

Review

Evolution of macromolecular import pathways in mitochondria, hydrogenosomes and mitosomes

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All eukaryotes require mitochondria for survival and growth. The origin of mitochondria can be traced down to a single endosymbiotic event between two probably prokaryotic organisms. Subsequent evolution has left mitochondria a collection of heterogeneous organelle variants. Most of these variants have retained their own genome and translation system. In hydrogenosomes and mitosomes, however, the entire genome was lost. All types of mitochondria import most of their proteome from the cytosol, irrespective of whether they have a genome or not. Moreover, in most eukaryotes, a variable number of tRNAs that are required for mitochondrial translation are also imported. Thus, import of macromolecules, both proteins and tRNA, is essential for mitochondrial biogenesis. Here, we review what is known about the evolutionary history of the two processes using a recently revised eukaryotic phylogeny as a framework. We discuss how the processes of protein import and tRNA import relate to each other in an evolutionary context.

Keywords: mitochondria; protein import; tRNA import; mitosomes; hydrogenosomes

1. INTRODUCTION

Mitochondria or mitochondria-like organelles are part of the essential inventory of eukaryotic cells. Their origin can be traced down to an endosymbiotic event between what were probably two prokaryotic organisms: a host cell of uncertain lineage and an intracellular symbiont with the characteristic features of an α proteobacterium (Lang *et al.* 1999; Andersson *et al.* 2003; Dyall *et al.* 2004).

Comparative genomics suggest that mitochondria and the mitochondria-like organelles—hydrogenosomes and mitosomes—share a monophyletic evolutionary origin, with the original endosymbiotic event dated to at least 1.5 Gyr ago (vanderGiezen & Tovar 2005; vanderGiezen *et al.* 2005). It has long been known that most proteins of mitochondria are nucleus-encoded and imported from the cytosol (Dolezal *et al.* 2006; Neupert & Herrmann 2007; Bolender *et al.* 2008; Hildenbeutel *et al.* 2008; Mokranjac & Neupert 2009). Less well known is that most mitochondria also import tRNAs (Salinas *et al.* 2008). Import of these macromolecules, both proteins and tRNAs, therefore represents a key aspect of mitochondrial biogenesis. In this review, we will outline what is known about the two processes. Moreover, we discuss how the two processes relate to each other in an evolutionary context.

The shared features of mitochondria and mitochondria-like organelles are a double membrane and the fact that most if not all of their proteome is acquired by import of nucleus-encoded proteins from the cytosol. The protein translocation machineries in the outer and the inner membrane consist, with some interesting variations that will be discussed here, of a set of conserved core components. However, in considering organelle morphology and metabolic capabilities, there are breath-taking differences between mitochondria and mitochondria-like organelles from different organisms. This should perhaps not be surprising, given that these organelles and their corresponding host cells have coevolved through a very long time (maybe up to 1.5 Gyr). The net result is a collection of morphologically, genetically and functionally heterogeneous organelle variants that can be divided into three main groups: ‘classic’ mitochondria, hydrogenosomes and mitosomes (vanderGiezen & Tovar 2005; vanderGiezen *et al.* 2005) (figure 1).

(a) *Mitochondria, hydrogenosomes and mitosomes*

Classic mitochondria are found in all aerobic eukaryotes. These organelles oxidize pyruvate and house the citric acid cycle. Their major metabolic activity is to produce ATP by oxidative phosphorylation. However, while mitochondria devote much of their proteome either directly or indirectly to oxidative phosphorylation, this is not the mitochondrial function that is essential for life under all conditions. There are micro-organisms such as *Saccharomyces*

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One contribution of 12 to a Theme Issue ‘Evolution of organellar metabolism in unicellular eukaryotes’.

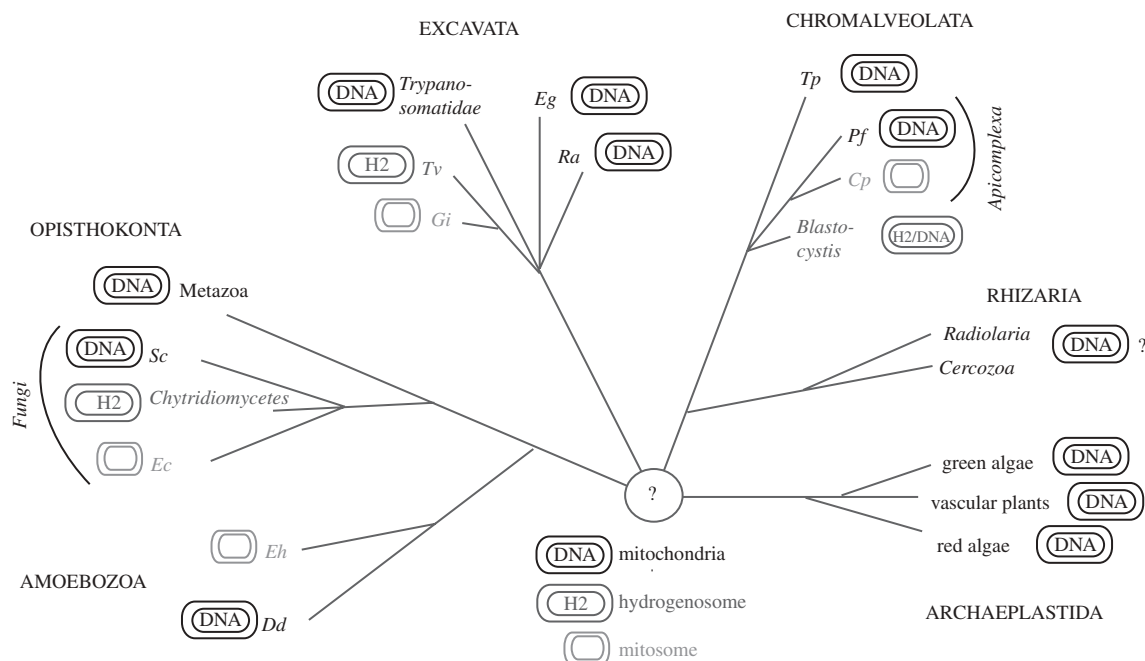


Figure 1. Occurrence of mitochondria, hydrogenosomes and mitosomes within the six eukaryotic supergroups. Branching order reflects the phylogenetic relationship of taxa but branch length is not to scale. All mitochondria-like organelles derive from the same endosymbiotic event. Hydrogenosomes and mitosomes evolved at least three and four times independently. The mitochondrial-like organelle in *Blastocystis* shows feature of mitochondria and hydrogenosomes and therefore blurs the distinction between these two organelles. Acronyms for species: *Sc*, *Sacharomyces cerevisiae*; *Ec*, *Encephalitozoon cuniculi*; *Eh*, *Entamoeba histolytica*; *Dd*, *Dictyostelium discoideum*; *Tv*, *Trichomonas vaginalis*; *Gi*, *Giardia intestinalis*; *Eg*, *Euglena gracilis*; *Ra*, *Reclinomonas americana*; *Tp*, *Tetrahymena pyriformis*; *Pf*, *Plasmodium falciparum*; *Cp*, *Cryptosporidium parvum*.

cerevisiae or the bloodstream form of *Trypanosoma brucei* that can live without oxidative phosphorylation—these organisms nevertheless require mitochondria. This is due to the fact that mitochondria are the only site for the biogenesis of FeS clusters, essential cofactors in mitochondrial and cytosolic proteins (Lill & Mühlenhoff 2008). Mitochondria also play key roles in haem biosynthesis, Ca^{2+} homeostasis and, in metazoans, in accentuating apoptosis. Perhaps the most striking feature of classic mitochondria is that they all house a vestige of the genome that belonged to the endosymbiont ancestor (Burger *et al.* 2003).

Hydrogenosomes too produce ATP. They ferment pyruvate to acetate, CO_2 and molecular hydrogen (hence the name) and produce ATP by substrate level phosphorylation. Thus, hydrogenosomes can be considered as the anaerobic equivalent of mitochondria (Müller 1993; Hackstein *et al.* 1999). That said, it is important to emphasize that while hydrogenosomes decarboxylate pyruvate, they do this by pyruvate:ferredoxin oxidoreductase, an enzyme that is not related to the pyruvate dehydrogenase complex found in mitochondria. Moreover, hydrogenosomes do not have cytochromes and most importantly—with very notable exceptions (Boxma *et al.* 2005)—they do not contain a genome or a translation machinery. Hydrogenosomes are also the site of FeS-cluster biogenesis. Hydrogenosomes are found in a number of phylogenetically unrelated microbial eukaryotes that inhabit anaerobic environments such as rumen-dwelling ciliates and fungi, as well as free living and parasitic flagellates that do not have classical mitochondria (figure 1).

Mitosomes represent the third class of mitochondria-like organelles and, characteristically, mitosomes do not produce ATP and do not have an organellar genome. Mitosomes have been found in anaerobic or micro-aerophilic organisms that do not have classic mitochondria including the archamoeba *Entamoeba histolytica* (Mai *et al.* 1999), the diplomonad *Giardia intestinalis* (Tovar *et al.* 2003), the microsporidian *Encephalitozoon cuniculi* (Katinka *et al.* 2001) and the apicomplexan *Cryptosporidium parva* (Henriquez *et al.* 2005) (figure 1). Mitosomes are also expected to be present in many or most of the close relatives of these groups. The only function attributed to mitosomes so far is the synthesis of FeS clusters (Tovar *et al.* 2003; Goldberg *et al.* 2008), which therefore appears to be the only function that is shared between all three types of mitochondria-like organelles. The mitosomes of *E. histolytica* and *Mastigamoeba balamuthi* may represent an exception to this rule, as in these amoebae the FeS-cluster biogenesis pathway appears to localize outside of the mitosomes. The function of the *E. histolytica* mitosome therefore remains unknown (Gill *et al.* 2007; Aguilera *et al.* 2008).

