20 and Tom70 nor to the trypanosomal ATOM46 and ATOM69. The three

receptor pairs therefore present examples of convergent evolution over large phylogenetic distances (Mani et al., 2016).

In summary these results suggest the following evolutionary scenario for the evolution of the TOM complex and its protein import receptors (Fig 2). The last eukaryotic common ancestor (LECA) likely had a simplified import system consisting of ancestral forms of Tom40, Tom22 and Tom7 (Fig. 2A).

During evolution Tom7 was lost in most excavates, including all kinetoplastids (Fukasawa et al., 2017). Moreover, comparing the Tom22 orthologues in different eukaryotic supergroups indicates, that its ancestral form was lacking the presequence-binding cytosolic domain found in opisthokont Tom22 and therefore may have looked more similar to *T. brucei* ATOM14 and the plant Tom22 orthologue Tom9 (Maćasev et al., 2004). In line with this is was shown that the cytosolic domain of ATOM14 is dispensable (Mani et al., 2016) and that the one of Tom9 does not bind presequences (Rimmer et al., 2011). The ancestral Tom22 could therefore not have served as a primoridal import receptor. Instead the import substrates were likely directly recognized by Tom40 which contains conserved acidic and hydrophobic patches that line the import channel and that likely were already present in LECA (Fukasawa et al., 2017).

After a first divergence of eukaryotes two primary import receptors with substrate preferences for either presequence-containing or hydrophobic proteins evolved independently at least three times: in opisthokonts, plants and kinetoplastids. Interestingly, recent studies suggest that ATOM46 and ATOM69 homologues are present in *Euglena* (Ebenezer et al., 2019) (J. Lukes, University of South Bohemia in České Budějovice, personal communication). Moreover, an ATOM69-like receptor has been found in hydrogenosomes of *Trichomonas* (Makki et al., 2019). This suggests that the trypanosomal-type receptors are

not linked to a parasitic life-style and might be more widespread in the Excavate supergroup than initially thought. In summary, the receptor pairs with distinct substrates preferences appeared relatively late in evolution possibly because an increased number of proteins needed to be imported or because a higher specificity of targeting was required at this stage. It will be interesting to see whether there are even more examples of distinct protein import receptor pairs in other eukaryotic supergroups.

Outer membrane: ATOM complex and tRNA import

There is another unusual feature of the trypanosomal ATOM complex, it does not only import proteins but also cytosolic tRNAs. – In contrast to most other eukaryotes the trypanosomal mitochondrial genome does not encode any tRNAs. This means that all organellar tRNAs have to be imported from the cytosol. The process is not restricted to trypanosomes but import of a subset of cytosolic tRNAs into mitochondria has also been found in plants, some fungi and a number of protists (Alfonzo et al., 2009; Salinas et al., 2008; Schneider, 2011). Recently, it has been shown that plugging the ATOM40 import channel with a precursor protein inhibits both protein and tRNA import. Thus, ATOM40 serves as a pore for the translocation of both proteins and tRNAs. Interestingly however, it was shown that protein import could be uncoupled from tRNA translocation, since simultaneous ablation ATOM46 and ATOM69, the two protein import receptors, while completely abolishing protein import did not affect tRNA import (Niemann et al., 2017). Thus, despite the fact that proteins and tRNAs use the same import pore, protein and tRNA import are independent processes in trypanosomes. - *S. cerevisiae* imports a small fraction of a single cytosolic $tRNA^{Lys}$ isoacceptor into mitochondria even though the yeast mitochondrial genome encodes all tRNAs required for organellar translation. However, in

contrast to trypanosomes, it was suggested that the yeast $tRNA^{Lys}$ is co-imported in complex with the precursor of a mitochondrial aminoacyl-tRNA synthetase (Tarassov et al., 1995; Tarassov et al., 1995). Thus, while both the yeast TOM and the trypanosomal ATOM complex are able to translocate tRNAs they do it by a different mechanism.

Outer membrane: SAM complex

Sam50, the pore-forming subunit of the SAM complex, was commandeered from the bacterial endosymbiont that gave raise to mitochondria. In line with that we find a highly conserved Sam50 orthologue in *T. brucei* that as its yeast counterpart mediates insertion of β-barrel proteins into the OM (Sharma et al., 2010). In yeast Sam50 is associated on the cytosolic side with the peripheral OM proteins Sam35 and Sam37. While we do find an orthologue of Sam35 in trypanosomes there is no evidence that it is associated with the SAM complex (Niemann et al., 2013).

Outer membrane: pATOM36

Peripheral ATOM36 (pATOM36) is an abundant kinetoplastid-specific protein with probably two transmembrane domains whose N- and C-termini face the cytosol. RNAi-mediated ablation of pATOM36 essentially exclusively affects mitochondrial OM proteins that have classical α-helical transmembrane domains including six subunits of the ATOM complex. pATOM36 appears to facilitate membrane insertion for some substrates whereas for others it seems to mediate their assembly into protein complexes (Bruggisser et al., 2017; Käser et al., 2017; Pusnik et al., 2012). Thus, the function of pATOM36 is reminiscent of the MIM complex in yeast. However, pATOM36 has a different molecular weight and topology, and does not show any sequence similarity to either of the two subunits of the MIM complex.

Deletion of the two subunits, Mim1 and Mim2, of the MIM complex in yeast strongly inhibits growth of the cells and assembly of the TOM complex. Interestingly, expression of pATOM36 in the MIM complex deletion strain could essentially completely complement these phenotypes. Furthermore, the converse experiment also worked. Thus, expressing Mim1 and Mim2 in the induced pATOM36 RNAi cell line rescued the ATOM assembly defect observed in this cell line (Vitali et al., 2018). These results demonstrate that pATOM36 and the MIM complex are functional analogues and therefore represents another example of convergent evolution over large phylogenetic distances (Tokatlidis, 2018). The results furthermore suggest that neither pATOM36 nor Mim1/Mim2 need any trypanosome- or yeast-specific partner proteins to exert their function. Since the mechanism by which the MIM complex or pATOM36 promotes OM protein biogenesis is not known in detail, new results obtained in either of the two systems will be of immediate relevance for the other system.

