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REVIEW

Mitochondrial protein import in trypanosomes: Expect the unexpected

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Mitochondria have many different functions, the most important one of which is oxidative phosphorylation. They originated from an endosymbiotic event between a bacterium and an archaeal host cell. It was the evolution of a protein import system that marked the boundary between the endosymbiotic ancestor of the mitochondrion and a true organelle that is under the control of the nucleus. In present day mitochondria more than 95% of all proteins are imported from the cytosol in a proces mediated by hetero-oligomeric protein complexes in the outer and inner mitochondrial membranes. In this review we compare mitochondrial protein import in the best studied model system yeast and the parasitic protozoan *Trypanosoma brucei*. The 2 organisms are phylogenetically only remotely related. Despite the fact that mitochondrial protein import has the same function in both species, only very few subunits of their import machineries are conserved. Moreover, while yeast has 2 inner membrane protein translocases, one specialized for presequence-containing and one for mitochondrial carrier proteins, *T. brucei* has a single inner membrane translocase only, that mediates import of both types of substrates. The evolutionary implications of these findings are discussed.

KEYWORDS

evolutionary cell biology, mitochondrial biogenesis, protein translocase, *S. cerevisiae*, *T. brucei*, TIM complex, TOM complex

1 | INTRODUCTION

Mitochondria are double membrane-bounded, essentially ubiquitous organelles of the eukaryotic cytosol. They are responsible for a number of important cellular functions the most famous of which is oxidative phosphorylation.[1] The origin of mitochondria can be traced back to a single event approximately 1.6-2 billion years ago in which a bacterium was taken up by an archaeon. Interestingly, the bacterium was not digested but evolved a symbiotic relationship with its host cell.[2,3] The subsequent transformation of the endosymbiont into an organelle marks the advent of the eukaryotic cell, since the highly efficient energy production by mitochondria likely was a prerequisite for the evolution of other eukaryotic traits such as a large genome and the nucleus itself[4] (for a different opinion see Reference 5). The process of organellogenesis involved the loss of some of the endosymbiont's genes while many others were transferred to the genome of the host.[6–8] However, the loss never went to completion and all present day mitochondria, capable of oxidative phosphorylation, have retained at least a small genome. In order for the mitochondrial ancestor to profit from the genes that had been transferred to the host genome it needed the capability to import proteins from the cytosol. Thus, the emergence of mitochondrial protein import systems defines the boundary between the endosymbiont and an organelle that is under the control of the host cell.[9–12] Understanding the evolutionary history of these import machines is therefore expected to provide insights into the origin of eukaryotes in general.

2 | WHAT IS TRUE FOR *E. COLI* IS TRUE FOR AN ELEPHANT—OR IS IT?

The use of model organisms to understand particular biological processes, with the expectation that discoveries made in these species will reveal basic features of the process that are valid for other organisms, has been a tremendously successful approach in cell and molecular biology. Mitochondrial protein import is no exception: it has mainly been studied in the yeast *Saccharomyces cerevisiae* and to a lesser extent in the fungal species *Neurospora crassa*. The results

obtained in these model systems provide a detailed view on the import signals, the architecture of the import machineries and the mechanisms of the import process.[13–17]

To underscore the value of model systems Jacques Monod once stated: what is true for *E. coli* is true for the elephant.[17] While this applies for many basic cellular functions, it must be obvious to everybody that there are large phenotypic differences between *E. coli* and an elephant. In fact diversity is one of the most striking features of life, and ultimately this diversity must be reflected at the cellular and molecular levels.

Thus, focusing cell biological studies on the few common model systems, which—except for plants—are phylogenetically closely related, provides only a very restricted view of this diversity. In order to change this, a new discipline, termed "evolutionary cell biology," has emerged which combines the power of cell biology with evolutionary biology, 2 fields of research which in the past did not interact much.[18,19] One aim of the new discipline is to map the molecular diversity of cellular processes in order to understand the chemical and physical constraints that shape them.

There have been numerous excellent reviews on various aspects of mitochondrial protein import which for the reasons mentioned above mainly dealt with yeast and related organisms.[13–17] With the aim to present an evolutionary cell biology perspective we focus our review on *Trypanosoma brucei*, a parasitic protozoan of clinical importance that causes human sleeping sickness and nagana in cattle.[20] *T. brucei* belongs to the eukaryotic supergroup Excavata and is essentially unrelated to fungi and metazoans, which are part of the supergroup Opisthokonts.[21] Moreover, the mitochondrial protein import systems of trypanosomes have over the last few years been studied in quite some detail allowing for a comparative analysis with yeast.[12,22]

3 | LIMITS OF BIOINFORMATICS

A number of comparative studies regarding the phylogenetic distribution of subunits of the mitochondrial protein import systems that focused on bioinformatic methods have been published.[23–25] Bioinformatics allowed to identify orthologues of specific subunits of the various protein import systems in a wide variety of organisms and to reconstruct their evolutionary history. However, it also has limitations: thus, it will not allow the identification of highly dissimilar subunits that may have evolved in isolation from the already known components. Moreover, it cannot confidently assign functions to proteins, since even proteins with similar sequences may have different functions. One example of the latter are the putative trypanosomal orthologues of yeast ER mitochondria encounter structure (ERMES) subunits[26] which upon experimental analysis proved not be localized at ER mitochondrial contact sites.[27]

Here we provide a comparative analysis that, rather than being based on sequence comparisons, focuses on functional aspects of mitochondrial protein import and on import factors that, at least to some extent, have experimentally been analyzed in both yeast and trypanosomes. It is important to note that yeast and mammals belong to the same eukaryotic supergroup of the Opisthokonts. In line with this the composition of their mitochondrial protein import systems is highly similar[28] although some taxon-specific subunits do exist.[29] We have recently published a comparative analysis of the translocases of the outer membrane (TOM) from yeast, plants and trypanosomes.[12] Our emphasis in this review will therefore be on the protein translocases of the inner membrane (TIM).

4 | MITOCHONDRIAL PROTEIN IMPORT IN YEAST—THE GOLDEN STANDARD

4.1 | Mitochondrial outer membrane

Three hetero-oligomeric protein translocases have been characterized in the mitochondrial outer membrane (OM) of yeast: the TOM complex, the general entry gate to the mitochondrion, and 2 complexes with more specific functions, the sorting and assembly machinery (SAM) and the mitochondrial import (MIM) complex[14] (Figure 1; Table 1).