The grouping of mitochondria-like organelles into classic mitochondria, hydrogenosomes and mitosomes is useful to appreciate the metabolic diversity of these organelles. Given the numerous times that these metabolic specializations have occurred, it is perhaps unsurprising that there are some mitochondria-like organelles that cannot easily be categorized. For example, the recent characterization of mitochondria-like organelles in the anaerobic ciliate *Nyctotherus ovalis* and the stramenopile *Blastocystis* revealed features of both classic mitochondria and

hydrogenosomes (Boxma *et al.* 2005; Pérez-Brocal & Clark 2008; Stechmann *et al.* 2008; Wawrzyniak *et al.* 2008). In many respects, an acceptance that mitochondria are found in all eukaryotes, allowing for extreme variation in metabolic differences and specializations, would be a more reasonable and useful definition for this organelle than the tripartite categorization in common use.

(b) Eukaryotic phylogeny

In order to understand the evolutionary history of mitochondrial protein and tRNA import, it is important to understand the evolutionary relationships between the different organisms that are studied. This is not a trivial task; the more we know, the more we realize what we do not know about the evolutionary relationship of microbial eukaryotes. Previous models for the 'tree of life' that are still very popular have placed many micro-organisms that contain hydrogenosomes or mitosomes (e.g. *Trichomonas*, *Giardia* and Microsporidia) as well as the mitochondria-containing trypanosomatids at the base of the eukaryotic evolutionary tree (Sogin 1991). In this model, these select groups of organisms diverged very early from the rest of the eukaryotes which only much later radiated into the so-called crown group. The early diverging eukaryotes were considered to be primitive and hence expected to have retained ancestral traits. Accumulation of much more molecular data and their integration with morphological data led to a paradigm shift in the relationships (Simpson & Roger 2004; Adl *et al.* 2005): six major supergroups. Amoebozoa, Opisthokonta, Excavata, Chromalveolata, Rhizaria and Archaeplastida are now recognized which arose more or less simultaneously from an already complex last universal common ancestor at an unknown time (figure 1). The root of this new phylogeny and how the six supergroups are related to each other is unknown. In this new phylogeny, protozoa such as trypanosomatids, *Giardia* and *Trichomonas* are not more early diverging, nor more primitive, than other organisms (Dacks *et al.* 2008).

It is fair to say that a debate continues on whether or not the new more 'egalitarian' view of eukaryotic phylogeny is more appropriate than the old 'ladder-type model'. In this review, we decided to adopt the new concept of eukaryotic phylogeny as it offers a fresh view on the evolution of mitochondrial protein and tRNA import that has not been discussed before.

Mitochondria are found in species of all six supergroups. Hydrogenosomes are found in at least three of the supergroups and mitosomes are found in four of the supergroups (figure 1). This illustrates that while all mitochondria-like organelles derive from a single endosymbiotic event, what we describe as hydrogenosomes and mitosomes evolved independently more than once in different branches of the eukaryotic evolutionary tree.

There are two important consequences of this new phylogeny. First, it means that the absence of specific traits in any of the previously defined 'early diverging' eukaryotes is in most cases better explained by secondary loss of traits rather than reflecting the ancestral state

(Dacks *et al.* 2008). Second, it is important to realize that yeast and metazoans, which include most of the popular model systems in biology such as *Drosophila*, yeasts, mouse, etc., belong to the same supergroup of the Opisthokonta. Thus, studies in these organisms only reflect a small part of eukaryotic diversity.

(c) Gradual loss of endosymbiont genes

There is little doubt that acquiring a bacterial endosymbiont and its subsequent transformation into a mitochondrion was a key event in the early evolutionary history of eukaryotes. How natural selection has favoured the maintenance of the symbiosis and the conversion of the endosymbiont to an organelle is hotly debated, and a number of scenarios that differ by the suggested metabolic nature of the endosymbiosis are being discussed (Embley & Martin 2006).

A major driving force of this transition was the gradual loss of genes from the genome of the endosymbiont (Adams & Palmer 2003). Interestingly, however, in the case of classic mitochondria, this loss never went to completion and all have retained a genome. The coding content of these genomes varies between five genes in apicomplexans (Feagin 2000) to 97 genes in the protozoan *Reclinomonas americana* (Lang *et al.* 1997). With few exceptions, proteins encoded on mitochondrial genomes are components of the respiratory chain or factors of the mitochondrial translation system that produces them. Having a mitochondrial genome is costly as it needs to be maintained, replicated and expressed. Mitochondrial translation alone requires well over 100 proteins (e.g. approx. 80 ribosomal proteins, 20 aminoacyl-tRNA synthetases and translation factors) as well as at least 22–24 different structural RNAs (rRNAs and tRNAs). Given the fact that only few proteins—in the case of some apicomplexans, only three—are actually produced inside the organelle, the continued existence of a mitochondrial genome appears as an incredible waste of resources (Feagin 2000). However, for reasons that we still do not fully understand, it seems that it is not possible to sustain oxidative phosphorylation without producing at least a few proteins inside the organelle. In order to explain this surprising fact, two main hypotheses, the 'hydrophobicity' theory (von-Heijne 1986) and the 'co-location for redox regulation theory' (Allen & Raven 1996), have been proposed.

Most of the genes that were lost from the endosymbiont genome were essential for the survival of the symbiont and their absence had to be compensated for. This was achieved by two strategies. The most important one was that many mitochondrial protein genes did not disappear for good but were functionally transferred to the nucleus. In order for the organelle to make use of the transferred genes, they needed to acquire regulatory sequences that allowed their transcription in the nucleus as well as their translation in the cytosol. Furthermore and most importantly, the resulting proteins needed to be imported into mitochondria.

A relatively small group of essential protein genes of the endosymbiont did indeed disappear, which was

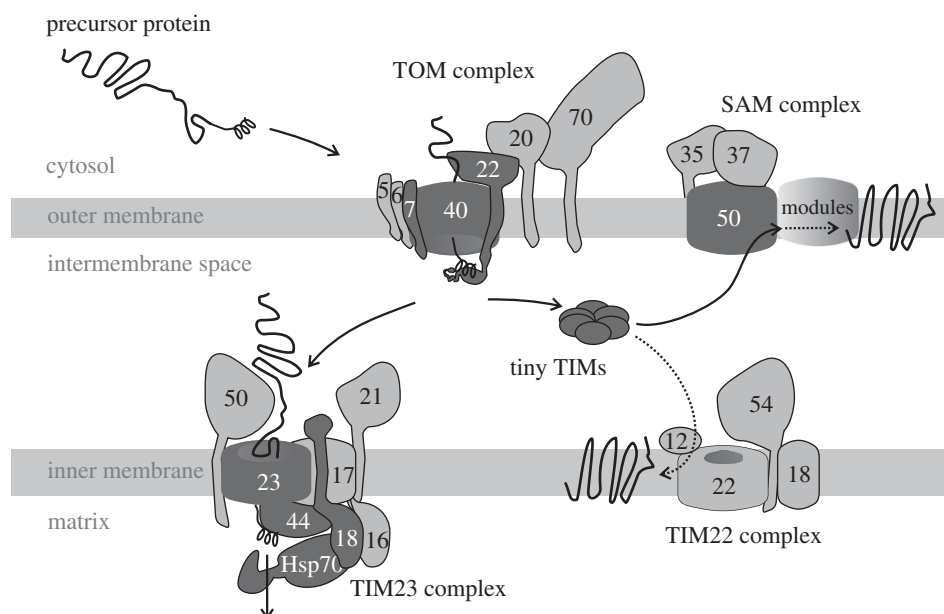


Figure 2. The range in complexity of mitochondrial protein transport machines. The protein import machinery from yeast (*Saccharomyces cerevisiae*) is depicted. Protein substrates (black) translated on cytoplasmic ribosomes contain *N*-terminal, helical targeting segments that enable their binding to the TOM complex on the mitochondrial surface. There are seven components of the TOM complex, with the Tom40, Tom7 and Tom22 subunits (dark grey) being found generally in eukaryotes, while the other subunits (light grey) are found only in opisthokont. Similarly, the Sam50 subunit of the SAM complex has a common occurrence in eukaryotes, while the other subunits of this complex are more restricted. Four subunits of the TIM23 complex (dark grey) are common to the eukaryotic kingdoms, while the subunits shown in light grey are restricted in their occurrence and probably evolved later. The TIM22 complex might be considered a later addition to the protein import pathway (Schneider *et al.* 2008). Protein substrates pass from the TOM complex to either the SAM, TIM22 or TIM23 complexes for their transport to the outer membrane, inner membrane or matrix, respectively.

made possible because their role was taken over by pre-existing nuclear-encoded proteins with equivalent functions. In contrast to proteins encoded by genes transferred to the nucleus, most of these were only in part imported into mitochondria and their dual localization allowed them to simultaneously perform their newly acquired mitochondrial as well as their traditional cytosolic function. How many proteins are imported into mitochondria is revealed by comprehensive mitochondrial proteomic studies in various organisms which have identified up to 1000 or more different proteins (Sickmann *et al.* 2003; DaCruz *et al.* 2005; Millar *et al.* 2005; Panigrahi *et al.* 2009). Most of these are nuclear-encoded, synthesized in the cytosol and therefore need to be imported into mitochondria.

Not only protein but also tRNA genes were lost during mitochondrial evolution. This loss was very variable: some mitochondria have kept the total set of mitochondrial tRNA genes, many have lost a subset and in a few cases the process went to completion and the entire set was lost (Schneider & Marechal-Drouard 2000; Salinas *et al.* 2008).

2. MITOCHONDRIAL PROTEIN IMPORT

(a) Occurrence of protein import

After 30 years of research into mitochondrial protein import, much of it focused on the model yeast *S. cerevisiae*, a clear picture is emerging of how mitochondrial proteins are recognized and imported by the organelle. As detailed in the following sections, a

series of molecular machines serve as protein translocases to import nuclear-encoded mitochondrial proteins from their site of synthesis in the cytosol to the internal compartments of mitochondria. Translocation across the outer membrane is mediated by the TOM complex (translocase of the outer membrane of mitochondria), and translocation across the inner membrane is mediated by a TIM complex (translocase of the inner membrane of mitochondria). Another ubiquitous complex, the sorting and assembly machinery (SAM) complex, receives imported proteins destined for the outer membrane from the TOM complex, and catalyses integration and assembly of this select set of mitochondrial proteins (figure 2).

