

The direct route: a simplified pathway for protein import into the mitochondrion of trypanosomes

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Trypanosoma brucei is a unicellular eukaryote that causes the deadly human African trypanosomiasis ('sleeping sickness') in humans. The parasite has a complicated lifestyle, it developmentally changes aspects of its mitochondrial function as it alternates from forms in the tsetse fly to forms adapted for life in the human bloodstream. The single mitochondrion found in each trypanosome has to be duplicated precisely in each round of the cell cycle in order for parasites to replicate, and this depends on the import of proteins from the cytosol. Here we review what is known about the mitochondrial protein import pathway in *T. brucei*, how it compares with the process in humans, and how the distinguishing features seen in *T. brucei* and humans promise new understanding of the mitochondrial protein import process in all eukaryotes.

Introduction

The mitochondrion is an essential organelle carried by all eukaryotes [1,2]. Although the morphology of the organelle varies greatly from species to species, and even in different tissues of a single species, most cells have numerous mitochondria that form dynamic weblike structures throughout the cytoplasm. Mitochondria house the systems for energy production through oxidative phosphorylation, synthesis of key metabolites and iron-sulfur clusters, and can be the reservoir for factors that amplify signals for programmed cell death [3]. There is little doubt that acquiring a bacterial endosymbiont and the subsequent transformation of this endosymbiont into the mitochondrion was a key – perhaps 'the' key, event in the early evolutionary history of eukaryotes. The mitochondrial proteome varies from organism to organism and even the largest mitochondrial genomes are vestigial, encoding only very few of the proteins on which the function of organelle depends. Thus, a crucial evolutionary step was the establishment of a protein import system in the bacterium that gave rise to mitochondria.

Most phylogenetic trees based on molecular data suggest that the parasitic protists *Giardia intestinalis*, *Trichomonas vaginalis* and the trypanosomatids diverged early from other eukaryotes [1]. *Trypanosoma brucei* is the

best studied trypanosomatid species and the causative agent of the deadly human African trypanosomiasis ('sleeping sickness'); transmitted by the tsetse fly, it kills 300 000 people each year in sub-Saharan Africa [4]. Besides its clinical importance *T. brucei* has attracted interest because of its rich biology in general, and the many intriguing features of its mitochondrion in particular (Box 1). *Trypanosoma brucei* has only a single mitochondrion whose genome is physically connected to the flagellar basal bodies [5] by way of proteinaceous connections that span the two mitochondrial membranes (Figure 1). Whereas most organisms have a single type of mitochondrial chromosome, the mitochondrial DNA in *T. brucei* consists of two genetic elements, the maxicircles and the minicircles, which are topologically intertwined. Replication of the genome is restricted to a 'mitochondrial S-phase' that is strictly coordinated with the S-phase of the cell cycle [6]. Segregation of the replicated mitochondrial DNA is directly linked to the segregation of the flagellar basal body, and mitochondrial division seems to be a checkpoint for cytokinesis [7]. Mitochondrial gene expression in *T. brucei* is complex; many genes are composed of incomplete open reading frames whose primary transcripts have to be edited to convert them into translatable mRNAs. This RNA editing is mediated by small, mitochondrially-encoded transcripts called guide RNAs [8]. Translation is also unconventional in the mitochondrion of *T. brucei*: the mitochondrial rRNAs of *T. brucei* are among the smallest found in nature; the mitochondrial genome has no tRNA genes; mitochondrial protein synthesis requires tRNAs that are imported from the cytosol in a process we are only beginning to understand [9].