The TOM complex consists of 7 subunits.[12,32] Except for the β-barrel protein Tom40 all have a single α-helical transmembrane domain. The most conserved TOM complex subunits are Tom40 and Tom22 which have been bioinformatically detected in essentially all eukaryotes[12] excluding the recently identified eukaryotic species that completely lacks a mitochondria-like organelle.[33] The β-barrel structure of Tom40 indicates its bacterial origin although no direct orthologue could be identified among bacterial β-barrel proteins. Tom40 forms the pore across which all mitochondrial proteins are translocated across the OM. Tom22 is involved in biogenesis and stabilization of the TOM complex and also functions as secondary receptor which recognizes substrate proteins and transfers them to the Tom40 pore.[31,34–36] More peripherally associated with the TOM complex are Tom20 and Tom70. Both have large cytosolic domains containing TPR repeats and function as import receptors. Tom20 mainly recognizes N-terminal targeting sequences of matrix and inner membrane (IM) proteins, whereas Tom70 is specialized to recognize internal targeting sequences in hydrophobic substrates, such as the mitochondrial carrier proteins (MCPs).[37] Orthologues of Tom20 and Tom70 are present in essentially all Opisthokonts.[12,38] While Tom20 is restricted to this supergroup, orthologues of Tom70 have been detected in some Stramenopiles and Haptophytes.[39] Finally, there are 3 small TOM subunits, Tom5, Tom6 and Tom7 which have been implicated in the regulation of TOM complex assembly.[40–44] Tom7 appears to be widely conserved since it is found in most but not all eukaryotic supergroups.[45]

The main subunit of the SAM complex is the essential β-barrel protein Sam50 (Figure 1; Table 1). It forms the pore that mediates insertion of β-barrel membrane proteins into the OM, after they have been translocated into the intermembrane space (IMS) by the TOM complex.[46] In order to prevent their aggregation in the IMS β-barrel proteins associate with members of the tiny TIM chaperone protein family.[47,48] Sam50 is found in essentially all eukaryotes and is an orthologue of BamA, which mediates membrane insertion of β-barrel proteins into the bacterial OM.[46] Thus, the evolutionary origin of Sam50 explains why mitochondrial β-barrel proteins are inserted into

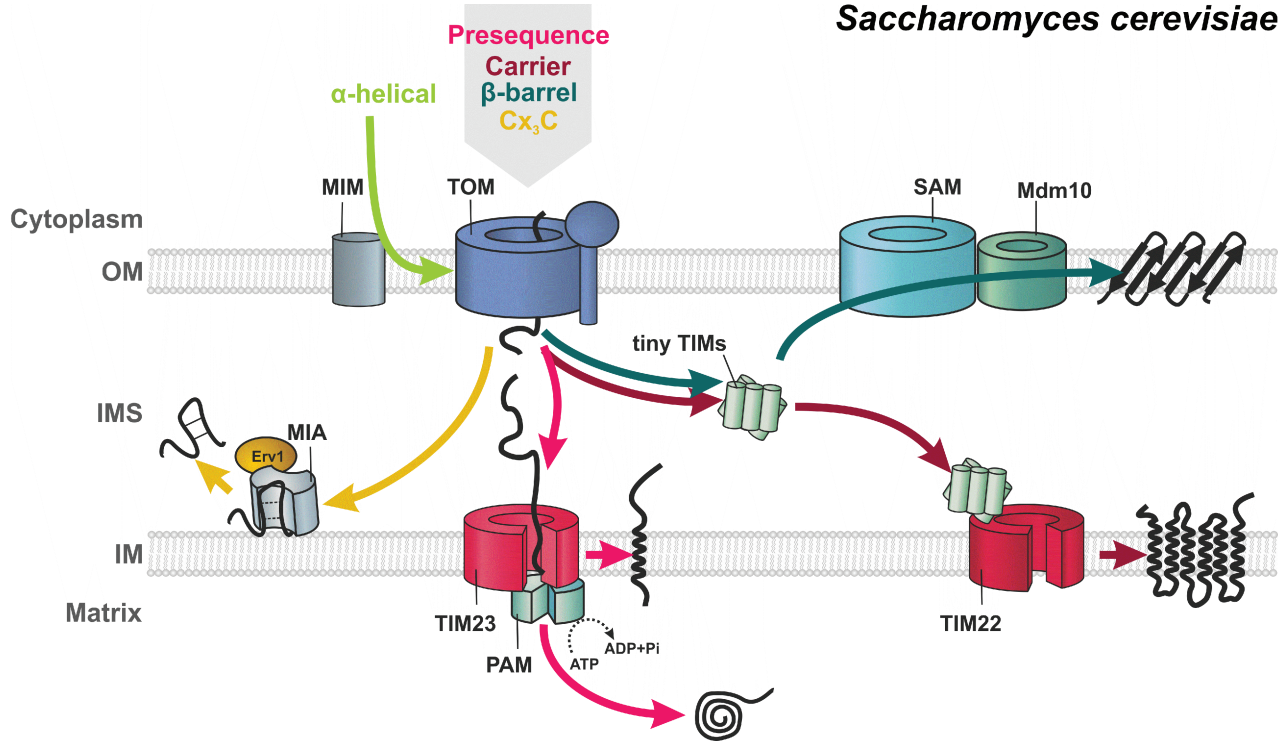

FIGURE 1 Overview of mitochondrial protein import pathways in *S. cerevisiae*. The biogenesis of a small number of α-helically anchored OM proteins (green arrow), including subunits of the TOM complex, depends on MIM complex. All other mitochondrial proteins are initially imported by the heterooligomeric TOM complex (gray arrow). After OM translocation the import pathways diverge depending on the class of substrates. The hydrophobic β-barrel proteins (green arrows) interact with the tiny TIM chaperones in the IMS and are then inserted into the OM by the SAM complex. Mdm10 dynamically associates with the SAM complex and assists TOM complex assembly. Small IMS-localized proteins (yellow arrows) with twin cysteine motifs are retained in the IMS by the formation of disulfide bonds catalyzed by the MIA-pathway. The function of Erv1 is to regenerate Mia40 by oxidation and to transfer the electrons to the mitochondrial electron transport chain. Presequence-containing proteins (bright red arrows) are handed over to the TIM23 complex by which they are either laterally released into to IM, in the case of α-helical IM proteins, or pulled into the matrix by the PAM module for matrix-localized proteins. The hydrophobic MCPs (dark red arrows) associate with the tiny TIM chaperones in the IMS and subsequently are inserted into the IM by the TIM22 complex.