Analysis of genome sequence data shows that representative organisms, from across the major supergroups, all have a SAM complex. The core subunit of the SAM complex is a protein called Sam50 (Kozjak *et al.* 2003; Paschen *et al.* 2003; Gentle *et al.* 2004), which is related to the bacterial protein Bama (Omp85) and was inherited from the α -proteobacterial ancestor to mitochondria. The beta-barrel assembly machinery (BAM) complex, containing Bama, assembles bacterial proteins into the outer membrane (Misra 2007; Gatsos *et al.* 2008; Knowles *et al.* 2009), just as the SAM complex assembles mitochondrial proteins into the outer membrane. Whereas the evolutionary progenitor to the SAM complex is clear, there are no obvious bacterial protein translocases that could have given rise to the TOM and TIM complexes. Bacteria are not known to import proteins across their outer or inner membranes. There are hints that evolution

may have cobbled together these complexes from various bacterial protein parts, using proteins with no previous function in protein transport. Taken together, the best model suggests that the TOM and TIM complexes were purpose-built to respond to new pressures arising from developments in the endosymbiotic relationship. Perhaps the strongest evidence to date that the hydrogenosomes, mitosomes and mitochondria have all arisen from a common organelle comes from the observations that they all share this common protein import machinery, composed of the TOM and TIM complexes, which evolved specifically to import nuclear-encoded proteins into mitochondria (Dolezal *et al.* 2006). While this evidence is largely derived from 'in silico' studies, components of the TIM complex are found in mitosomes and hydrogenosomes (Dolezal *et al.* 2005), and a recent study on the TOM complex in *Giardia* shows that it localized to mitosomes and related to the core TOM complex from the mitochondria of yeast (Dagley *et al.* 2009).

(b) Targeting of proteins to mitochondria

Work done in yeast and other organisms shows that targeting sequences can be found at the *N*-terminus, *C*-terminus or internally in proteins destined for mitochondria. Many membrane proteins, for example, do not have the otherwise typical *N*-terminal targeting sequence that has been well studied and found to occur on almost all soluble proteins and on many membrane proteins too. It remains, however, a useful generalization to posit that mitochondrial proteins are designated by an *N*-terminal, positively charged, amphipathic sequence that can form an α helix, thereby displaying surface features that are recognized by the TOM and TIM complexes. While the nature of mitochondrial targeting sequences is more complex, with as many exceptions as there are cases following the rule, a simple *N*-terminal, basic, amphipathic, helical sequence will direct even a non-mitochondrial protein passenger along the protein import pathway and into the mitochondrial matrix (Lemire *et al.* 1989). The hydrophobic surface of the amphipathic helix is recognized by Tom20 and a positively charged surface recognized by Tom22 (Brix *et al.* 1999; Moberg *et al.* 2004; Saitoh *et al.* 2007; Yamano *et al.* 2008). These same segments of the protein that adopt an α -helical conformation in contact with Tom20 (Saitoh *et al.* 2007) adopt an extended conformation when bound by the matrix-located processing peptidase (MPP).

How mitochondrial targeting sequences evolved has been a difficult question to address. Detailed analyses using comparative genomics made clear the later stages of mitochondrial evolution, e.g. how escape of genes (either the DNA or an RNA copy of the gene) from the endosymbiont could enable transfer to the nucleus and integration in the genome, how adaptive rearrangements could allow gene expression and how exon shuffling and other recombination events could create a mitochondrial targeting sequence (Brennicke *et al.* 1993; Kadowaki *et al.* 1996; Kurland & Andersson 2000; Gray *et al.* 2001). A major impetus for the development of the TOM complex, however,

was likely to have relied on pre-adaptive features, literally basic amphipathic *N*-terminal segments, in many bacterial proteins that would serve as mitochondrial targeting information (Lucattini *et al.* 2004).

The structural aspects in mitochondrial targeting sequences are conserved across all six supergroups of eukaryotes: mitochondrial proteins from animals or fungi are targeted to mitochondria in organisms from other kingdoms, and vice versa (Bowler *et al.* 1989; Häusler *et al.* 1997; Alvarez-Forbes *et al.* 1998; Bhaduri-McIntosh & Vaidya 1998; Murcha *et al.* 2003; Dolezal *et al.* 2005; Burri *et al.* 2006; Uboldi *et al.* 2006). In all cases, the targeting sequences studied feature positively charged residues in a context that could adopt an *N*-terminal, amphipathic helix. In many cases, the targeting sequence also includes features that might assist its folding to a helical structure, and extensions that would allow for processing by the highly conserved inner membrane protease (IMP) or mitochondrial processing protease (MPP) peptidases in the intermembrane space and matrix, respectively. These proteases evolved from bacterial processing peptidases: (i) IMP is related to the signal sequence processing leader peptidase and (ii) MPP is related to single subunit peptidases from α proteobacteria (Gakh *et al.* 2002; Burri *et al.* 2005; Brown *et al.* 2007; Smíd *et al.* 2008).

The same targeting sequences can serve to target proteins between all three classes of mitochondria-like organelles: hydrogenosomal sequences target proteins into mitosomes or mitochondria, and mitosomal sequences target proteins to mitochondria and hydrogenosomes (Häusler *et al.* 1997; Dolezal *et al.* 2005; Burri *et al.* 2006; Uboldi *et al.* 2006). In all three cases, the core targeting sequence can be a short segment corresponding to just two to three turns of an α helix. Work done in *Leishmania major* showed that a simple sequence motif of only eight residues, which described a basic amphipathic sequence, was sufficient to predict up to 100 of the proteins targeted to mitochondria from genome sequence alone (Uboldi *et al.* 2006). A tailored bioinformatic approach based on the recognition site for MPP processing (RXF/[ILF-SAGQ] or R[FNESG]/[ILFSAGQ]) detected 147 proteins from the proteome of *T. vaginalis* that might be targeted into hydrogenosomes, with the majority of these putative targeting sequences being 10 residues or less (Smíd *et al.* 2008). Using random DNA sequences, Lemire *et al.* (1989) showed that a large collection of synthetic sequences of 10–14 residues is necessary and sufficient to target passenger proteins to mitochondria. The summary picture then is that while the extension to be proteolytically cleaved from a mitochondrial protein can in some cases be very long, the information needed for mitochondrial targeting is housed within a portion of the sequence, in just two to three turns of an α -helical segment.

(c) Membrane translocation of proteins

Mitochondrial targeting sequences are recognized sequentially by a series of protein translocases (Neupert & Brunner 2002; Rehling *et al.* 2003;

Koehler 2004; Dolezal *et al.* 2006; Bolender *et al.* 2008) (figure 2). Translocation across the outer membrane is mediated by the TOM complex, a molecular machine composed of several integral membrane protein components. The receptors Tom70 and Tom20 (Endo & Kohda 2002; Perry & Lithgow 2005) recognize mitochondrial proteins and then release them into a translocation channel provided by the beta-barrel protein Tom40. In yeast, there are four additional subunits docked onto Tom40 to form the 'core' TOM complex: Tom5, Tom6, Tom7 and Tom22 (Meisinger *et al.* 2001). Structural studies using NMR and electron microscopy, as well as electrophysiology and a range of biochemical assays, have been used to investigate and define the steps of protein import at the level of the outer membrane (Verschoor & Lithgow 1999; Herrmann & Neupert 2000; Endo & Kohda 2002; Neupert & Brunner 2002; Koehler 2004; Rehling *et al.* 2004; Perry & Lithgow 2005; Bolender *et al.* 2008).

Studies done in yeast have shown that after passing through the TOM complex, imported proteins can interact with one of the two distinct machines in the inner membrane (figure 2). One of these, the TIM23 complex, is built around a channel formed from Tim23, with this channel allowing for substrate entry to the mitochondrial matrix. Translocation through the TIM23 complex is driven by a motor complex built around a mitochondrial Hsp70. The molecular chaperone Hsp70 is anchored to the membrane by J proteins, Tim16/Pam16 and Tim14/Pam18 (Koehler 2004; Rehling *et al.* 2004) and the peripheral inner membrane protein Tim44: this tethering of Hsp70 to the translocation channel harnesses its activity to drive substrate proteins into the matrix.

The second TIM complex is built around the Tim22 subunit, and this TIM22 complex functions in the insertion of multi-topic inner membrane proteins. Interestingly, Tim22 and Tim23 appear to have come about through gene duplication events in many organisms: but some organisms, including 'excavates' such as *T. brucei* (Schneider *et al.* 2008) and opisthokonts like *E. cuniculi* (Waller *et al.* 2009), have only a single 'TimX' protein and probably therefore a single TIM complex to function both for translocation through the inner membrane and for assembly into the inner membrane. Even in yeast, the TIM23 complex is known to sort some proteins into the inner membrane by a 'stop-transfer' mechanism (Neupert & Brunner 2002; Koehler 2004; Kutik *et al.* 2007), demonstrating the versatility of this machine.

A small TIM complex operates in the mitochondrial intermembrane space to deliver imported membrane proteins from the TOM complex to the SAM complex (in the case of proteins destined for the outer membrane) or the TIM22 complex (in the case of proteins destined for the inner membrane) (Koehler 2004). The small TIM proteins function in partnerships, forming hetero-oligomeric hexamers that serve as chaperones for the membrane proteins en route through the intermembrane space. When catalogued across the major eukaryotic kingdoms, there are four small TIM families: Tim8, Tim9, Tim10 and Tim13 (Gentle *et al.* 2007). In yeast and in

humans, the Tim8 and Tim13 subunits form a hetero-hexameric complex (i.e. Tim8₃:Tim13₃) and the Tim9 and Tim10 subunits form a distinct hetero-hexameric complex (i.e. Tim9₃:Tim10₃) (Webb *et al.* 2006; Baker *et al.* 2007). A fifth small TIM, Tim12, was derived recently through a gene duplication (from Tim10 in yeast, and from Tim9 in animals), and an equivalent scenario of 'four-plus-one' small TIMs is found in plants and in apicomplexans suggesting that the four TIM families arose early in the evolution of eukaryotes, with the fifth small TIM having been derived independently in many lineages. However, more simple sets of small TIMs can be seen with the mitosomes of the apicomplexan *Cryptosporidium* having a single small TIM protein (Gentle *et al.* 2007). Whether this orphan protein forms a homo-hexamer or functions as a smaller chaperone is not clear.