From considerations of genome sequences, it is likely that several hundred proteins are imported into the mitochondrion of trypanosomes and related trypanosomatids, a proteome size comparable to other eukaryotes including humans. Comparative sequence analysis has been used to address the extent to which the machines that drive protein import into the mitochondrion of *T. brucei* are distinct from those that function in humans (Box 2). The protein import machinery found in animals and fungi is shown in Figure 2. Here we discuss what is known about the mitochondrial protein import pathway in *T. brucei* and how it compares with the process in humans. We propose

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Box 1. The extreme biology of trypanosomes

Although the mitochondrial biology of *T. brucei* appears to have unique features [9], history has shown that many 'unique' biological phenomena first discovered in trypanosomes are in fact more widespread, but cryptic, in other eukaryotes. Examples of this include trans-splicing of nuclear RNAs, GPI anchoring of membrane proteins and RNA editing. All were first described in trypanosomes, regarded as curiosities, but subsequently shown to be general features of eukaryotes. We expect that this will also be true for many apparently unique aspects of the mitochondrial biology of *T. brucei*. For example, recent work suggests a membrane-associated mitochondrial DNA segregation apparatus, like that seen in *T. brucei*, might be a more general feature of mitochondria. Furthermore, coordination of mitochondrial division with the cell cycle might indeed occur in other eukaryotes, but with large numbers of individual and interconnected mitochondria this is currently difficult to determine.

that several apparently distinguishing features of the *T. brucei* machinery provide a means to better understand the mitochondrial protein import process in all eukaryotes.

Primitive targeting sequences: the bare bones

Experimental studies and bioinformatic analyses have indicated that mitochondrial targeting sequences in trypanosomatids are usually much shorter than those found on mitochondrial proteins in animals, plants and fungi [10–16]. Shortened targeting sequences are also found in microsporidians, a group of organisms that phylogenetically group with fungi [17], and in *Giardia* and *Trichomonas*, organisms that have metabolically-specialized organelles,

Box 2. Protein import into mitochondria: animals and fungi

In humans and other animals, the mitochondrial genome is small; in humans it codes for only 13 proteins so that almost all of the ~1000 proteins that constitute the mitochondria are synthesized on ribosomes in the cytosol and targeted to mitochondria post-translationally [41]. Mitochondrial targeting information is coded in hydrophobic and basic residues, often present at the N-terminus in the form of a basic, amphipathic helix referred to as a presequence. Such presequences are 30–50 residues long and are proteolytically removed by peptidases: the matrix processing peptidase (MPP) or inner membrane located peptidase (IMP), once the imported protein has reached its final destination within the mitochondrion [2,35,41].

Mitochondrial targeting signals are recognized by receptors on the mitochondrial surface. These receptors are part of the TOM complex: the protein translocase in the outer mitochondrial membrane (Figure 2). After translocation through the outer membrane, proteins with appropriate N-terminal extensions are passed to the TIM23 complex and can be pulled into the inner membrane or through to the matrix by the action of the presequence-associated motor, a module of the TIM23 complex built from the subunits Tim44, Pam18 (Tim14), Pam16 (Tim16) and mitochondrial Hsp70 (mHsp70). Membrane proteins are transferred instead to the TIM22 complex in the inner membrane or the sorting and assembly machinery (SAM complex) in the outer membrane for membrane insertion and assembly. A set of 'tiny TIMs' in the intermembrane space can drive this transfer of substrate protein from the TOM complex [26,27,32].

termed mitosomes and hydrogenosomes, that are derived from mitochondria [1]. These short targeting sequences were shown to be functionally interchangeable: they can be swapped between proteins from *Giardia* and *Trichomo-*