the OM from the IMS side. The 2 peripheral subunits of the SAM complex are Sam35 and Sam37. Sam35 is essential, it functions as a receptor recognizing the β-signal found in the last β-strand of mitochondrial β-barrel proteins and assists the Sam50-mediated membrane insertion of these proteins.[49,50] The nonessential Sam37 promotes the formation of a TOM-SAM supercomplex that helps to transfer β-barrel proteins from the TOM to the SAM complex.[51–54] A fraction of the SAM complex associates with the β-barrel protein Mdm10 where it assists in assembly of Tom40 into the TOM complex.[55] Mdm10 is also a subunit of ERMES complex that forms mitochondria-ER contact sites.[56]

The MIM complex mediates the insertion and/or assembly of multi-pass OM proteins. It may also be involved in the biogenesis of single-spanning membrane proteins as it stimulates the biogenesis of Tom20 and Tom70 (Figure 1). The MIM complex consists of multiple copies of Mim1 and Mim2, neither of which has orthologues outside the fungal lineage (Table 1). The molecular mechanism by which the MIM complex exerts its function is presently unknown.[57–59]

4.2 | Intermembrane space

Import of IMS-localized proteins that have cysteine-rich motifs, such as the Cx_3C motifs of tiny Tim chaperones, use the Tom40 pore in the OM. Their import is driven by oxidative folding which traps the proteins in the IMS. The process is mediated by the mitochondrial IMS assembly (MIA)-pathway consisting of Mia40 and Erv1. Mia40 forms transient disulfide bridges with its substrates (Figure 1; Table 1). Finally, Mia40 is re-oxidized by Erv1 which transfers the electrons to cytochrome *c* of the respiratory chain.[60]

4.3 | Mitochondrial inner membrane

There are 2 main heterooligomeric protein translocases in the IM of yeast mitochondria termed TIM23 and TIM22 complex. The former mediates import of presequence-containing proteins whereas the latter is required for insertion of multi-spanning IM proteins such as MCPs which have 6 membrane-spanning domains (Figure 1).[15,61,62]

The essential membrane-embedded core of the TIM23 complex consists of Tim23, Tim17 and Tim50 (Table 2).[63] Tim23 forms the channel across which presequence-containing proteins are imported.[64] Tim17 which shows sequence similarity to Tim23 may contribute to channel formation and regulates the pore structure of TIM23.[65] Tim50 blocks the inactive Tim23 channel and its large IMS-exposed soluble domain serves as a preprotein receptor.[66–68] In addition to the 3 essential subunits the TIM23 complex also contains the nonessential subunits Tim21 and Mgr2. Tim21 which binds to the

TABLE 1 Protein import machineries of the mitochondrial OM and the IMS in *S. cerevisiae*

Subunit	Essential	Function
TOM		
Tom40	+	β-barrel translocation pore
Tom22	(+)[1]	Receptor at cis and trans side of OM, TOM organizer
Tom20	–	Primary receptor for presequence proteins
Tom70	–	Primary receptor for hydrophobic precursors
Tom5	–	regulation of TOM complex assembly
Tom6	–	regulation of TOM complex assembly
Tom7	–	regulation of TOM complex assembly
SAM		
Sam50	+	β-barrel insertase
Sam35	+	Precursor recognition
Sam37	–	Complex stability and TOM/SAM supercomplex formation
Mdm10	–	β-barrel assembly, mitochondrial inheritance/morphology
MIM		
Mim1	–	Biogenesis of α-helical OM proteins
Mim2	–	Biogenesis of α-helical OM proteins
MIA		
Mia40	+	Receptor in IMS, transfers disulfide bonds to IMS precursors
Erv1	+	sulfhydryl oxidase oxidizing and cooperating with Mia40

[1] Initially shown to be essential,[30] later shown to have severe growth and spore germination defects.[31]

IMS domain of Tom22 and thus contributes to TOM-TIM23 supercomplex formation.[69] Moreover, Tim21 together with Mgr2 mediate binding of the TIM23 complex to respiratory chain supercomplexes.[70–72] Mgr2 has also been shown to function as a gate keeper regulating the lateral release of precursor proteins into the IM.

The TIM23 complex is dynamically associated with the matrix-exposed presequence translocase-associated import motor (PAM; Table 2).[15,73] Assembly of the PAM complex is initiated by Pam17[74,75] which preferentially binds to the TIM23 complex lacking Tim21.[76] The core subunit of PAM is mitochondrial Hsp70 (mHsp70) which in addition to the driving force provided by the membrane potential mediates ATP-dependent vectorial transfer of preproteins into the matrix.[73,77–79] The activity of mHsp70 is regulated by the 2 PAM complex subunits, the co-chaperone Pam18 and its binding partner Pam16, both of which contain J-like domains.[80–82] Moreover, mHsp70 is regulated by the matrix-localized nucleotide exchange factor Mge1.[83,84] The PAM complex subunit Tim44 positions mHsp70 at the exit site of the translocation pore of the TIM23 complex.[74,85]

MCPs and few other IM proteins with multiple membrane-spanning regions use a dedicated translocase, the TIM22 complex for their insertion into the IM (Figure 1; Table 2). It consists of the 4 integral membrane proteins Tim22, Tim54, Tim18 and Sdh3 and the peripherally associated IMS proteins Tim9, Tim10 and Tim12 which belong to the tiny TIM chaperone family (Table 2).[61,86,87] A soluble hexameric chaperone complex consisting of Tim9/Tim10 associates with the hydrophobic TIM22 substrates in the IMS and prevents their aggregation.[88–91] This complex is coupled via Tim12 to the TIM22 membrane complex.[90,92] An alternative tiny Tim complex composed of Tim8 and Tim13 delivers a subset of hydrophobic substrates to TIM22.[93,94] Tiny Tim-like proteins are found in all eukaryotes.[95] Tim22 forms the insertion pore,[96] it shows sequence similarity to the Tim23 and Tim17 subunits of the TIM23 complex suggesting that all 3 proteins derive from a common ancestor. The Tim17/22/23 proteins are the most conserved membrane subunits of the TIM22 and TIM23 complexes and at least 1 member of this family is found in essentially all eukaryotes.[97] Tim54 has large IMS-exposed domain that binds the Tim9/Tim10/Tim12 complex.[96,98,99] Tim18 and Sdh3 finally assist in the assembly of the TIM22 complex.[98–101] The function of Sdh3 as a subunit of the TIM22 complex is surprising, as it is primarily known as an obligatory component of complex II of the respiratory chain. Thus, Sdh3 depending on whether it associates with Sdh4 or with Tim18 either functions in oxidative phosphorylation or in mitochondrial protein import, respectively.[101]

5 | EXTREME MITOCHONDRIAL BIOLOGY OF TRYPANOSOMES

Compared to Opisthokonts many aspects of the mitochondrial biology of *T. brucei* appear extreme[102,103] (Figure 2). It has a single mitochondrion only that, except prior to cytokinesis, is not subject to fission and fusion events.[104] The disc-shaped single unit mitochondrial genome, termed kinetoplast DNA (kDNA), consists of multiple copies of highly topologically interlocked DNA elements, termed maxi- and minicircles.[105] The kDNA disc is physically linked across the 2 mitochondrial membranes with the basal body of the flagellum, via the tripartite attachment complex (TAC).[106,107] Unlike in other eukaryotes replication of the mitochondrial genome occurs at a specific time during the cell cycle prior to the nuclear S-phase.[108] It is the function of the TAC to couple the segregation of the replicated kDNA disks to the segregation of the old and the newly formed flagellum.[109] Consequently, a defective TAC prevents inheritance of kDNA to daughter cells.