A set of highly conserved subunits in the SAM, TOM and TIM complexes are found in enough representative supergroups that they can be considered ubiquitous in eukaryotes (figure 3). It has been proposed, therefore, that one of the early events in the evolution of the first eukaryotic cells was to establish a protein import pathway, using relatively simple protein translocases (Lucattini *et al.* 2004; Dolezal *et al.* 2006). Through the course of evolution, lineage-specific components have been added on to these primary machines, with the best case in point being the Tom20 subunit of the TOM complex. Tom20 is shaded grey in figure 2, because hidden Markov models based on the biochemically defined receptor characterized in fungi (Söllner *et al.* 1989; Ramage *et al.* 1993) find homologues only in fungi and animals (Likić *et al.* 2005). The simplest interpretation is that this protein was developed as an import receptor in the early stage of divergence of the opisthokont lineage. This yeast/animal Tom20 is not an essential component of a viable TOM complex, with yeast mutants lacking Tom20 growing only slightly less well than wild-type yeast (Lithgow *et al.* 1994). In addition, it is now clear that microsporidians have lost the gene encoding Tom20 secondarily (Burri *et al.* 2006; Waller *et al.* 2009). Evidence that other lineages of eukaryotes independently acquired a Tom20-like receptor to improve protein import efficiency comes from analysis of the 20 kDa subunit of the TOM complex in plants (Werhahn *et al.* 2001): this protein functions as an import receptor, but structural analysis shows that the protein is coded in reverse with respect to the yeast Tom20, clearly demonstrating that this distinct import receptor arose by convergent evolution (Perry *et al.* 2006).

More extreme cases of gene loss are starting to become apparent in select, unrelated, parasites. Figure 3 shows what on the surface appears to be a radical gene loss for components of the import apparatus found in the microsporidian *Encephalozoon cuniculi* and the amoeba *E. histolytica*. As with any sequence-based analysis, one must be aware of the potential for confounding negative results that might simply be explained by extreme sequence divergence. But the analyses that figure 3 summarizes made use of sensitive hidden Markov models, and very close

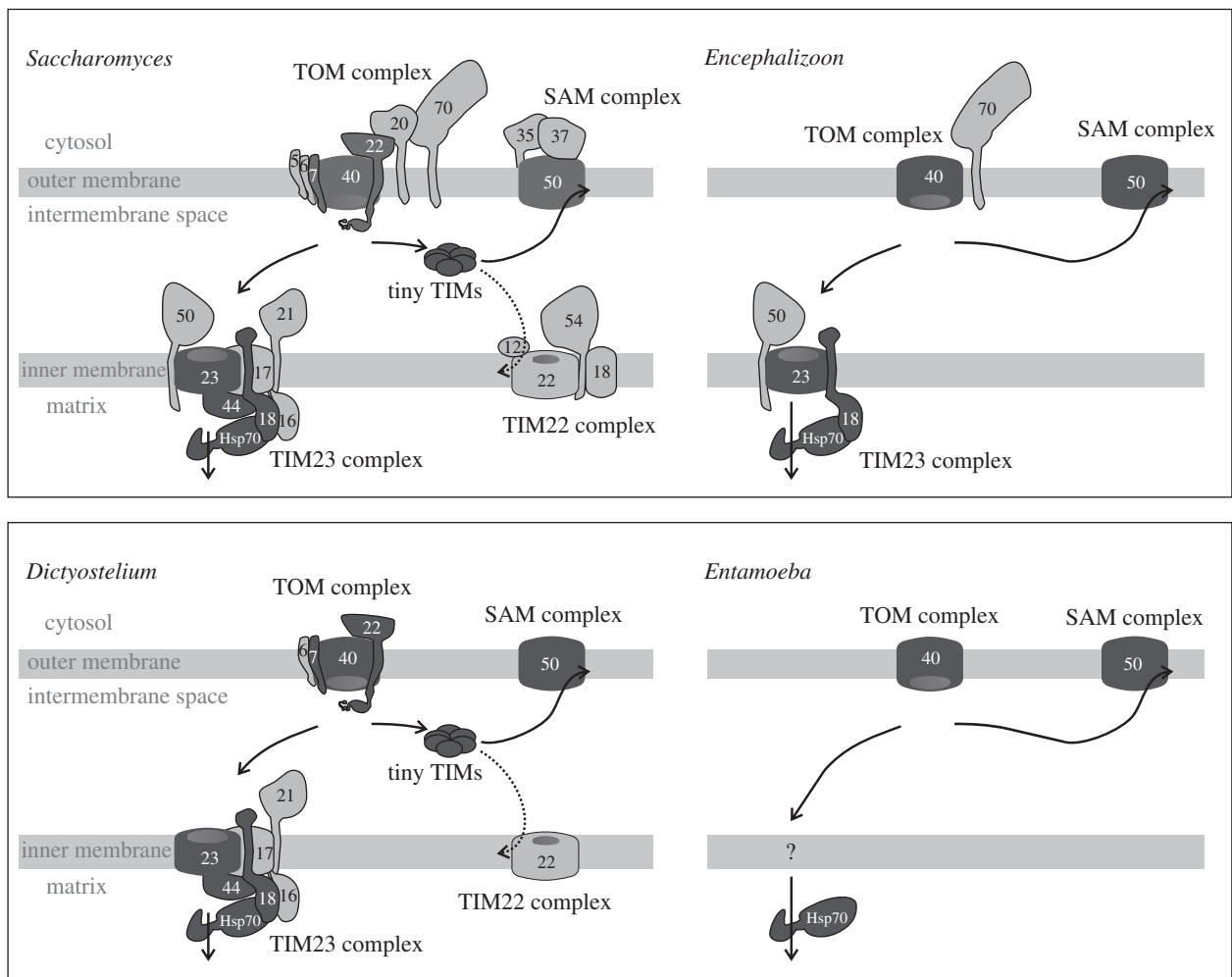


Figure 3. Protein transport machines in ‘classic’ mitochondria and mitosomes: a tale from two kingdoms. The opisthokont kingdom includes fungi and microsporidia. Protein sequence similarity between species of fungi and microsporidia is high, enabling confident predictions of mitochondrial protein import components in microsporidia (Burri *et al.* 2006; Waller *et al.* 2009), and leading to the suggestion that microsporidia have managed a secondary loss of numerous modules from the protein import machinery. At least in the context of the small proteome likely for the mitosomes, small TIM chaperones and the TIM22 complex can be dispensed with and the TOM, SAM and TIM23 complexes can be highly simplified. In the case of Amoebozoa, we might anticipate that further components of the protein import machinery, specific to this lineage, might be found. In *Dictyostelium discoideum*, the common ‘core’ components in the TOM, TIM23 and TIM22 complexes and the small TIM chaperones are recognizable (Dolezal *et al.* 2006). However, much of this machinery appears to have been dispensed with secondarily in the amoeba *E. histolytica*.

sequence relationships exist for the other components of the protein import machinery in these organisms. Furthermore, microsporidians are opisthokonts, and the pairwise sequence similarities are very high between the components of the import machinery from the yeast *Saccharomyces* and the microsporidian—yet none of the other components of the machinery were identified even with the most sensitive hidden Markov model searches (Waller *et al.* 2009). *Dictyostelium discoideum* is a well-studied model organism and the genome of this amoeba encodes all the expected core subunits of the TOM, SAM and TIM complexes (Barth *et al.* 2007). This would argue that sequence divergence between the amoebozoa and opisthokonts is not a barrier to detection of components of the mitochondrial import pathway. However, while the related amoeba *E. histolytica* has mitosomes with clear matrix-located chaperones and Tom40 and Sam50 proteins to serve in a TOM and SAM complex, further import machinery was not

detectable (Tovar *et al.* 1999; Bakatselou *et al.* 2003; Tovar *et al.* 2007; Likić *et al.* in press).

3. MITOCHONDRIAL RNA IMPORT

Just as the loss of protein-coding genes to the nucleus instigated a need for protein import, loss of tRNA-coding genes is coupled with tRNA import into mitochondria. Translation of the few proteins encoded in the mitochondrial genome requires, depending on the genetic code and the wobble rules, at least 20–22 different tRNAs. Experimental analysis in a number of different species confirmed that this has been achieved with an import pathway for tRNAs (Schneider & Marechal-Drouard 2000; Entelis *et al.* 2001b; Tarassov *et al.* 2007; Salinas *et al.* 2008). While it would be possible in principle, there is no evidence in any system for a functional transfer of mitochondrial tRNA genes to the nucleus; instead, imported tRNAs derive from