The MIM complex is restricted to the fungi and pATOM36 has only been found in kinetoplastids, although a recent study showed its presence in *E. gracilis* suggesting it might be more widely distributed (Ebenezer et al., 2019) (M. Hammond and J. Lukes, University of South Bohemia in České Budějovice, personal communication). We don't know which membrane factors mediate the biogenesis of α-helically anchored OM proteins in other systems such as mammals or plants. However, we expect that insertion of OM proteins is a protein-assisted process in these systems as well. The most parsimonious interpretation of the available data suggests the following evolutionary model (Fig. 3). The primordial TOM complex in LECA might not have required a dedicated OM protein biogenesis factors (Fig. 3A). However, such a system became a requirement later in evolution possibly because more and more α-helically anchored OM proteins evolved, including the two receptor pairs

of the different TOM complexes. Thus, completely unrelated but functionally essentially identical systems evolved in the ancestors of all fungi and all *Euglenozoa* (Fig. 3B and C). Again it will be interesting to see which factors mediate OM biogenesis in mammals and other eukaryotic taxons (Fig. 3D). Complementation of the yeast MIM complex deletion strain with cDNA libraries of the corresponding organisms may be a way to identify these postulated factors.

Surprisingly pATOM36, unlike the MIM complex in yeast, has a second function that is unrelated to mitochondrial protein import. It was shown that pATOM36 is not only localized all over the mitochondrial surface but concentrated at the tripartite attachment complex (TAC), the kinetoplastid-specific structure that links the single unit mitochondrial genome with the basal body of the flagellum. The function of the TAC is to couple the segregation of the replicated mitochondrial genomes to the segregation of the old and the new flagellum in dividing cells (Schneider et al., 2018). Thus RNAi-mediated depletion of pATOM36 not only affected biogenesis of OM proteins but also resulted in a loss of the mitochondrial genome and an increase in the distance between the basal body and the mitochondrial OM indicating that it is an essential subunit of the TAC (Käser et al., 2016). pATOM36 therefore integrates mitochondrial protein import and mitochondrial DNA inheritance.

Intermembrane space: small TIMs

The small TIM proteins are a highly conserved protein family found in all eukaryotes. This includes trypanosomes which contains six different small TIM proteins (Gentle et al., 2007; Harsman et al., 2017). However, sequence comparisons do not allow a one to one assignment of the trypanosomal Tims to their counterparts in yeast and human. The structure of one trypanosomal TIM, TbTim12, is unusual since it has an incomplete Cx3C

small Tim signature motif and thus can only form a single intramolecular disulfide bond (Wenger et al., 2017).

Ablation small Tim proteins as expected affects import of TbTim17, an integral membrane subunits of the TIM complex (Basu et al., 2013; Eckers et al., 2013; Smith et al., 2018; Wenger et al., 2017). Tim13 however is an exception, it does not appear to be required for import of TbTim17 but rather plays are role in the assembly and/or maintenance of the TIM complex.

Trypanosomal small Tims are present in two complexes. One is associated with the single bifunctional trypanosomal TIM complex (see below), the other one is soluble in the IMS (Fig. 1). The latter has a molecular weight of approximately 70 kDa consistent with the hexameric assemblies of small Tims found in yeast and humans. However, pulldown experiments with tagged variants of specific small TIMs always recovers all six small Tims of trypanosomes(Wenger et al., 2017). This suggests that, unlike in yeast and humans, the postulated hexamers in trypanosomes do not consist of specific alternating pairs of small Tim proteins but are composed of all six small TIM proteins.

Intermembrane space: MIA system

Only an incomplete MIA system is present in *T. brucei*. While the sulfhydryl oxidase Erv1 has been found an orthologue of the Mia40 oxidoreductase is absent (Basu et al., 2013; Eckers et al., 2013). RNAi-mediated ablation of Erv1 reduces the abundance of IMS proteins, such as small Tims, or other proteins rich in cysteines that are arranged in either twin-Cx3C or -Cx9C motifs found in many IMS proteins of yeast (Peikert et al., 2017). Thus, Erv1 as in yeast mediates import of IMS proteins in *T. brucei*. This raises the question of how the disulfide relay can function in the absence of a Mia40? Two different scenarios have been

proposed to explain this. It could be that the requirement of Mia40 is bypassed and that the job is done by Erv1 alone. In support of this, it has been shown that in *Arabidopsis* which has both Erv1 and Mia40, but in which Mia40 is not essential (Carrie et al., 2010), Erv1 can directly transfer disulfide bonds to import substrates at least to a limited extent (Peleh et al., 2017). Alternatively, there might be another unrelated protein that functions as a Mia40 analogue. Recent studies of the unusual trypanosomal mitochondrial contact site and cristae organizing system (MICOS), that mediates the formation cristae in the IM, supports the later scenario. Mic20 a subunit of the trypanosomal MICOS is a thioredoxin-like protein, whose ablation to a large part phenocopies the effects seen after ablation of Erv1, suggesting it may replace the lacking Mia40 (Eichenberger et al., 2019; Kaurov et al., 2018). Should this be the case part of the trypanosomal IMS disulfide relay system would be similar to disulfide bond formation in the bacterial periplasm, which is catalyzed by the thioredoxin-like protein DsbA (Lu et al., 2014). However, the evidence that Mic20 functions a Mia40 analogue in trypanosomes is still indirect and further evidence is required to exclude alternative explanations.