The kDNA encodes 18 protein-coding genes[110] many of which are cryptogenes, meaning that their primary transcripts have to be edited by multiple uridine insertions and/or deletions to become functional mRNAs.[111] It was in the trypanosomal mitochondrion where the process of RNA editing was initially discovered.[112] Furthermore the kDNA encodes the 2 ribosomal RNAs, which are the shortest such molecules known.[113,114] They are much shorter than the already much reduced rRNAs in mammalian mitochondria, indicating *T. brucei* has very unusual mitochondrial ribosomes.

Finally, the kDNA shows a striking absence of tRNA genes.[115–117] The trypanosomal mitochondrion therefore has to import all of its organellar tRNAs from the cytosol. This contrasts with humans whose mitochondrial genome appears to encode a complete set of tRNAs.[118]

However while the *T. brucei* mitochondrion has many unique features it also shares common traits with mitochondria of *S. cerevisiae*.

TABLE 2 Protein import machinery in the mitochondrial IM of *S. cerevisiae*

	Presequence translocase[1]	Carrier translocase[2]	Essential	Function
Transmembrane components	**TIM23**			
	Tim23		+	Channel-forming subunit of TIM23
	Tim17		+	Contributes to and regulates channel formation
	Tim50		+	IMS receptor, Tim23 channel regulator
	Tim21		–	Modulator of TIM23 and supercomplex formation
	Mgr2		–	Modulator of TIM23, lateral gate keeper
		TIM22		
		Tim22	+	Channel-forming subunit of TIM22
		Tim54	–	IMS domain binds Tim9/10/12 complex
		Tim18	–	Involved in assembly of TIM22
		Sdh3	–	Important for assembly and stability of TIM22
Motor components	**PAM**			
	mHsp70 (Ssc1)		+	Hsp70 family ATPase, import motor
	Pam18		+	J-protein stimulating ATPase activity of mHsp70
	Tim44		+	Membrane anchor for mHsp70, preprotein binding
	Mge1		+	Mitochondrial ATP exchange factor for mHsp70
	Pam16		+	J-like protein controling Pam18
	Pam17		–	Modulates interaction of TIM23 and PAM
IMS chaperones		Tim12	+	IMS chaperone tethering precursors to TIM22
		Tim9	+	IMS chaperone for hydrophobic proteins
		Tim10	+	IMS chaperone for hydrophobic proteins
		Tim8	–	IMS chaperone for hydrophobic proteins
		Tim13	–	IMS chaperone for hydrophobic proteins

[1] TIM23 complex and PAM complex dynamically associate with each other.

[2] TIM22 complex and tiny Tims interact dynamically.

Their mitochondrial proteomes are of similar size (ca. 1000 proteins) and the import signals of the different protein classes (presequence-containing proteins, β-barrel protein, MCPs, etc.) are largely functionally interchangeable between the 2 systems.[12] Thus the functional requirements the 2 mitochondrial protein import systems have to fulfill are essentially identical. There is some evidence that, at least in the case of the IM, the protein import system of trypanosomes might participate in tRNA import in an as yet unknown way.[119] However, the situation in yeast is comparable: a small fraction of a single $tRNA^{Lys}$ isoacceptor is co-imported in complex with a precursor protein along the presequence pathway.[120] Thus, the differences between the protein import machineries of trypanosomes and yeast that are discussed below, are not due to different functional selection but rather reflect their distinct evolutionary history.

6 | MITOCHONDRIAL PROTEIN IMPORT IN TRYPANOSOMES

6.1 | Mitochondrial outer membrane

The OM protein translocase of *T. brucei* was termed archaic translocase of the OM (ATOM) (Figure 3). Like the TOM complex in yeast it is a high molecular weight complex composed of 7 subunits termed ATOM40, ATOM19, ATOM14, ATOM12, ATOM11, ATOM69 and ATOM46[121,122] (Table 3). Except for ATOM46 all are essential for viability and mitochondrial protein import. In yeast, in contrast, only Tom40 is essential although growth of Tom22 deletion strains is strongly reduced.[31] Only 2 ATOM subunits, ATOM40 and ATOM14, show homology to yeast TOM subunits[12] (Table 3). The β-barrel protein ATOM40,[123] which forms the import channel, likely is a highly diverged orthologue of yeast Tom40 as revealed by HHPred analysis.[133] However, the protein also shows sequence similarity to Omp85-like proteins of bacteria[123] and in electrophysiological experiments its pore behaves more like the one in Omp85-like proteins than the 1 in Tom40.[124] ATOM14 is a remote orthologue of yeast Tom22 that has a much shorter cytosolic domain which, similar to the plant Tom22 orthologue Tom9, lacks the clusters of acidic residues found in the yeast protein.[12] Unlike for Tom22 the short cytosolic domain of ATOM14 is not essential for its function.[125]

Up to now no orthologues of the remaining 5 ATOM subunits were found outside the kinetoplastid lineage. However, there are functional similarities between ATOM and TOM subunits. Thus, the small subunits ATOM11 and ATOM12 positively and negatively regulate the assembly of ATOM40 with its peripheral receptor subunits, a role that is similar to Tom6 and Tom7 in the yeast TOM complex.[122] ATOM69 and ATOM46 are peripherally associated with the ATOM complex. Both have large cytosolic domains and function as protein import receptors with overlapping but distinct substrate specificities. Their function is essentially identical to Tom20 and Tom70 of yeast. ATOM69 and Tom70 share TPR repeats. However, they are not orthologues since, unlike Tom70