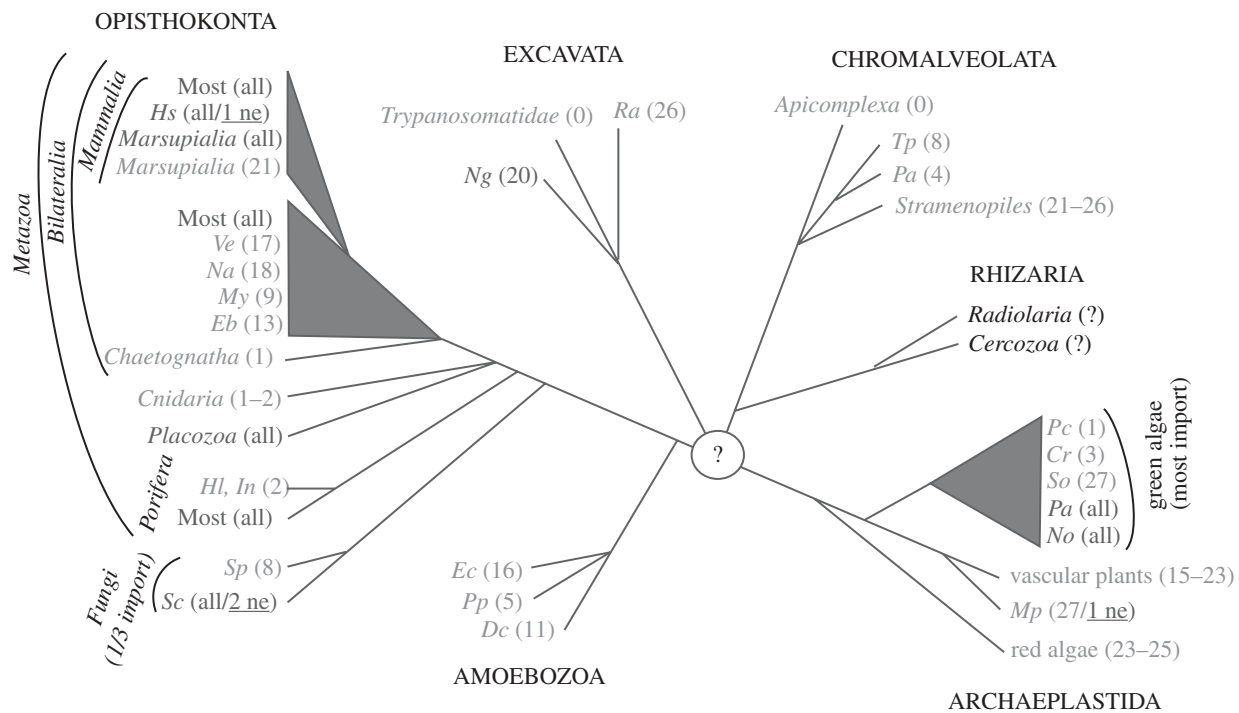


Figure 4. Occurrence of mitochondrial tRNA import. Unrooted phylogenetic tree of the six eukaryotic supergroups (indicated in capitals). Branching order reflects the phylogenetic relationship of taxa but branch length is not to scale. Bioinformatic analysis of complete mitochondrial genome sequences allows to predict whether the encoded tRNAs are sufficient to read all codons that are used by the corresponding mitochondrial translation systems. Based on this analysis, eukaryotes were divided into two groups: the ones having a complete set of mitochondrial tRNA genes (shown in dark grey) and the ones that lack a variable number of apparently essential mitochondrial tRNA genes (shown in light grey). Taxons shown were chosen to represent organisms having the most complete as well as the most reduced mitochondrial tRNA gene contents, respectively. The numbers of tRNA genes encoded in the different mitochondrial genomes are indicated. ‘All’ indicates that the mitochondrial-encoded tRNA gene set is complete and ‘0’ indicates complete absence of mitochondrial tRNA genes. If organisms retain a single mitochondrial tRNA gene only it is always the tRNA^{Met}. The minimal number of tRNAs required for mitochondrial translation is, depending on the wobble rules and the genetic code variations, between 20–22. Thus, all organisms having 20 or less mitochondrial tRNA genes must import at least some tRNAs from the cytosol. However, even in organisms having more than 22 mitochondrial tRNA genes, the set of mitochondrial-encoded tRNA is often not complete and import of cytosolic tRNAs is required. In most of these cases, it is the tRNA^{Thr} that is imported. In a few systems, import of a cytosolic tRNA that has the same decoding capacity as a still existing mitochondrial-encoded tRNA gene has been shown experimentally (shown underlined). Import of these tRNAs is expected to be redundant. Acronyms for species: *Hs*, *Homo sapiens*; *Vē*, *Vanhornia eucnemidarum*; *Na*, *Neomaskellia andropogonis*; *My*, *Mizuhopecten yessoensis*; *Eb*, *Epiperipatus biolleyi*; *Hl*, *Hypospongia lachne*; *In*, *Igornella notabilis*; *Sp*, *Spizellomyces punctatus*; *Sc*, *Saccharomyces cerevisiae*; *Ng*, *Naegleria gruberi*; *Ra*, *Reclinomonas americana*; *Tp*, *Tetrahymena pyriformis*; *Pa*, *Paramecium aurelia*; *Pc*, *Polytomella capuana*; *Cr*, *Chlamydomonas reinhardtii*; *Sc*, *Scenedesmus obliquus*; *Pa*, *Pseudoclonium akinetum*; *No*, *Nephroselmis olivacea*; *Mp*, *Marchantia polymorpha*; *Ec*, *Entamoeba castelani*; *Pp*, *Physarum polycephalum*; *Dc*, *Dictyostelium citrinum*.

cytosolic tRNAs that are also essential for cytosolic translation. The import of cytosolic tRNAs therefore must have preceded the loss of mitochondrial tRNA genes.

(a) Occurrence of mitochondrial tRNA import

Complete mitochondrial genome sequences are available for more than a 1000 different species of eukaryotes (O’Brien *et al.* 2009). Bioinformatic analysis of these sequences can predict the number of mitochondrial tRNA genes and match these to the codons that are used by the corresponding mitochondrial translation systems. The conclusion of such an analysis is that most eukaryotes lack some of the essential mitochondrial tRNA genes in their mitochondrial genomes (figure 4). Experimental analysis in a number of these systems has shown that this lack is compensated for by import of the corresponding

cytosolic tRNAs. Thus, imported tRNAs always represent a small fraction of the cytosolic tRNA pool. Exclusive mitochondrial localization of a nucleus-encoded tRNA has so far not been found, though a tantalizing possibility exists in the recently described *Chlamydomonas reinhardtii* tRNA^{Lys}UUU (Vinogradova *et al.* 2009).

The phylogenetic distribution of mitochondrial tRNA, as predicted by bioinformatics, shows that contrary to popular belief the process is very widespread. At least some tRNAs are imported in the vast majority of species in probably all six eukaryotic supergroups (mitochondrial genome sequences for Rhizaria are still missing) (figure 4). It is organisms with a complete set of mitochondrial tRNA genes that are exceptional rather than the ones lacking them! Essentially, all these exceptions are opisthokont species and yet even within the opisthokonts, we find taxa such as the Cnidaria and the Chaetognatha (arrow worms) that

have lost all but one or two mitochondrial tRNA genes. In addition, within the Fungi and the Bilateria, there are many examples of individual species that have lost at least some mitochondrial tRNA genes whereas their close relatives have kept the whole set (figure 4).

It is likely that the original endosymbiont had a complete set of tRNA genes, and loss of mitochondrial tRNA genes is expected to be irreversible. Thus, having a complete set of mitochondrial tRNA genes represents the ancestral situation. It follows that the loss of mitochondrial tRNA genes and import of the corresponding cytosolic tRNAs are derived traits. Based on this, we conclude that mitochondrial tRNA import has a polyphyletic evolutionary origin: it was invented many times in different branches of the eukaryotic evolutionary tree.

(b) Limits of bioinformatic analysis

One of the caveats on bioinformatic analyses aimed at the occurrence of mitochondrial tRNA import is that modifications of the wobble nucleotide, which cannot be predicted, can change the decoding capacity of a tRNA, making it difficult to match it to a specific codon set. Some mitochondrial tRNAs have unconventional structures (Wolstenholme *et al.* 1987) or undergo RNA editing (Bullerwell & Gray 2005) and are therefore hard to recognize. Moreover, the existence of a mitochondrial tRNA gene does not preclude mitochondrial import of a cytosolic tRNA that is able to read the same codons. In fact, a scenario like this may even have been an obligatory evolutionary intermediate that subsequently allowed the loss of the corresponding mitochondrial tRNA genes.

The limitations to predict scenarios for tRNA import can best be illustrated in the yeast *S. cerevisiae*. The yeast mitochondrial genome encodes an apparently complete set of mitochondrial tRNAs. Nevertheless, yeast mitochondria import a small fraction of one of two cytosolic tRNA^{Lys} isoacceptors (Tarassov *et al.* 1995b). Import of this tRNA is redundant under standard growth conditions but becomes essential when cells are grown at elevated temperature (Kamenski *et al.* 2007). Import of the tRNA^{Lys} represents the best studied case of mitochondrial tRNA import in any species (discussed below). A recent study suggests that besides the tRNA^{Lys} also a small fraction of the cytosolic tRNA^{Gln} is imported into yeast mitochondria (Rinehart *et al.* 2005). The function of the imported tRNA^{Gln} is presently unknown. However, surprisingly, it seems to be imported by a different pathway than the tRNA^{Lys}.

Moreover, a complex situation is also found in humans. Just as in yeast, the human mitochondrial genome encodes a complete set of mitochondrial tRNAs. Recent evidence, however, suggests that a fraction of the cytosolic tRNA^{Gln} is imported into mitochondria (Rubio *et al.* 2008). In addition to tRNA import, import of the RNA subunits of RNase P (Puranam & Attardi 2001) and RNase MRP (Chang & Clayton 1989) as well as the 5S rRNA (Magalhaes *et al.* 1998) has been suggested. In the case of RNase P, these claims are highly controversial

as mammalian mitochondrial RNase P has recently been shown to lack an RNA subunit (Holzmann *et al.* 2008). Import of 5S rRNA is also surprising as no 5S rRNA has been found in mitochondrial ribosomes of mammals (Sharma *et al.* 2003). However, import of 5S rRNA has been analysed in some detail using an *in vitro* system and it has been proposed that the RNA might be co-imported with an as-yet-unknown protein (Entelis *et al.* 2001a).

While it is clear that the described cases are of great interest and need to be further investigated, they are exceptions and, at least in the case of tRNAs, bioinformatics remains a valid tool to analyse the distribution of mitochondrial import on a global scale.