Based on the lack of Mia40 in some species and the non-essential nature of the protein in *Arabidopsis* it has been proposed that the mitochondrial IMS disulfide relay may have evolved in a stepwise fashion, from an ancestral system requiring Erv1 alone to the more complex one composed of Erv1 and Mia40 found in most extant eukaryotes (Carrie et al., 2017; Peleh et al., 2017). However, this model is inconsistent with the wide phylogenetic distribution of Mia40, which is found in a number of different supergroups (opisthokonts, amoebozoans, Archaeplastida and in a few excavates) indicating that the protein was already present in LECA (Backes et al., 2019; Munoz-Gomez et al., 2015). Thus, the ancestral system likely contained both Erv1 and Mia40 (Fig. 4A). It then appears that in the

ancestor of the kinetoplastids Mia40 was replaced by the thioredoxin-like protein Mic20 (Fig. 4B), whereas in plants Mia40 is still present but at least in *Arabidopsis* dispensable. We have no information how the members of the SAR group (stramenopiles, alveolates, and rhizarians), which also lack a Mia40, compensate for this absence but the plant example shows that a system solely based on Erv1 would in principle feasible (Fig. 4D). Moreover, it is likely that at an intermediate stage, before Mia40 was lost, it became dispensable as is observed in *Arabidopsis*.

Inner membrane: a single unique TIM complex

Rather than having a TIM23 and a TIM22 complex each specialized for different substrates such as yeast and mammals, trypanosomes have a single TIM complex only that imports or inserts all imported IM or matrix proteins (Fig. 1). The evidence for this comes from tagged substrates, that in yeast would be typical for either the TIM23 (presequence-containing proteins) or the TIM22 complex (carrier proteins), that are stuck in their respective import machineries. Pulldown of both of these import intermediates recovers the same four integral membrane proteins. Only two rhomboid-like proteins, TimRhom I and TimRhom II, were specifically associated with the presequence substrate (Fig .1). Furthermore, the pulldown also recovered all six small TIM proteins. Thus, trypanosomes have a single bifunctional TIM complex that is tightly associated with small TIM proteins and that with minor compositional variations mediates import of both presequence-containing and mitochondrial carrier proteins (Harsman et al., 2016).

The only subunit of the trypanosomal TIM complex that shows homology to any subunit of the yeast TIM23 and TIM22 complexes is TbTim17 (Gentle et al., 2007; Singha et al., 2008), which despite its name is an orthologue of yeast Tim22, the core subunit of the TIM22

complex (Fukasawa et al., 2017; Pyrihova et al., 2018; Zarsky et al., 2016). Interestingly, ablation of TbTim17 not only abolished protein but also mitochondrial tRNA import (Tschopp et al., 2011), suggesting that - as for the OM membrane – subunits of the protein translocase are involved in the translocation of tRNAs across the IM. The remaining TIM subunits of trypanosomes are Tim42 (Harsman et al., 2016), Tim62 and ACAD, an orthologue of a medium chain length acyl-CoA dehydrogenase (Harsman et al., 2016; Singha et al., 2015; Singha et al., 2012). Except for Tim42 which has a single predicted transmembrane domain all trypanosomal TIM subunits have multiple transmembrane regions. A previous study identified a putative trypanosomal orthologue of yeast Tim50, a subunit of the TIM23 complex (Duncan et al., 2013). However, the significance of the observed similarity was disputed in two recent reviews, since the transmembrane domain of the protein would disrupt the conserved C-terminal phosphatase motif. Moreover, the protein was not recovered in any of three reciprocal immunoprecipitations using tagged Tim subunits (Harsman et al., 2016).

Whereas the ATOM46 and ATOM69, the receptor subunits of the ATOM complex, and the functional MIM complex analogue pATOM36 each have orthologues in *E. gracilis*, this is not case for the subunits of the bifunctional trypanosomal TIM complex, if we discount the universally conserved Tim22-homologue TbTim17. In fact *E. gracilis* has orthologues of both Tim23 and Tim22, respectively (M. Hammond and J. Lukes, University of South Bohemia in České Budějovice, personal communication). This demonstrates that the evolution of a single bifunctional TIM complex occurred independently of the ATOM-like OM translocase. There is ample bioinformatic evidence that orthologues of Tim23/Tim17 and Tim22, the core subunits of the TIM23 and TIM22 complex, respectively, are present in essentially all eukaryotes (Fukasawa et al., 2017; Pyrihova et al., 2018; Zarsky et al., 2016). Thus, two

distinct specialized IM protein translocases were likely already present in LECA. Having a single TIM complex only is therefore a derived trait.

Inner membrane: a unique PAM module

One function of the single TIM complex of trypanosomes is to translocate presequence-containing substrates across the mitochondrial IM, before their presequences get processed by a conventional matrix protease (Desy et al., 2012; Smíd et al., 2008). It should therefore be associated with a PAM module. Indeed if we search the *T. brucei* genome we find ORFs homologous to mHsp70, Mge1, Pam18 and Pam16, all of which are subunits of the yeast PAM module (Fig. 1). The only yeast PAM subunit orthologue missing is the highly conserved Tim44 (Clements et al., 2009; Fukasawa et al., 2017), even though a protein having a very limited similarity to Tim44 is found. Furthermore, previous work has shown that, as might be expected, the single trypanosomal mHsp70 is required for mitochondrial protein import (Tschopp et al., 2011). However, while the trypanosomal J-domain containing Pam18 and Pam16 orthologues are required for normal growth, they are neither associated with, nor required for the formation of, the presequence intermediate indicating that the two proteins are not involved in mitochondrial protein import (von Känel et al., 2020). - Pulldown of the presequence intermediate did however recover another kinetoplastid-specific J-domain containing protein, that was termed TbPam27. Ablation of this protein selectively inhibits the formation of the presequence intermediate and preferentially affects import of presequence-containing but not of mitochondrial carrier proteins. Moreover, as for Pam18 an intact J-domain was essential for TbPam27 function (von Känel et al., 2020). In summary, these results indicate that in trypanosomes the function of Pam18 was replaced by the unrelated J-domain protein TbPam27. In line with these results TbPam27 has a C-

terminal transmembrane domain whereas Pam18 orthologues are C-terminally anchored in the membrane.