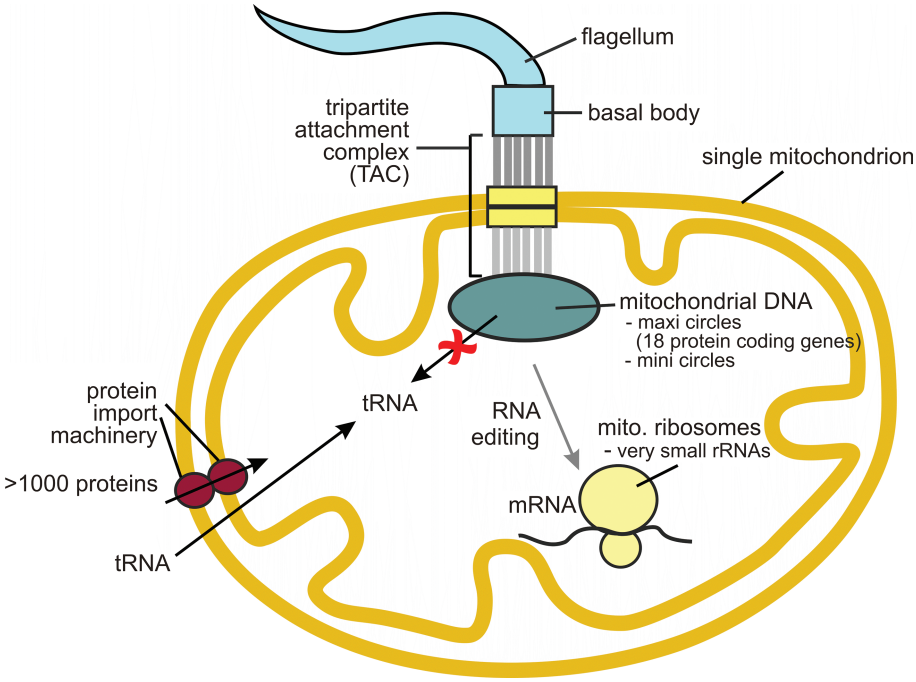

FIGURE 2 Extreme mitochondrial biology of trypanosomes. *T. brucei* has a single mitochondrion with a single unit mitochondrial genome, termed kDNA. The kDNA is physically linked across the 2 mitochondrial membranes with the basal body of the single flagellum. The linkage is formed by the TAC, which consists of intramitochondrial filaments, differentiated membranes and cytosolic filaments. The TAC couples the segregation of the replicated kDNA to the segregation of the old and the newly formed flagellum. The maxicircles of the kDNA encode 18 protein-coding genes, of which 12 are cryptogenes, meaning that their primary transcripts have to be edited in order to be become functional mRNAs. The information for RNA editing is provided by guide RNAs most of which are encoded on the minicircle DNA. The maxicircles also encode the 2 mitochondrial rRNAs which are among the shortest found in nature, indicating that trypanosomes have highly unusual mitochondrial ribosomes. Unlike in most other eukaryotes the mitochondrial DNA of trypanosomes does not encode any tRNAs. Instead all organellar tRNAs derive from a small fraction of cytosolic tRNAs that are imported into mitochondria. The size of the mitochondrial proteome of *T. brucei* is comparable to the one of yeast, indicating that in both system more than 1000 proteins are imported into the organelle.

which is a signal-anchored protein, ATOM69 is inserted into the OM with its C-terminus. Furthermore it contains an Hsp20-like domain that is not found in Tom70. ATOM46 unlike any other ATOM or TOM subunits has armadillo-repeats.[122]

Finally, a recent study reported the discovery of an additional ATOM subunit, termed ATOM19.[121] The protein had previously been identified as a factor required for the maintenance of mitochondrial morphology and function.[134] However, a more detailed analysis showed that the protein is tightly associated with the ATOM complex. Furthermore, its ablation affected the assembly and the stability of the ATOM complex and as a consequence abolished mitochondrial protein import. ATOM19 is unusual since unlike any other TOM or ATOM subunits it contains 2 transmembrane domains.

Except for ATOM40 and ATOM14 all ATOM subunits are unique to the Kinetoplastids. Thus, the observed functional similarities between the kinetoplastid-specific ATOM subunits and the TOM subunits of yeast are best explained by convergent evolution rather than by common ancestry.[12]

T. brucei contains a SAM complex in the mitochondrial OM and its core subunit Sam50/Tob55 is highly conserved.[126] As in other eukaryotes *T. brucei* Sam50 mediates the biogenesis of β-barrel membrane proteins (Figure 3). An orthologue of Sam35 has been detected in the OM of trypanosomes,[127] however, whether it forms a complex with Sam50 is presently unknown.

T. brucei does not have a MIM complex as it lacks orthologues for Mim1 and Mim2. However, an essential mitochondrial OM protein, termed peripheral ATOM36 (pATOM36),[128] has been characterized that is required for the assembly of many ATOM complex subunits and some other signal-anchored OM proteins[129] (Figure 3). Thus pATOM36 may have an analogous function to the yeast MIM complex.[58] However, besides the presence of GxxxG protein-protein interaction motifs in its transmembrane domains it neither shows structural similarity to Mim1 nor to Mim2. Interestingly, pATOM36 is not only localized all over the OM but is also concentrated in the TAC (Figure 2) and was found to interact with bona fide TAC components. In line with this, ablation of pATOM36 not only abolishes ATOM complex assembly but also disrupts the segregation of the replicated mitochondrial genome.[129] In that respect the protein resembles the fungi-specific Mdm10 which accelerates TOM complex assembly[55] and independently, as subunit of the ERMES complex, influences mitochondrial genome inheritance.[56]

6.2 | Intermembrane space

Bioinformatic analysis reveals that trypanosomes have at least 6 tiny Tim-like IMS proteins that have cysteine-rich motifs[95,135,136] (Table 2). Thus, orthologues of the yeast substrates of the IMS oxidative folding machinery are present in *T. brucei*. However, while an