(c) Non-random loss of mitochondrial tRNA genes

The extent of tRNA gene loss from the mitochondrial genome is variable (figure 4). The most extreme situations are found in some poriferan species, Cnidaria, Chaetognatha and the green algae *Pseudendoclonium akinetum*, which have retained only one or two mitochondrial tRNA genes, and in the trypanosomatids and apicomplexans that lack mitochondrial tRNA genes altogether (O'Brien *et al.* 2009).

Even though the loss of specific mitochondrial tRNA genes does not show a defined phylogenetic pattern, it follows a distinct order when analysed across all taxa. This can best be explained by the fact that lost mitochondrial tRNA genes of bacterial genetic origin are compensated for by import of a fraction of cytosolic tRNAs that are of eukaryotic origin. The evolutionary origin of tRNAs can formally be demonstrated only for few species that show domain specific features. However, circumstantial evidence such as the fact that nucleus-encoded tRNAs are transcribed by RNA polymerase III suggests that all imported tRNAs are of eukaryotic descent.

Compensation can only be successful if the imported tRNA can be functionally integrated into the bacterial-type translation system of mitochondria. For the initiator tRNA^{Met}, this is difficult. Translation initiation is very different in eukaryotes and in systems of bacterial origin, the latter requiring a specific initiator tRNAs^{Met} that carries a formylated methionine (Mayer *et al.* 2001). This means that even if a cytosolic eukaryotic initiator tRNA^{Met} would be imported into mitochondria, it would not be functional as it could not be formylated. This explains why the single tRNA gene that has been retained in mitochondria of many Cnidaria, Chaetognatha and *P. akinetum* is the tRNA^{Met}.

Many mitochondria show variations from the classic genetic code, the most frequent one being a reassignment of the stop codon UGA to tryptophane (Knight *et al.* 2001). Decoding of the reassigned codon requires an anticodon change in the mitochondrial tRNA^{Trp} which for that reason cannot simply be replaced by its cytosolic counterpart that lacks this change. Thus, most mitochondrial genomes have kept the gene for the tRNA^{Trp}.

Can the concept that the overall frequency of mitochondrial tRNA gene loss is determined by the

efficiency of functional integration of an imported tRNA into the mitochondrial translation system be extended to all tRNAs? It has been suggested that overall the loss of mitochondrial tRNA genes follows a specific order which could be explained by the differential capabilities of mitochondrial aminoacyl-tRNA synthetases to recognize imported eukaryotic-type tRNAs (Schneider 2001a). In such a scenario, the loss of a mitochondrial tRNA gene would essentially be driven by how good its imported counterpart can be aminoacylated in the mitochondrion. The model is supported by the observation that the frequency of the loss of a specific mitochondrial tRNA gene is positively correlated with the similarities of the corresponding bacterial-type and eukaryotic-type aminoacyl-tRNA synthetases as well as the similarities of their corresponding identity elements on the tRNA (Schneider 2001a).

Thus, the difficulty to functionally integrate specific imported tRNAs into the mitochondrial translation system represents a barrier for mitochondrial tRNA gene loss. Translation initiation and mitochondrial codon reassignments appear to be especially strong handicaps for imported tRNAs. Surprisingly, however, two taxonomic groups, trypanosomatids (Schneider 2001b) and apicomplexans (Feagin 2000; Crausaz-Esseiva *et al.* 2004b), have lost all mitochondrial tRNA genes, indicating that these barriers are not absolute (see below).

(d) Mitochondrial targeting of tRNAs

The mechanisms of mitochondrial tRNA import in the various systems have recently been expertly reviewed (Salinas *et al.* 2008). Here, we provide a condensed discussion of this subject with the emphasis on aspects we consider important in the evolutionary context. To discuss how tRNAs are imported into mitochondria, it is helpful to subdivide the process into three temporally and spatially ordered steps: (i) targeting of the tRNA to the mitochondrion, (ii) membrane translocation, and (iii) integration of the imported tRNA into the mitochondrial translation system.

The number of imported tRNAs ranges from one only to the whole set and is species-specific. Interestingly, in some taxons such as plants, the import specificity can differ even in closely related species. However, in all species, at least a few tRNAs still exist that are exclusively cytosolic indicating the need of the cell to select a subset of tRNAs for mitochondrial import. This selection depends on targeting signals on the tRNAs and is mediated by proteins. There are only three systems where the signals that are both necessary and sufficient for mitochondrial tRNA import have been characterized in detail.

(i) The first case concerns import of a fraction of one of two tRNA^{Lys} isoacceptors into yeast mitochondria (Martin *et al.* 1979). Mitochondrial targeting of the tRNA^{Lys} requires specific binding to Eno2p, an isoform of the glycolytic enzyme enolase (Entelis *et al.* 2006). Enolase delivers the tRNA to the surface of mitochondria. There, the tRNA is released and can now bind to the precursor of mitochondrial lysyl-tRNA synthetase to which it has a higher affinity

than to enolase. The precursor of mitochondrial lysyl-tRNA synthetase is translated in the vicinity of mitochondria and acts as a carrier for import (see below). The specific binding of the two proteins to imported tRNA^{Lys} is primarily determined by acceptor stem and anticodon nucleotides that differ between the two tRNA^{Lys} isoacceptors (Entelis *et al.* 1998).

(ii) The second example is the trypanosomatid *T. brucei* in which all mitochondrial tRNAs are derived from cytosolic ones (Hancock & Hajduk 1990; Tan *et al.* 2002b). The initiator tRNA^{Met} and the tRNA^{Sec}, however, are not imported (Bouzaidi-Tiali *et al.* 2007). An *in vivo* analysis has shown that the single T-stem nucleotide pair at position 51:63 is both necessary and sufficient to determine the localization of trypanosomal tRNAs (Crausaz-Esseiva *et al.* 2004a). Thus, a U:A nucleotide pair at this position, found in the initiator tRNA^{Met}, specifies a cytosolic localization, whereas any other base pair indicates a mitochondrial localization. Interestingly, the U51:A63 nucleotide pair in the cytosolic initiator tRNA^{Met} has previously been characterized as an anti-determinant for elongation factor 1a (eEF1a) binding (Drabkin *et al.* 1998). In line with this, it was shown that mitochondrial targeting of trypanosomal tRNAs requires interaction with eEF1a. This also explains the cytosolic localization of the tRNA^{Sec} which lacks the U51:A63 cytosolic localization signal but nevertheless does not bind to eEF1a as it has its own specialized elongation factor. Thus, in *T. brucei*, eEF1a besides its housekeeping function in translation elongation has a second function in selecting a subpopulation of cytosolic tRNAs for mitochondrial import. Moreover, *in vivo* analysis has shown that eEF1a-mediated targeting of tRNAs to the mitochondria is an obligatory step for membrane translocation of tRNA to occur.

(iii) The third example is *Tetrahymena* which contains three very similar tRNA^{Gln} isoacceptors. Two of them with the anticodons UUA and CUA are cytosol-specific and recognize the stop codon UCA which has been reassigned to glutamine in the nucleus of *Tetrahymena*. The third tRNA^{Gln} with the anticodon UUG recognizes the standard glutamine codons and is in part imported into mitochondria (Rusconi & Cech 1996a). In an *in vivo* analysis, it was shown that the anticodon UUG of the imported tRNA^{Gln} is both necessary and sufficient to induce import of any of the three tRNA^{Gln} molecules (Rusconi & Cech 1996b). However, no protein interacting with the import signal has been identified yet.

These three examples illustrate that the targeting signals on the tRNA and the targeting factors are not conserved between the different systems. This is no surprise because it reflects the very different specificities of mitochondrial tRNA import in the three systems. Moreover, finding different targeting signals and mechanisms is in line with the presumed polyphyletic origin of mitochondrial tRNA import. However, despite these differences, there are also some striking similarities between yeast and trypanosomatids. Targeting of the tRNAs to the mitochondria is in both cases essential for subsequent membrane translocation of the tRNA. Moreover, targeting is mediated by cytosolic housekeeping proteins that perform a

second function. Further, research is needed to show whether this common principle of mitochondrial tRNA targeting can be extended to even more organisms.

There are a number of other *in vivo* studies that have attempted to identify the *cis*-elements on the tRNAs that induce their import into mitochondria (Dietrich *et al.* 1996; Lima & Simpson 1996; Delage *et al.* 2003). However, these studies are generally less complete and thus their interpretation is difficult. Moreover, targeting has also been analysed by *in vitro* import assays (Mahapatra *et al.* 1998; Rubio *et al.* 2000; Bhattacharyya *et al.* 2002). These assays were in most cases done in the absence of cytosol and therefore in this respect may not reflect the *in vivo* situation. Overall, the studies mentioned above identified all major tRNA domains as being important for mitochondrial tRNA targeting in one or the other system and therefore further illustrate the non-conserved nature of the tRNA targeting signals.

(e) *Extent of mitochondrial tRNA localization*

Studies in trypanosomatids (Tan *et al.* 2002b) and *Chlamydomonas* (Vinogradova *et al.* 2009), which import all or nearly all of their mitochondrial tRNAs, revealed large variations between the extent of mitochondrial localization of individual tRNAs. In both organisms, these variations were not correlated with the cytosolic concentration of the tRNAs. This raises two questions: why is the extent of mitochondrial localization of different tRNAs so variable and how is it regulated?

(i) The steady-state levels of *Chlamydomonas* cytosolic tRNAs correlate with the codon usage of nuclear genes as has been shown in other organisms as well. However, in *Chlamydomonas*, the same is true in mitochondria and the frequency of specific codons in mitochondrial genes appears to correlate with the levels of imported tRNAs that read them (Vinogradova *et al.* 2009). As the nuclear and the mitochondrial codon usage are different, this requires differential mitochondrial localization of tRNAs. Interestingly, in *T. brucei*, a similar study failed to reveal such a correlation (Tan *et al.* 2002b). However, as in this study only a subset of tRNAs was analysed, the question may need to be reinvestigated. Thus, at least in *Chlamydomonas*, the extent of mitochondrial localization may serve to adapt tRNA abundance to the mitochondrial codon usage.