We propose the following evolutionary scenario to explain the observed homologue replacement (von Känel et al., 2020). As explained above, LECA likely already had both a TIM23-like and a TIM22-like TIM complex (Fig. 5A). Kinetoplastids however have a single bifunctional TIM complex whose core subunit TbTim17 is an orthologue of yeast Tim22 (Fig. 5D). This indicates that the trypanosomal TIM complex derives from a TIM22-type complex, which in addition to its primary function in mitochondrial carrier protein biogenesis has acquired the capability to translocate presequence-containing precursor protein. We propose that in the ancient pro-kinetoplastid the ancestor of TbPam27, a J-domain-containing protein with a C-terminal transmembrane domain, that possibly arose by gene duplication, was recruited to the IM and interacted with the TIM22 complex (Fig. 5C). Initially this interaction was neutral. However, since the J-domain is known to bind to Hsp70s to regulate their ATPase activity, TbPam27 allowed binding of mHsp70 to the TIM22 complex. With time, possibly by the addition of further subunits including the presequence pathway-specific TimRhom I and TimRhom II, this prepared the way for the evolution of a TIM22-type complex that could translocate presequence-containing substrates (Fig. 5C). This in turn made the TIM23 complex obsolete: its subunits were free the accumulate deleterious mutations and eventually disappeared. - However, why did Pam18 and Pam16 not disappear? One possibility is that they acquired novel as yet unknown functions unrelated to mitochondrial protein import. Alternatively, and perhaps more interestingly, they may always have had a second function, that was masked by their well established role in mitochondrial protein import. The proposed scenario ties the evolution of the kinetoplastid-specific PAM subunit TbPam27 to the evolution of the single bifunctional

Tim22-like TIM complex of trypanosomes. Moreover, it can explain why trypanosomes lack the otherwise highly conserved Tim44 (Clements et al., 2009). In yeast Tim44 connects the PAM module to the TIM23 complex (Fig. 1). In the absence of a TIM23 complex it therefore becomes redundant.

Having a single member only of the Tim17/22/23 protein family is not restricted to trypanosomes, but has also been found in a number of other unicellular eukaryotes including *Giardia, Cryptosporidium, Microsporidium* and *Trimastix* (Heinz et al., 2013; Pyrihova et al., 2018). All of these organisms have mitosomes, mitochondria-related organelles that have lost the organellar genome and the capability to perform oxidative phosphorylation (Makiuchi et al., 2014). It is unknow how the postulated single TIM complexes evolved in mitosomes of *Microsporidium* and *Trimastix* but it has been suggested that their core subunits are most closely related to yeast Tim22 indicating that, as in trypanosomes, they derive from the TIM22 complex. Interestingly, a different scenario applies for the evolution of the single TIM complex in *Giardia*, since its core subunit appears to be most similar to yeast Tim17 which together with Tim23 forms the protein-conducting channel of the TIM23 complex. In line with this, orthologues of Pam18, Pam16 as well as of Tim44 have been found in the *Giardia* mitosome (Martincova et al., 2015; Pyrihova et al., 2018), suggesting it has a more conventional PAM module than trypanosomes. - It is important to keep in mind that in mitosomes the reduction of the IM protein translocases to a single complex coincides with the massive reduction of their proteomes (50-100 proteins in *Giardia*) (Heinz et al., 2013; Jedelsky et al., 2011) when compared to bona fide mitochondria (1000-1500 proteins) (Pagliarini et al., 2008; Rao et al., 2017). This is different in trypanosomes, they have an organellar genome and a fully functional mitochondrion capable of oxidative phosphorylation whose mitochondrial proteome (ca.

1200 proteins) (Peikert et al., 2017) is even a bit bigger than the one of yeast (ca. 1000 proteins) (Morgenstern et al., 2017; Vogtle et al., 2017). Thus, in *T. brucei* a reduced number of import substrates cannot explain why two distinct TIM complexes were reduced to a single one.

Concluding remarks

The comparative analysis of the mitochondrial protein import systems of yeast and trypanosomes helped to determine which import factors are universally conserved and therefore likely formed the primitive import systems present in LECA. In addition, it also revealed a surprising number of differences including at least three examples of convergent evolution over large phylogenetic distances. Furthermore, it showed that in some cases orthologues of yeast protein import factors, while still being present in trypanosomes, do not function in mitochondrial protein import. This illustrates the limit of bioinformatic analyses that cannot with certainty infer functions of homologous proteins. I'm convinced that we have just scratched the surface. Future studies of the trypanosomal protein import system should focus on assigning specific functions to the individual subunits of the trypanosomal ATOM and TIM complexes and compare them with their yeast counterparts. This will likely provide more insights into the very fundamental features of mitochondrial protein import that are "conserved" not due to common descent but due to the same selective forces. Moreover, future experimental studies of the mitochondrial protein import systems should be extended to representatives of other supergroups than opisthokonts and excavates. Extrapolating from our findings in trypanosomes this will without doubt uncover further lineage-specific features of mitochondrial protein import. Because protein import was one of the first mitochondria-specific trait to evolve, such studies will likely provide

further insight into the early evolutionary history of mitochondria and the origin of eukaryotes in general.

Acknowledgements

I would like to thank my whole group for helpful discussions and E. K. Horn for technical assistance. Work in my lab is supported by the NCCR "RNA & Disease" and by grant 175563 both funded by the Swiss National Science Foundation.

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Figure legends

Figure 1. Comparison of protein complexes and their subunits involved in mitochondrial protein import between *S. cerevisiae* and *T. brucei*.