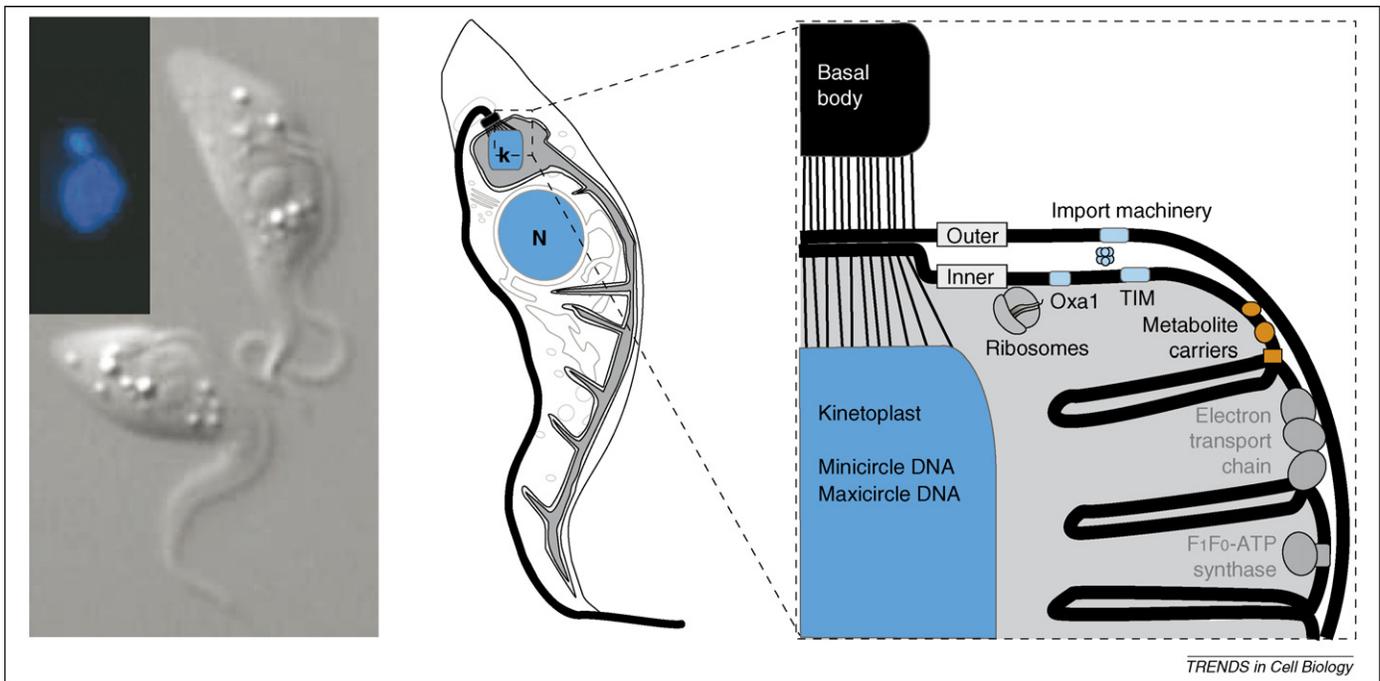


Figure 1. Architecture of the mitochondrion of *T. brucei*. (a) Two parasites viewed by Nomarski optics. The blue DNA staining (inset) reveals two genomic compartments: the large nucleus (N) and the mitochondrial genome, termed kinetoplast (k). (b) Schematic drawing of a *T. brucei* cell with an enlarged kinetoplast region. The kinetoplast DNA is contained within the mitochondrial matrix and represents the mitochondrial genome. The flagellum (black) is attached to the cell surface and extends along the entire length of the cell body. The branched single mitochondrion is shaded grey. The mitochondrial genome consists of minicircle and maxicircle DNA that are topologically interlocked among themselves and among each other. It is directly associated with the flagellar basal body (black) through proteinaceous structures that cross both mitochondrial membranes. In the soluble matrix compartment, mitochondrial ribosomes translate proteins that assemble together with proteins imported from the cytoplasm by way of the protein translocases in the outer and inner membranes. Note that only in insect stage of the parasite are components of the electron transport chain expressed: in the forms adapted to live in the glucose-rich environment of the human blood stream, these components are absent from the mitochondrial membranes.