(ii) What is responsible for the differential localization of imported tRNAs is not known in any system. Different mitochondrial steady-state levels of tRNAs could in principle be achieved by different import efficiencies or by regulating tRNA stability after import. A study in *Leishmania* suggests that it might be the former. Cytosolic leishmanial tRNA^{Glu} and tRNA^{Gln} have a thiomodified uridine at the wobble position whereas their imported counterparts are lacking this modification (Kaneko *et al.* 2003). Thus, it was proposed that the thiomodified uridine acts as an antideterminant that prevents mitochondrial tRNA import. However, *in vivo* evidence for this attractive proposal is yet to emerge.

(f) *Membrane translocation of tRNAs*

tRNAs destined to be imported into mitochondria must be translocated across the outer and the inner mitochondrial membranes. From *in vitro* import experiments, we know that this process requires ATP and in most but not all cases the membrane potential. Moreover, protease treatment of mitochondria prevents tRNA import indicating that it is mediated by proteins. Information on the nature of these protein factors is available in three systems: *S. cerevisiae*, *Leishmania* and plants.

(i) *Saccharomyces cerevisiae*

After targeting to the mitochondrial surface, the tRNA^{Lys} is released from enolase and binds to the precursor of mitochondrial lysyl-tRNA synthetase (pre-MSK) (Tarassov *et al.* 1995b). Subsequently, the folded tRNA is co-imported together with pre-MSK across the protein import channel (Entelis *et al.* 1998). This may seem surprising as it is difficult to see how the interaction between pre-MSK and the imported tRNA^{Lys} can be maintained during mitochondrial protein import which requires unfolding of the transported protein. However, the evidence for the co-import model is very convincing. *In vivo* and *in vitro* import of the tRNA^{Lys} strictly depends on the precursor of pre-MSK. The involvement of Tom20 and Tim44, two components of the protein import machinery, has directly been shown *in vivo* and *in vitro* (Tarassov *et al.* 1995a). Thus, in *S. cerevisiae*, pre-MSK has two functions, it aminoacylates the mitochondria-encoded tRNA^{Lys} and it is responsible for import of the cytosolic tRNA^{Lys}. Recent experiments have shown that the two functions can be separated and are associated with distinct regions of the pre-MSK molecule (Kamenski *et al.* 2007). The imported tRNA^{Lys} cannot be aminoacylated inside mitochondria. Yet, as it can only bind to pre-MSK when aminoacylated, it is imported as a functional tRNA that can take part in protein synthesis, even though recycling is not possible. Interestingly, the other tRNA that is imported into mitochondria of *S. cerevisiae*, the tRNA^{Gln}, is not co-imported with protein but by an as-yet-unknown mechanism (Rinehart *et al.* 2005).

(ii) *Leishmania*

The tRNA import pathway in *Leishmania tropica* has been elucidated in great detail by the group of S. Adhya. It can only be summarized here, for more information refer to Bhattacharyya & Adhya (2004), Mirande (2007) and Adhya (2008). The inner membrane tRNA import machinery of *L. tropica* appears to consist of an unconventional protein complex of approximately 580 kDa, termed tRNA import complex (RIC), that was initially characterized by affinity chromatography using an *in vitro*-defined tRNA-import signal (Goswami *et al.* 2006). Mass spectrometry analysis revealed that RIC consists of 11 major subunits, eight of which are nucleus-encoded and three that are mitochondria-encoded (Mukherjee *et al.* 2007). Six of the former are essential for tRNA import and four are identical to subunits of different respiratory

complexes (iron sulphur protein and subunit 6b of complex III, cytochrome oxidase subunit 6 of complex IV and F1 α subunit of complex V, the ATP synthase complex). Ablation of either of the six essential subunits by an unusual conditional antisense knockdown strategy reduced the level of mitochondrial tRNAs to zero within 24 h. Moreover, the functional RIC complex could be reconstituted into liposomes with recombinant subunits expressed in *Escherichia coli*. Omission of any of the six essential factors abolished ATP-dependent import of tRNAs into liposomes. How tRNAs are transported across the outer membrane has not been addressed.

Characterization of the tRNA import machinery of the *Leishmania* mitochondrial inner membrane is a truly amazing feat. However, a closer analysis of the published data raises a number of questions. Conditional ablation of specific leishmanial mRNAs has been tried by many groups using various strategies without success. Yet, in the *L. tropica* strain used by the Adhya group, the antisense strategy seems to work extremely efficiently. Moreover, reconstitution of the RIC complex—consisting of six different proteins—into liposomes was done starting from denatured proteins that were eluted from sodium dodecyl sulphate (SDS) gels and refolded (Mukherjee *et al.* 2007). While this might not be impossible, we are not aware of any precedent where such an approach has worked. Finally, *L. tropica* and *T. brucei* are closely related. We therefore would expect to find the same tRNA import machinery in both species. However, while the tRNA import machinery has not been identified in *T. brucei*, at least three of the trypanosomal RIC orthologues are not expressed in the bloodstream stage of the parasite (Panigrahi *et al.* 2009), even though it does import tRNAs. Thus, there is much left to be sorted out regarding the tRNA import machinery of trypanosomatids.

(iii) Plants

Recently, it was shown that antibodies against the voltage-dependent anion channel (VDAC), the metabolite transporter of the outer mitochondrial membrane, inhibited import of tRNAs into isolated plant mitochondria (Salinas *et al.* 2006). Consistent with these results, recombinantly expressed VDAC was able to bind tRNAs. *In vitro* import of tRNAs was also inhibited using antisera against Tom20 and Tom40, two conserved components of the mitochondrial outer membrane protein translocation machinery. However, the fact that *in vitro* import does not require a mitochondrial precursor protein together with tRNA import competition studies showed that, unlike in yeast, tRNAs and proteins are not co-imported. Based on these results, it was suggested that VDAC may be a major component of the tRNA import channel whereas Tom20 and Tom40 may function as import receptors (Salinas *et al.* 2006).

In summary, it appears clear that, in agreement with the postulated polyphyletic origin of mitochondrial tRNA import, the membrane translocation mechanism of tRNAs is not conserved between the different organisms. Moreover, there is no evidence

for a dedicated tRNA import machinery in any system. Instead, its components, just as the ones required for tRNA targeting, appear to be house-keeping components performing a second function.

(g) Functional integration of imported tRNAs

Imported tRNAs are always of the eukaryotic type, whereas the mitochondrial translation system is of bacterial descent. Many tRNAs might in principle be functionally interchangeable between the cytosol and the mitochondria. However, for the initiator tRNA^{Met} and for tRNAs that read codons that differ from the standard genetic code, this is not the case. Trypanosomatids and apicomplexans did not retain any mitochondrial tRNA genes. Their mitochondrial translation system therefore depends exclusively on imported eukaryotic-type tRNAs and as a consequence requires unique evolutionary adaptations. Some of these adaptations have been characterized in *T. brucei* and are discussed below.

Even though there is no bacterial-type initiator tRNA^{Met} in trypanosome mitochondria, translation initiation requires a formylated tRNA^{Met}. The tRNA that becomes formylated is a fraction of the imported eukaryotic-type elongator tRNA^{Met}. Formylation is catalysed by an unusual tRNA^{Met}-formyl-transferase that selectively formylates elongator-type tRNA^{Met} and therefore has a substrate specificity diametrically opposed to conventional formyl-transferases (Tan *et al.* 2002a). Thus, in *T. brucei*, the elongator tRNA^{Met} has three distinct functions that depend on its localization. In the cytosol, it functions as a conventional eukaryotic elongator tRNA^{Met}, whereas after import in the bacterial-type translation system of mitochondria it is used as both initiator and elongator tRNA^{Met}, depending on whether it is formylated or not (Martin 2002). The formylated methionine on the imported elongator tRNA^{Met}, but no specific feature of the tRNA itself, is the main determinant that is recognized by an apparently conventional bacterial-type initiation factor 2 (Charrière *et al.* 2005). Thus, the unusual formyl-transferase seems to be the only required adaptation allowing the use of elongator tRNA^{Met} in translation initiation.

In mitochondria of trypanosomatids, the stop codon UGA has been reassigned to tryptophane. The organellar tRNA^{Trp} therefore has to decode UGA in addition to the normal tryptophane codon UGG. It is not obvious how this can be achieved by an imported cytosolic tRNA^{Trp} that does not recognize the UGA stop codon.

Trypanosomatids solve this problem by a mitochondria-specific RNA editing event that converts the CCA anticodon of the imported tRNA^{Trp} to UCA (Alfonzo *et al.* 1999; Charrière *et al.* 2006). This allows the tRNA to decode both UGG and UGA codons. However, the CCA anticodon is an identity determinant for the eukaryotic tryptophanyl-tRNA synthetase. Thus, unlike most other imported tRNAs of trypanosomes, the edited tRNA^{Trp} in mitochondria cannot be charged by an aminoacyl-tRNA synthetase that is dually targeted

to the cytosol and the mitochondrion. Instead, trypanosomatids evolved a highly diverged eukaryotic-type tryptophanyl-tRNA synthetase that is specific for mitochondria and that, unlike its cytosolic counterpart, can aminoacylate both edited and unedited tRNA^{Trp} (Charrière *et al.* 2006). For trypanosomatids losing the mitochondrial tRNA^{Trp} gene was therefore very costly as it required the evolution of a specific enzyme that edits the tRNA^{Trp} as well as of a novel type of eukaryotic tryptophanyl-tRNA synthetase.

Many more adaptations of the mitochondrial translation system to imported tRNAs are likely to exist. Studies of how imported tRNAs are functionally integrated into the mitochondrial translation system have so far been restricted to trypanosomatids. It would be interesting to extend them to apicomplexans which are faced with the same problems. Apicomplexans belong to a different eukaryotic supergroup than trypanosomatids and therefore may have found different solutions. We believe that investigating the consequences tRNA import imposes on mitochondrial translation is of great interest. Most trypanosomatids and apicomplexans are clinically important pathogens. Thus, the parasite-specific adaptations of the mitochondrial translation system may offer novel drug targets. Moreover, exploring the limits of adaptation of a bacterial-type translation systems to eukaryotic components will help to reveal the fundamental requirements of translation.