The subunits of each protein complex (name indicated in boxes) involved in mitochondrial protein import are outlined the identical colours. Complexes that have the same function in yeast and *T. brucei* are outlined with the same colour. Evolutionary conserved subunits between yeast and *T. brucei* are filled in orange. Subunits which have distinct evolutionary origins in yeast and *T. brucei* are filled with grey. Blue broken outlines indicate that TimRhom I and TimRhom II are specifically associated with the trypanosomal presequence translocase.

Figure 2. Scenario for the convergent evolution of different OM protein import receptor pairs in the TOM complexes of different phylogenetic groups.

(**A),** shows the situation in LECA whose ancestral TOM complex likely consisted of a Tom40-like and a Tom22-like protein (shown in grey). (Tom7 which is also conserved in most eukaryotes and therefore likely was also present in the ancestral TOM complex is not shown). The cytosolic, presequence-binding domain of Tom22 probably evolved in Opisthokonts since it is absent in other phylogenetic groups. (**B**, **C**, **D),** illustrate the independent evolution of the three receptor pairs (indicated by different colours and shapes) in opisthokonts, Euglenozoa and the Archaeplastida. Except for the TPR motifs found in most but not all receptors they do not share sequence similarities and also have different topologies. The two receptors in each system appear to have preferences for either presequence-containing or hydrophobic import substrates, respectively. (**E**), The

broken arrow indicates that other phylogenetic groups may have yet different receptor pairs that have not been discovered yet.

Figure 3. Scenario for convergent evolution of different systems for the biogenesis of α-helically anchored OM proteins in different phylogenetic groups.
(**A**), the situation in LECA is unknown. It could be that due to the simpler composition of its TOM complex it did not need a dedicated biogenesis factor for α-helically anchored OM proteins. (**B**), in fungi biogenesis of many α-helically anchored OM proteins is mediated by Mim1 and Mim2 (orange), which form the MIM complex. (**C**), pATOM36 (yellow) is a functional analogue of Mim1 and Mim2 in kinetoplastids. (It contains at least two, possibly more, transmembrane domains). (**D**), the broken arrows indicate that mammals and other phylogenetic groups, in which the biogenesis of α-helically anchored OM proteins is likely also protein-mediated, may require factors (blue) that are different to both Mim1/Mim2 and pATOM36, respectively.

Figure 4. Scenario for convergent evolution of different MIA systems in different phylogenetic groups.
(**A**), shows the situation in LECA whose ancestral MIA system likely consisted of a Mia40-like and Erv1-like protein (shown in grey). (**B),** shows that opisthokonts and Archaeplastida have retained the ancestral system. (**C),** depicts the proposed situation in Kinetoplastids in which the lacking Mia40 might have been replaced by the thioredoxin-like protein Mic20 (yellow), a subunit of the non-canonical trypanosomal MICOS complex. (**D),** and (**E**) show two possibilities (indicated by broken arrows) of how the MIA systems may work in other

phylogenetic groups that lack Mia40, such as he SAR group. (**D)**, Erv1 alone is sufficient to do the job. (**E**), another protein (blue) different from Mic20 might replace Mia40.

Figure 5. Scenario for the evolution of a single TIM complex.

(**A)**, shows the situation in LECA which likely already had a TIM23-like complex, associated with a PAM module, as well as a TIM22-like complex, associated with small TIM complex(es). The ancestral TIM23 complex contained at least the Tim23-like and Tim17-like subunits, and the ancestral TIM22 complex at least the Tim22-like subunit. The ancestral PAM module included Hsp70-like, Pam16-like, Pam18-like and Tim44-like subunits. Ancestral subunits of the various complexes are depicted in grey throughout the figure. **(B)**, The TIM and PAM complexes in opisthokonts and most other eukaryotes look essentially identical to their ancestral counterparts in LECA. **(C)**, and **(D)** show the postulated evolutionary scenario for the kinetoplastid lineage that links the replacement of Pam18 by TbPam27 (yellow) to the transition from the ancestral state of two TIM complexes in LECA to a single bifunctional TIM22-like complex in extant kinetoplastids. **(C)** The J-domain containing integral membrane protein TbPam27 was recruited to the TIM22 complex in the ancient pro-Kinetoplastid. This was initially neutral but allowed mHsp70 to interact with the TIM22 complex via TbPam27. **(D),** subsequently, the TIM22 complex acquired to capability to translocate presequence-containing precursors, possibly by recruitment of other subunits such as TbRhom I and TbRhom II (blue) which are specifically associated with the presequence translocase. This rendered the TIM23 complex redundant and it eventually disappeared. Pam18 and Pam16, however, were retained because they either acquired a new as yet unknown function, or have an overlooked ancestral role. **(E)**, mitosomes of *Giardia* and *Cryptosporidium* likely have a single TIM23-like complex organized around a

Tim17-like core subunit. *Giardia* appears to have orthologues of Pam16, Pam18, mHsp70 and Tim44. **(F)**, mitosomes of *Microsporidium* and *Trimastix* appear to have a single TIM complex organized around a Tim22-like protein.