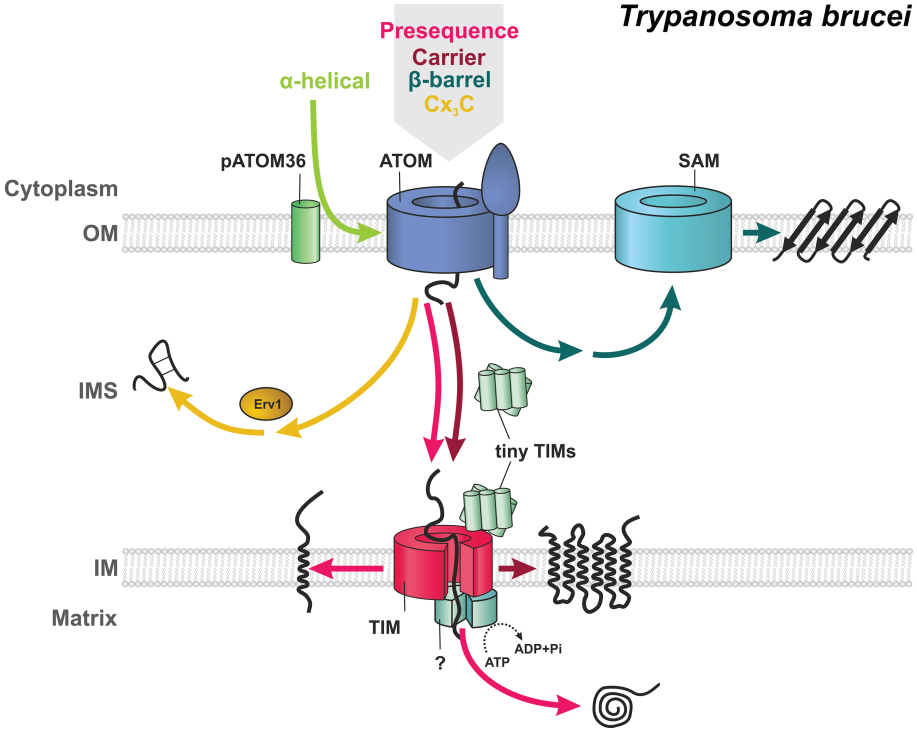

FIGURE 3 Overview of mitochondrial protein import pathways in *T. brucei*. The biogenesis of a small number of α-helically anchored OM proteins (green arrow), including subunits of the ATOM complex, depends on pATOM36. All other mitochondrial proteins are initially imported by the heterooligomeric ATOM complex (gray arrow). After OM translocation the import pathways diverge depending on the class of substrates. As in yeast, the hydrophobic β-barrel proteins (green arrows) likely interact with the tiny TIM chaperones in the IMS and are then inserted into the OM by the SAM complex. No Mia40 orthologue exists in *T. brucei*. Thus, import of small IMS-localized proteins (orange arrows) with Cx_3C signatures likely is mediated by Erv1 alone or in combination with as yet unknown factor(s). In contrast to the yeast system presequence-containing proteins (bright red arrows) and MCPs (dark red arrows), probably, with the help of tiny TIM chaperones that are tightly associated with the TIM complex, are handed over to the single TIM complex that with compositional variations mediates their insertion and lateral release into the IM or their complete translocation into the matrix. The last step requires an import motor whose identity is unclear at the moment.

TABLE 3 Protein import machineries of the OM and the IMS in *T. brucei*

Subunit	Accession number[1]	Essential[2]	Function	Potential orthologues in *S. cerevisiae*	References
ATOM					
ATOM40	Tb927.9.9660	+[3]	β-barrel translocation pore	Tom40	122–124
ATOM14	Tb11.02.5660	+	ATOM organizer, secondary receptor	Tom22	122,125
ATOM46	Tb11.02.5660	–	Receptor protein		122
ATOM69	Tb11.01.3290	+	Receptor protein		122
ATOM11	Tb927.10.11030	+	regulation of ATOM complex assembly		122
ATOM12	Tb927.8.4380	+	regulation of ATOM complex assembly		122
ATOM19	Tb927.9.10560	+	regulation of ATOM complex assembly		121
SAM					
Sam50[4]	Tb927.3.4380	+[3]	β-barrel insertase	Sam50	126
Sam35	Tb927.8.6600	n.a.	n.a.	Sam35	127
OTHERS					
pATOM36	Tb927.7.5700	+	ATOM assembly and kDNA inheritance		128,129
Erv1	Tb927.9.6060	+[5]	IMS protein import and cytosolic Fe-S assembly	Erv1	130,131

[1] www.tritrypdb.org.

[2] Tested by RNAi-mediated knockdown in procyclic and bloodstream forms, sometimes including a bloodstream form cell line that can grow in the absence of kDNA.[132]

[3] Only tested in procyclic form.

[4] Alias Tob55.

[5] Only tested in procyclic form and bloodstream form.

essential trypanosomal Erv1 has recently been characterized, a Mia40 homolog is missing[130,131,137] (Figure 3). Interestingly, the situation appears to be the same in Chromalveolates which despite having typical Mia40 substrates lack Mia40 orthologues.[137] This raises the question whether *T. brucei* and Chromalveolates do not require Mia40, because their Erv1 orthologues may directly perform oxidative folding of cysteine-rich IMS proteins, or whether as yet undiscovered proteins take over Mia40 function.

6.3 | Inner membrane

Unlike in most other eukaryotes only a single gene encoding a member of the Tim17/Tim22/Tim23 protein family can be detected in the *T. brucei* genome.[22,95,138] It is difficult to assign this protein to a specific subclass of the protein family, which is why it was termed TbTim17 based on its predicted molecular weight. TbTim17 is present in a high molecular weight complex and its ablation by RNAi shows phenotypes consistent with its predicted role in mitochondrial protein import (Table 4). [136,139] Moreover, expression of yeast Tim17 but not of Tim23 could complement for the ablation of the trypanosomal TbTim17.[140] However, expression of TbTim17 in the corresponding yeast mutants had no effect. Unfortunately, yeast Tim22 while being expressed in trypanosomes was not correctly localized. It is therefore unclear whether it also might complement for the lack of TbTim17. Based on the existence of only one Tim17/Tim22/Tim23 orthologue it had been suggested that trypanosomes may have a single TIM complex only that would import both presequence-containing and MCPs[22,95,138] (Figure 3).