4. CONCLUSIONS

Mitochondrial protein and tRNA import are key processes required for mitochondrial biogenesis. The problems faced in establishing each transport system are similar: most proteins and all tRNAs have to be translocated across the two mitochondrial membranes without disrupting the inner membrane potential. Import requires ATP, is protein-mediated and shows specificity: only a subset of the total cellular complement of proteins, or of tRNAs, are imported. There are of course some differences too: mitochondria in many, perhaps most, organisms import an estimated 500–1000 proteins with varying chemical properties. The number of imported tRNA molecules ranges from 1 to 30 depending on the species, and tRNAs have uniform structures and similar molecular weights (approx. 25 kDa). Also, the final localization of all imported tRNAs is the matrix, whereas proteins need to be sorted to the various mitochondrial subcompartments. It is clear that the evolution of mitochondrial import of macromolecules, both protein and tRNAs, was tightly linked to the gradual loss of genes from the genome of the endosymbiont (Adams & Palmer 2003). However, whereas the loss of most mitochondrial protein genes was shaped by their functional transfer to the nucleus, the disappearance of mitochondrial tRNA genes was largely governed by import of cytosolic tRNAs and their functional integration into the mitochondrial translation system. Moreover, whereas dual targeting to the cytosol

and mitochondria is exceptional for proteins, it is the rule for imported tRNAs.

Mitochondrial import and sorting of proteins have been studied for many years. They require one or more of four distinct hetero-oligomeric membrane protein complexes, depending on the nature and the intramitochondrial destination of the transported substrates (Dolezal *et al.* 2006; Neupert & Herrmann 2007; Bolender *et al.* 2008; Hildenbeutel *et al.* 2008; Mokranjac & Neupert 2009). These protein complexes contain a set of core components that are conserved in mitochondria, hydrogenosomes and mitosomes of all species indicating the monophyletic origin of mitochondrial protein import. While our knowledge is at an early stage, it does now appear that a universal pathway handles proteins imported into mitochondria and mitochondria-like organelles, one which already existed in the last common ancestor of all eukaryotes (Dolezal *et al.* 2006).

Compared with mitochondrial protein import, we know very little about mitochondrial tRNA import. Its phylogenetic distribution suggests that, in contrast to mitochondrial protein import, tRNA import was probably not present in the last common ancestor of all eukaryotes. Instead, it evolved multiple times in most branches of at least four of the supergroups of eukaryotes. Consistent with this idea, the mechanisms for tRNA import vary in the different lineages of eukaryotes (figure 5). Yet, despite these variations, there is a common theme: tRNAs appear to be transported by hitch hiking. They make use of distinct housekeeping proteins in order to be targeted to and imported into mitochondria. The lack of a dedicated tRNA import machinery is in line with the recent evolutionary origins of the process. Moreover, the fact that there are several ways to import tRNAs into mitochondria is good news for people who would like to use mitochondrial tRNA import as a tool to treat diseases caused by mitochondrial tRNA mutations. And it may explain why attempts to transplant tRNA import systems from one organism to another to complement mutated mitochondrial tRNAs have been amazingly successful at least in cell cultures (Entelis *et al.* 2001*b*, 2002; Salinas *et al.* 2008).

It should be remembered that the information on the phylogenetic distribution of mitochondrial tRNA import is incomplete. Having a complete set of mitochondrial tRNA genes is essentially restricted to opisthokont species (figure 4), and yet even some of these organisms (including ourselves) are known to import at least some tRNAs. This redundant tRNA import cannot be predicted bioinformatically but only be discovered experimentally. Thus, we cannot yet exclude that the import of redundant tRNAs occurs in many or even all mitochondria that have a complete set of mitochondria-encoded tRNA genes.

Our knowledge of tRNA import machineries is fragmentary: the characterization of the tRNA import machinery in plants is in its initial stages and, while import of the yeast tRNA^{Lys} is well characterized, it is not known how the yeast tRNA^{Gln} is imported. Moreover, in trypanosomatids, the tRNA translocation machinery of the outer membrane has not been

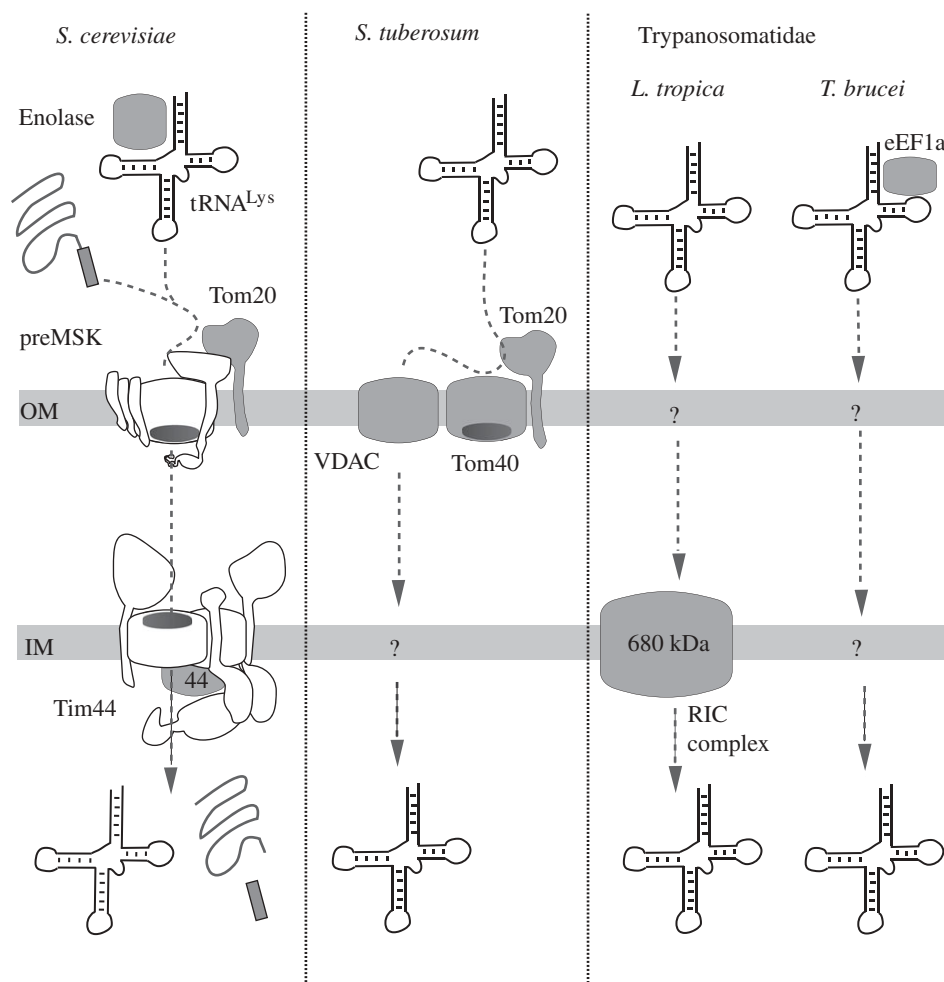


Figure 5. Current state of knowledge of mitochondrial tRNA import machineries. Components required for targeting and/or membrane translocation of tRNAs have been identified in yeast (*S. cerevisiae*), potato (*Solanum tuberosum*) and the Trypanosomatidae (*L. tropica* and *T. brucei*). Outer (OM) and inner mitochondrial membranes (IM) are indicated. Factors whose involvement in tRNA import has directly been shown are shaded in grey. *Saccharomyces cerevisiae*: the tRNA^{Lys} is targeted to mitochondria by enolase. Subsequently, the tRNA is co-imported with the precursor of mitochondrial lysyl-tRNA synthetase (pre-MSK). Pre-sequence of pre-MSK is shown as a box. Import of the tRNA^{Lys} is coupled to import of pre-MSK indicating that the entire protein import machinery is required for tRNA import. *Saccharomyces tuberosum*: the three outer membrane proteins VDAC, Tom40 and Tom20 are required to translocate tRNAs across the outer membrane. Unlike in yeast, tRNAs are not co-imported with proteins. Tom20 and Tom40 appear to function as receptors, whereas the VDAC may build the actual import channel. Trypanosomatidae: mitochondrial tRNA import has been analysed in detail in *L. tropica*. Inner membrane translocation of tRNAs appears to require a large protein complex termed RIC complex, consisting of six essential proteins (FeS-protein and subunit 6b of complex III, cytochrome oxidase subunit 6 of complex IV, F1a subunit of complex V and two unknown trypanosomatid specific proteins) and five non-essential proteins. In *T. brucei*, it was shown that eukaryotic elongation factor 1a (eEF1a) is essential for *in vivo* targeting of tRNAs to the mitochondria.

identified. It is therefore possible that when a more complete inventory of the tRNA import machineries is available, some conserved core proteins are identified. It is both striking and exciting that in plants and yeast, two of the three systems that have been studied, the tRNA import machinery includes components of the mitochondrial protein import system. Moreover, in yeast tRNA and protein import have even converged to a common macromolecule import pathway. It is of great interest that though both yeast tRNA^{Lys} and plant tRNAs require the TOM complex for import, the actual tRNA import mechanism is different and only the yeast tRNA^{Lys} is co-imported with proteins. Future studies on tRNA import promise better understanding of the mechanisms and capabilities of the mitochondrial protein import machinery too.

The suggestion that tRNAs might be imported into mitochondria was initially greeted with great scepticism. Later, it was thought to be restricted to only a few taxa of eukaryotes. Today, we know that it is a quasi-universal process. Should it in future turn out that mitochondrial tRNA import is a universal process and should further experimental work reinforce the connection between mitochondrial tRNA and protein import in different systems, one would be forced to consider that both mitochondrial macromolecular import pathways may have a monophyletic and common evolutionary origin.

This work was supported by grants 31003A_121937 (to A.S.) from the Swiss National Foundation and by a grant from the Australian Research Council (to T.L.).

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