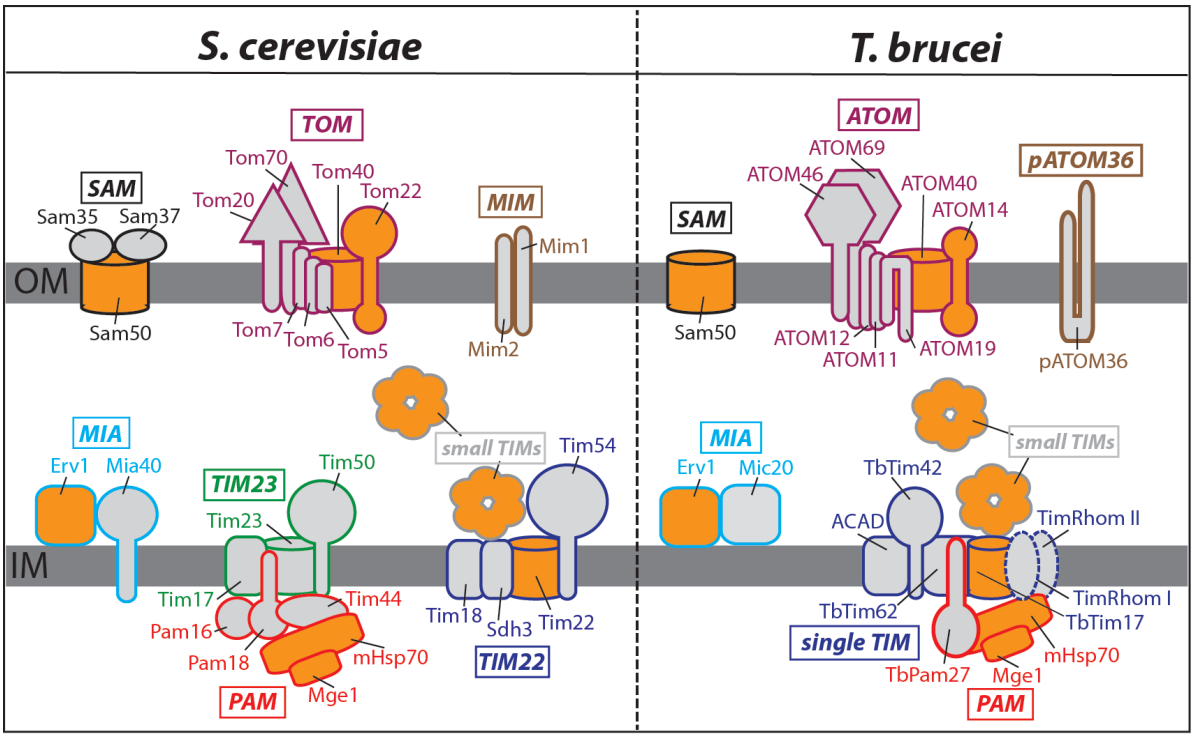
S. cerevisiae
T. brucei
TOM
Tom70
Tom40
Tom20
Tom22
SAM
Sam35
Sam37
MIM
Mim1
OM
Sam50
Tom7
Tom6
Tom5
Mim2
ATOM
ATOM69
ATOM46
ATOM40
ATOM14
pATOM36
SAM
Sam50
ATOM12
ATOM11
ATOM19
pATOM36
small TIMs
Tim54
MIA
Erv1
Mia40
TIM23
Tim50
Tim23
IM
Tim17
Tim44
Pam16
Pam18
mHsp70
Tim18
Sdh3
Tim22
TIM22
PAM
Mge1
MIA
Erv1
Mic20
small TIMs
TbTim42
ACAD
TimRhom II
TimRhom I
TbTim17
TbTim62
single TIM
mHsp70
TbPam27
Mge1
PAM

Figure 1

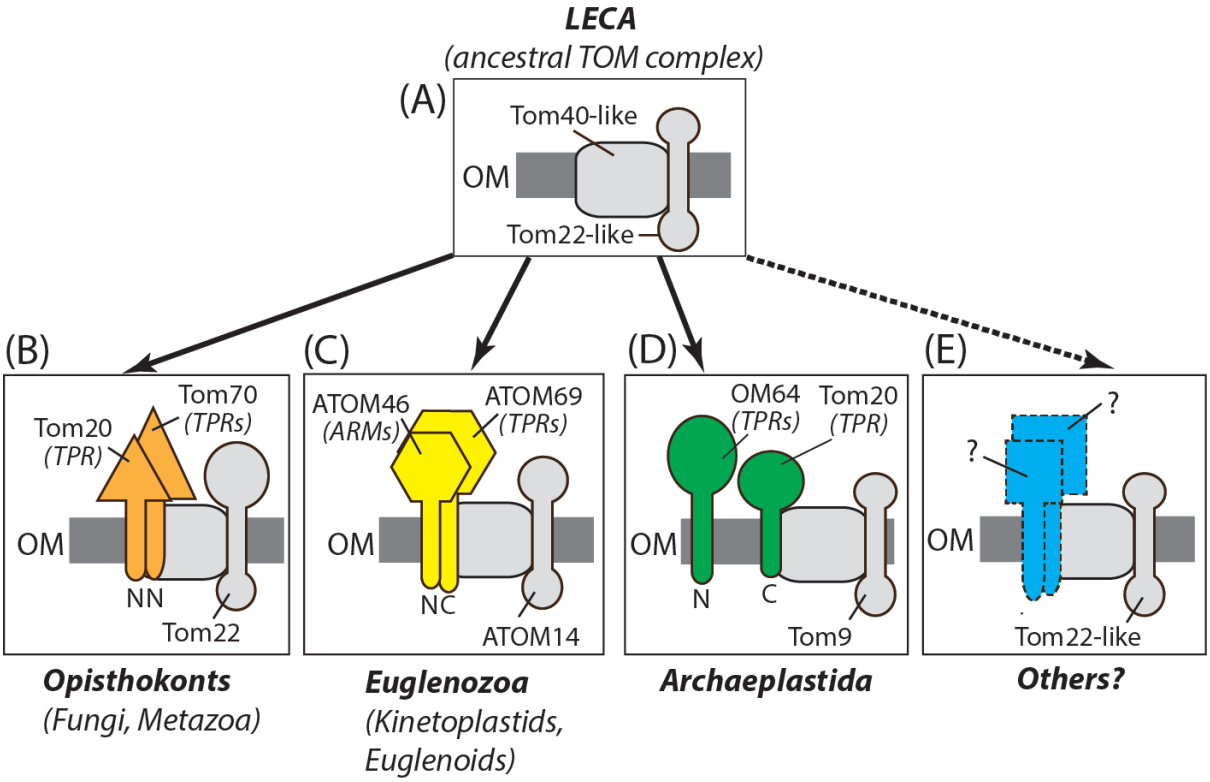
LECA
(ancestral TOM complex)
(A)
Tom40-like
OM
Tom22-like
(B)
Tom70
(TPRs)
Tom20
(TPR)
OM
NN
Tom22
(C)
ATOM46
(ARMs)
ATOM69
(TPRs)
OM
NC
ATOM14
(D)
OM64
(TPRs)
Tom20
(TPR)
OM
N
C
Tom9
(E)
?
?
OM
Tom22-like
Opisthokonts
(Fungi, Metazoa)
Euglenozoa
(Kinetoplastids,
Euglenoids)
Archaeplastida
Others?