The trypanosomal TIM complex has first been analyzed by TAP-purification of a C-terminally tagged TbTim17 and subsequent MS analysis identified 20 co-purifying proteins.[139] Four of the trypanosome-specific proteins were further analyzed and RNAi-mediated depletion of 3 of them—TbTim47, TbTim54 and TbTim62—inhibited in vitro import of proteins. RNAi against TbTim54 and TbTim62 furthermore resulted in reduced levels of a myc-tagged mitochondrial protein in vivo. Moreover, reciprocal pull down experiments of tagged TbTim54 and TbTim62 recovered TbTim17 confirming that the 3 proteins are in the same complex. Further functional analysis is only available for TbTim62 and indicates that it plays a role in the assembly and stability of the trypanosomal TIM complex.[141]

A gene encoding a putative orthologue of yeast and human Tim50 has recently been discovered in *T. brucei*.[142] Reciprocal pulldown experiments suggest that TbTim50 interacts with TbTim17, even though it was not recovered in the initial TAP tag purification of TbTim17.[139] Like human and yeast Tim50, TbTim50 contains a C-terminal phosphatase motif. However, strangely the predicted membrane-spanning domain of TbTim50 is in the middle of phosphatase motif whereas in human and yeast Tim50 the corresponding domain is in the N-terminal part. Based on in vitro import experiments it has been suggested that TbTim50 is involved in mitochondrial protein import.[142]

In a more recent study 3 reciprocal immunoprecipitations, in combination with stable isotope labeling with amino acids in cell culture (SILAC) and mass spectrometry (MS) analysis were performed. Using this method 10 proteins were identified to be core components of the trypanosomal TIM complex[136] (Table 4). In order to investigate whether trypanosomes indeed have a single TIM complex only, as has been proposed,[22,95,138] the following novel approach was used. Modified epitope tagged import substrates were expressed that could be arrested either within the presequence translocase[144] or at the carrier translocase.[145]

Pull down of the substrate associated with the presequence translocase and subsequent MS analysis identified all previously defined TIM complex core subunits. Moreover, as expected, since the arrested precursor spans both membranes, all ATOM subunits including the newly identified ATOM19[121] were also detected.[136]

Pull down of the substrate associated with the carrier translocase recovers 11 highly enriched proteins. Interestingly, 10 of these proteins, including TbTim17, are also found in the active presequence translocase. These results provide functional evidence that in *T. brucei* a single TIM complex with compositional variations mediates both import of presequence-containing proteins as well as MCPs.[136]

This is reminiscent of the 2 forms the yeast TIM23 complex can assume depending on the translocated substrate.[69,70] The core of both variants is formed by Tim23, Tim17, Tim50 and Mgr2. It associates either with Tim21 and respiratory chain components during lateral sorting of inner membrane proteins or with the import motor PAM for matrix protein import.[73]

In trypanosomes the common subunits of the presequence and the carrier translocase include the integral membrane proteins TbTim17, TbTim62 and an acyl-CoA dehydrogenase (ACAD), which were already detected in the first analysis of the trypanosomal TIM complex,[139] and the trypanosomatid-specific protein TbTim42 which was not detected in the previous study (Table 4). Moreover, 6 tiny TIM chaperones were also present in both translocases. However, the previously identified TIM subunits TbTim47, TbTim54[139] and TbTim50[142] were neither among the TIM core subunits nor were they present in the active presequence or carrier translocases.

Two rhomboid-like proteins, termed TimRhom I and TimRhom II were specifically associated with the presequence translocase. As expected for protein import factors they are essential in both procyclic and bloodstream form trypanosomes and an engineered bloodstream form cell line that can grow in the absence of mitochondrial DNA.[132] Furthermore, it was shown that ablation of these proteins results in the in vivo accumulation of unprocessed mitochondrial precursor proteins.[136]

The discovery that 2 inactive rhomboid-like proteins are subunits of the mitochondrial presequence translocase is striking since other inactive members of the rhomboid protease family were shown to be involved in protein translocation in the ER.

Rhomboid-like proteins, such as the yeast protein Der1p, are subunits of the endoplasmic reticulum-associated protein degradation (ERAD) system which exports misfolded proteins from the ER lumen to the cytosol for degradation by the proteasome.[146,147] Moreover, in plastids of red algae, that arose by secondary endosymbiosis and therefore are surrounded by 4 membranes, the ERAD system from the endosymbiont was commandered to the second outermost membrane to form a plastid-specific ERAD-like machinery, called SELMA.[148] This machinery was adapted such that protein translocation is no longer coupled to protein degradation. Rhomboid-like proteins are found in all 3 domains of life including α-proteobacteria[149] and TimRhom I and TimRhom II are not closer related to eukaryotic rhomboid-like proteins (eg, derlins, PARL) than to their bacterial

TABLE 4 Protein import machinery in the inner mitochondrial membrane of *T. brucei*

	Subunit	Accession number[1]	Tim17 pulldown (Singha et al.[2])	Presequence translocase (Harsman et al.[3])	Carrier translocase (Harsman et al.[4])	Essential[5]	Protein import defect	Potential yeast homolog	References
Transmembrane	TbTim17	Tb927.11.13290	X	X	X	+[6]	in vitro/in vivo	Tim17/22/23	119,136,138
Components	TbTim62	Tb927.8.1740	X	X	X	–	in vitro/in vivo		136,139,141
	ACAD[7]	Tb9278.1420	X	X	X	n.a.	n.a.		136,139
	TbTim42	Tb927.9.11220		X	X	+	in vivo		136
	TimRhom I	Tb927.9.8260		X		+	in vivo		136
	TimRhom II	Tb927.8.4150		X		+	in vivo		136
	TbTim50	Tb927.3.2110	X			–	in vitro/in vivo		142
	TbTim47[7]	Tb927.1.1310	X			–	In vitro		139
Others	TbTim54	Tb927.6.2470	X			–	in vitro/in vivo		139
	mHsp70	Tb927.6.3740	X			+[8]	In vitro	mHsp70	119,139
	Mge1	Tb927.6.2170				+[8]	n.a.	Mge1	143
		Tb927.8.3320[9]				n.a.	n.a.	Pam18	22
		Tb927.7.4620[9]				n.a.	n.a.	Tim44	22
IMS chaperones	Tim9[10]	Tb927.7.2200		X	X	n.a.	n.a.	Tim9	95,136
	Tim10	Tb927.3.1600		X	X	n.a.	n.a.	Tim10	95,136
	Tim8-Tim13	Tb927.11.5390		X	X	+[8]	n.a.	Tim8/Tim13	95,135,136
	TbTim11	Tb927.5.3340		X	X	n.a.	n.a.	Tiny Tim-like	135,136
	TbTim13	Tb927.10.11520		X	X	n.a.	n.a.	Tiny Tim-like	136
	Tiny Tim-like[10]	Tb927.4.3430		X	X	n.a.	n.a.	Tiny Tim-like	135,136

[1] www.tritrypdb.org.

[2] TAP-tag purification of TbTim17.[139]

[3] Core components detected in SILAC IP of a presequence-containing import intermediate.[136]

[4] Core components detected in SILAC IP of a carrier import intermediate.[136]

[5] Tested by RNAi-mediated knockdown in procyclic and bloodstream forms, sometimes including a bloodstream form cell line that can grow in the absence of kDNA.[132]

[6] Only tested in procyclic and bloodstream forms.