Figure 2

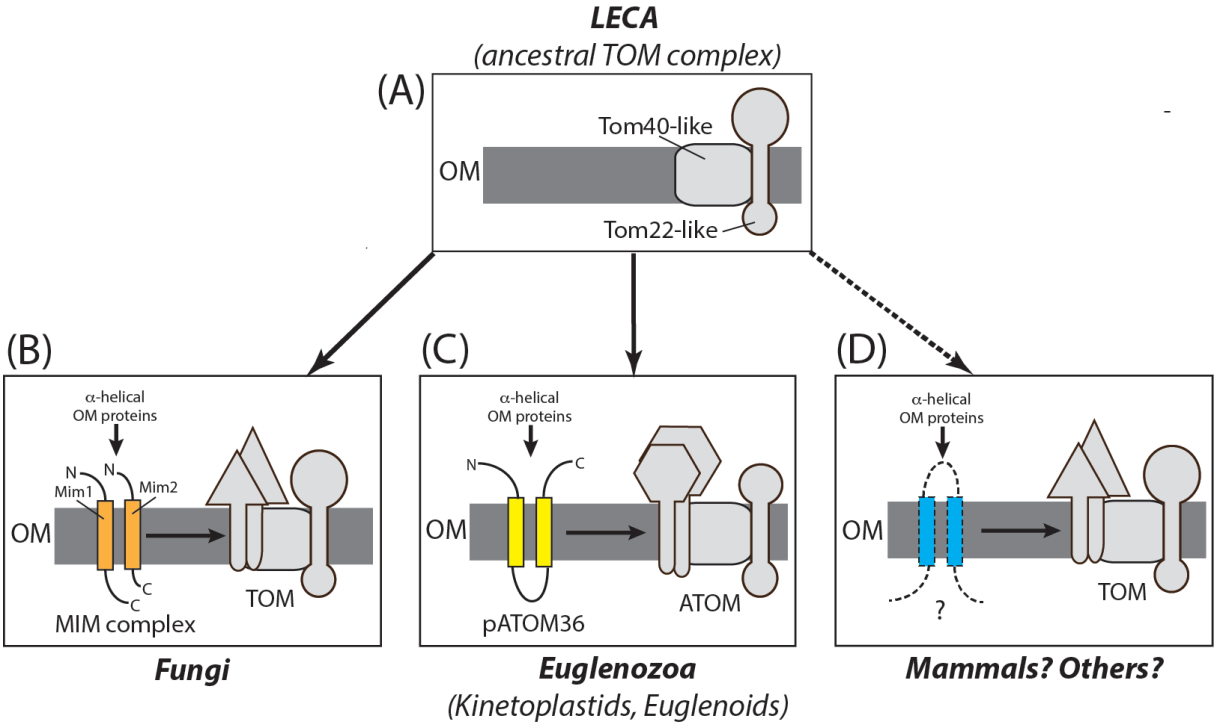
LECA
(ancestral TOM complex)
(A)
Tom40-like
OM
Tom22-like
(B)
α-helical
OM proteins
N
N
Mim1
Mim2
OM
C
C
MIM complex
TOM
Fungi
(C)
α-helical
OM proteins
N
C
OM
pATOM36
ATOM
Euglenozoa
(Kinetoplastids, Euglenoids)
(D)
α-helical
OM proteins
OM
?
TOM
Mammals? Others?

Figure 3

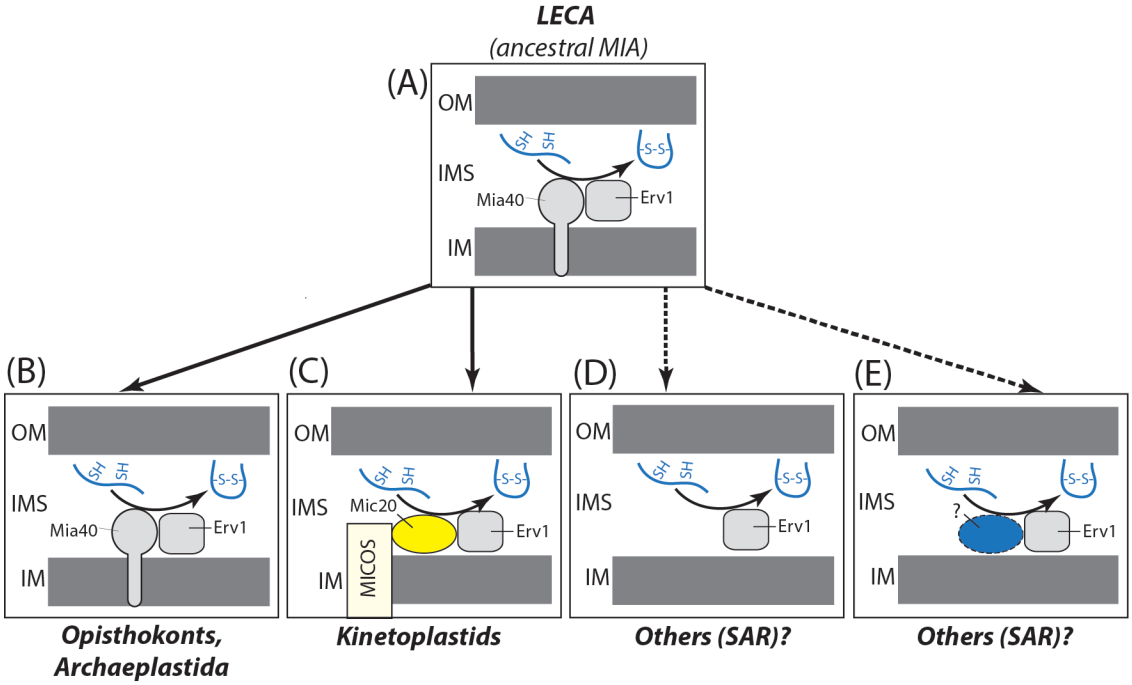
LECA
(ancestral MIA)
(A)
OM
IMS
IM
SH
SH
S-S
Mia40
Erv1
(B)
(C)
(D)
(E)
Mic20
MICOS
?
Opisthokonts,
Archaeplastida
Kinetoplastids
Others (SAR)?
Others (SAR)?

Figure 4

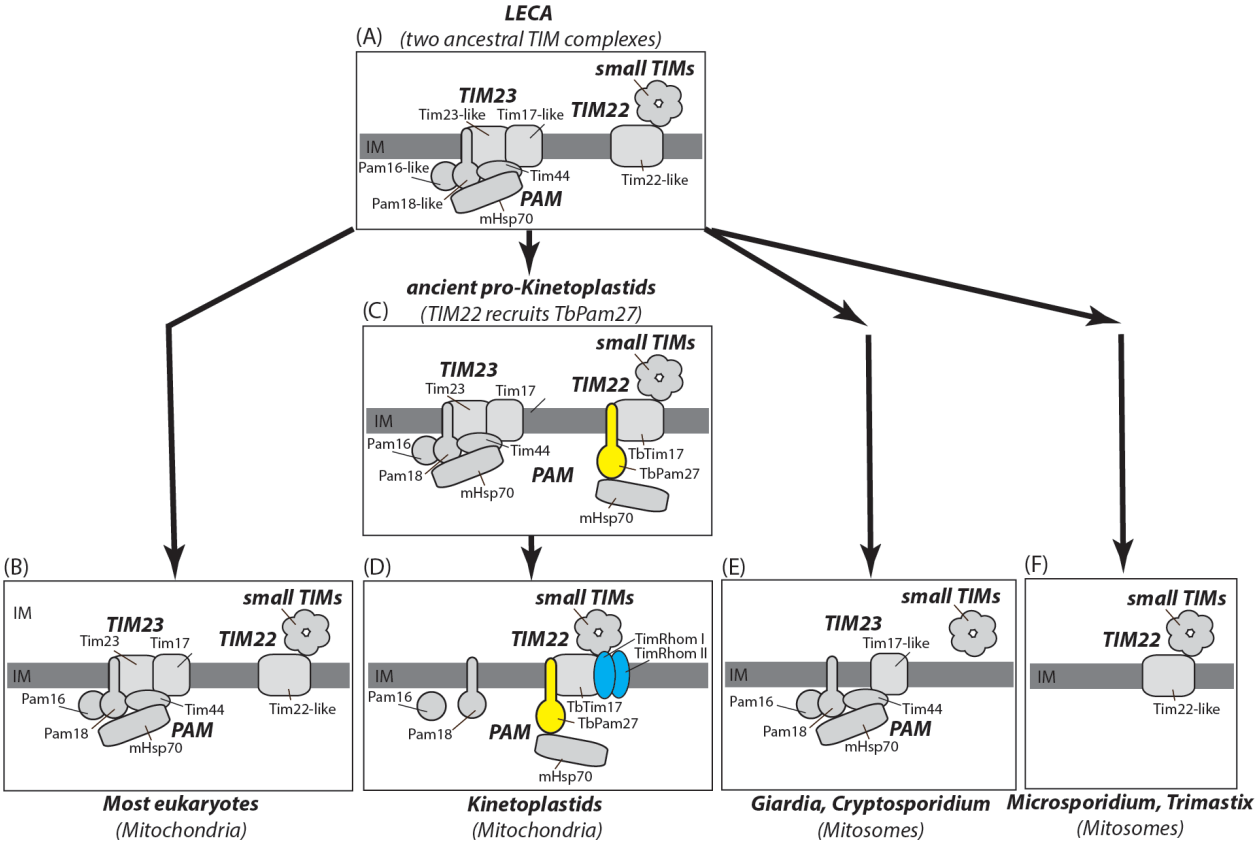
LECA
(A) (two ancestral TIM complexes)
small TIMs
TIM23
Tim23-like
Tim17-like
TIM22
IM
Pam16-like
Tim44
Tim22-like
Pam18-like
PAM
mHsp70
ancient pro-Kinetoplastids
(TIM22 recruits TbPam27)
(C)
small TIMs
TIM23
Tim23
Tim17
TIM22
IM
Pam16
Tim44
TbTim17
Pam18
PAM
TbPam27
mHsp70
mHsp70
(B)
small TIMs
IM
TIM23
Tim23
Tim17
TIM22
IM
Pam16
Tim44
Tim22-like
Pam18
PAM
mHsp70
Most eukaryotes
(Mitochondria)
(D)
small TIMs
TIM22
TimRhom I
TimRhom II
IM
Pam16
TbTim17
TbPam27
Pam18
PAM
mHsp70
Kinetoplastids
(Mitochondria)
(E)
small TIMs
TIM23
Tim17-like
IM
Pam16
Tim44
Pam18
PAM
mHsp70
Giardia, Cryptosporidium
(Mitosomes)
(F)
small TIMs
TIM22
IM
Tim22-like
Microsporidium, Trimastix
(Mitosomes)

Figure 5