[7] Contains predicted transmembrane domain(s), not experimentally verified.

[8] Only tested in procyclic form.

[9] BLAST analysis.

[10] Not in initially defined core components, but strongly enriched in both, presequence-containing and carrier import intermediates, respectively.

counterparts.[136] It is therefore possible that they may have been recruited for protein import from the original endosymbiont that gave rise to mitochondria.

The presequence translocase needs an import motor which in yeast is formed by the PAM module.[15] Orthologues of the PAM subunits Tim44, Pam18 and mHsp70 are found in *T. brucei* mitochondria[22] and ablation of mHsp70 prevents in vitro import of matrix proteins.[119] However, none of these orthologues were recovered in the active presequence translocase[136] (Table 4). It is possible that the PAM module got lost during purification, but in the light of its highly divergent presequence translocase it cannot be excluded that *T. brucei* utilizes a noncanonical motor complex for preprotein import.

7 | EVOLUTIONARY IMPLICATIONS AND OPEN QUESTIONS

The mitochondrial protein import systems of yeast and trypanosomes have the same function, namely to import and sort more than 1000 organellar proteins. The diversity in the compositions of their protein translocases is therefore unexpected. For the general translocases of the OM only 2, Tom40/ATOM40 and Tom22/ATOM14, out of 7 subunits each are conserved. Moreover, there is clear evidence that, despite the conservation of the various mitochondrial targeting sequences, the receptors recognizing these signals, Tom20/Tom70 and ATOM69/ATOM46, evolved independently in Opisthokonts and Kinetoplastids.[12]

For the TIM complexes the situation is even more extreme since, except for a single orthologue of the Tim17/Tim22/Tim23 protein family, none of their integral membrane subunits are conserved between the 2 systems.[136] Based on these observations we can define 3 integral membrane proteins that likely formed the core of the mitochondrial protein translocases in the last eukaryotic universal common ancestor (LECA). This core consisted of a Tom40-like and a Tom22-like protein in the OM and of a member of the Tim17/Tim22/Tim23 protein family in the IM. Moreover, a variable number of IMS-localized chaperones of the tiny TIM protein family, that in part are tightly associated with the TIM complex, are found in both systems and the same is the case for the mitochondrial import motor

mHsp70. Thus, it is likely that tiny TIM chaperones and mHsp70 already contributed to mitochondrial protein import in LECA.

There are 2 principal ways how the unique composition of the single trypanosomal TIM complex can be explained. It is possible that LECA had a very rudimentary IM import system, consisting of not much more than a single Tim17/Tim22/Tim23-like protein. Such a simple system might have been capable to import at least a few proteins. The need to import a larger number of substrates with high efficiency and specificity may have arisen only later in evolution, after the segregation of the branches leading to the ancestors of the Opisthokonts and the Kinetoplastids. This scenario would explain why we find complex, functionally essentially equivalent TIM complexes in yeast and trypanosomes, which however have a very different architecture and subunit composition. Alternatively, the yeast-type TIM complexes might be direct descendants of an ancestral TIM complex that in the lineage leading to the Kinetoplastids, for unknown reasons, was replaced by a single TIM complex with a unique subunit composition as we find it in trypanosomes today.

In order to get more insight into the evolution of the TIM complexes we need more information. It is known that orthologues of the yeast-type TIM complexes are found in essentially all Opisthokonts. However, it is presently unclear whether a trypanosome-type TIM complex occurs outside the Kinetoplastids. Bioinformatic analysis of other excavate species is only of limited use to address this question, since even if rhomboid-like proteins and ACAD orthologues are detected, it would not be possible to decide whether they might function as TIM complex subunits in the respective systems.

Moreover, it would be important to know more about the specific functions of the trypanosomal TIM subunits. The central questions concern the protein-translocating pore(s) of the single trypanosomal TIM complex. Is there 1 pore only, that translocates presequence-containing proteins and inserts MCPs, or are there 2 specialized pores that do the job? Furthermore, which TIM subunits are involved in pore formation? A prime candidate would be TbTim17 as it belongs to the Tim17/Tim22/Tim23 family of proteins. In yeast Tim23—possibly with the help of Tim17—forms the pore for presequence-containing import substrates and Tim22 provides the insertion pore for MCPs. However, at least in yeast, Tim17 alone is not able to build a protein-conducting pore.

The 2 catalytically inactive rhomboid-like proteins TimRhom I and TimRhom II were specifically associated with the trypanosomal presequence translocase.[136] Rhomboid-like proteins have been suggested to form the protein translocation pores in the ERAD and the SELMA systems.[146–148] Thus, TimRhom I and TimRhom II would be perfect candidates to form the pore of the trypanosomal presequence translocase, should it have a separate pore than the carrier translocase. Whatever the exact functions of rhomboid-like proteins in the trypanosomal TIM complex or the ERAD and SELMA systems are, it is clear that these proteins have been repeatedly recruited as subunits for protein translocation systems in different biological membranes.

An orthologue of an acyl-CoA dehydrogenase, termed ACAD, has been identified as a core subunit of the trypanosomal TIM complex, although its function in mitochondrial protein import has not yet been demonstrated.[136,139] This is reminiscent of the situation in the yeast TIM22 complex whose constituents include subunit 3 of succinate dehydrogenase, Sdh3,[101] which also functions in the citric acid cycle. Thus the recruitment of a metabolic enzyme to the TIM complex seems to be a common feature that is shared between trypanosomes and yeast.

Moreover, there are still many open questions concerning mitochondrial biogenesis in trypanosomes. How does the adaptation of the mitochondrion to the different life cycle stages work? From yeast it is known that phosphorylation of TOM complex subunits as well as the precursor proteins by cytosolic kinases is used to regulate protein import.[150,151] Do we find a similar situation in trypanosomes?

In summary, a comparative analysis of mitochondrial protein import in yeast and trypanosomes, which are only remotely related, has revealed an amazing diversity in the composition of their mitochondrial protein import machineries. This is surprising since the protein import systems essentially have the same function in all eukaryotes. The differences between the import systems therefore provide exciting examples of convergent evolution over large phylogenetic distances. Further work is required to find out whether trypanosomes are indeed that unique, or whether the protein import systems of other eukaryotes, outside the supergroups of the Opisthokonts and the Excavates, are different to both yeast and trypanosomes. Work in plants, which are representatives of the supergroup of the Archeaplastidae,[152] suggests that this might be the case. Thus, Monod might have been surprised since in the context of mitochondrial protein import, his saying "Anything found to be true of *E. coli* must also be true of elephants" does not apply.

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