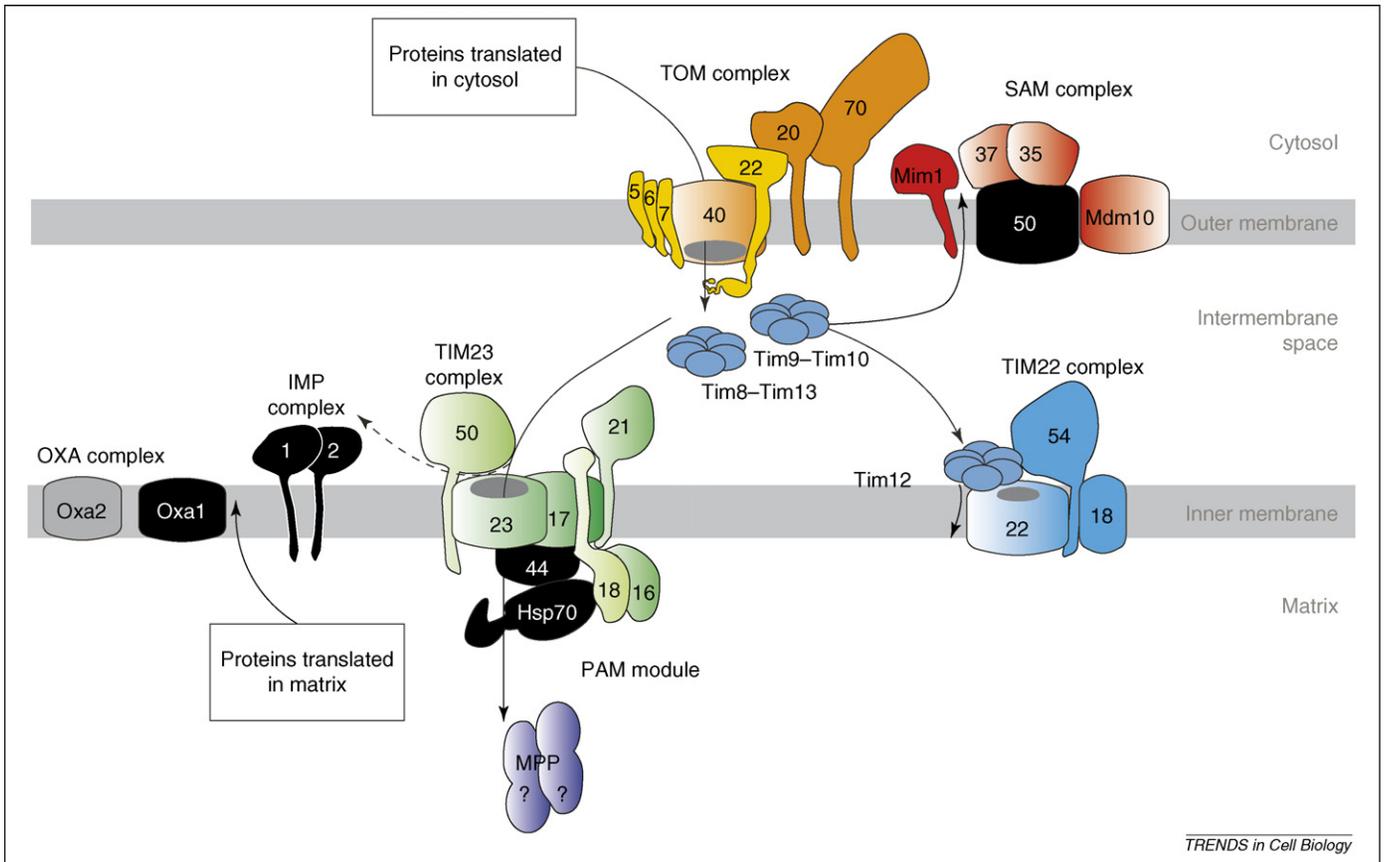


Figure 2. Protein import into mitochondria in animals and fungi. The components of each of the mitochondrial protein translocases that have been functionally defined in yeast are colour-coded, with black denoting those components inherited from the bacterial ancestor. The TOM and SAM complexes in the outer membrane are composed of multiple subunits, most of which are highly conserved in humans, but only few of which are conserved in other organisms. In the inner membrane, three distinct protein translocases are known: the TIM23 complex that drives protein import into the matrix, the TIM22 complex for assembly of most multi-spanning membrane proteins into the inner membrane, and the OXA complex that assembles proteins from the matrix space into the inner membrane. A membrane-embedded protease, the IMP complex, is composed of Imp1 and Imp2 subunits and processes some of the substrates released by the OXA complex and, for some of the substrates passed from the TIM23 complex, this processing liberates protein domains into the intermembrane space. Transfer of membrane proteins from the TOM complex to either the SAM complex or to the TIM22 complex is mediated by hexameric tiny TIM complexes: one composed of three subunits of Tim10 and three subunits of Tim9, the other composed of three subunits of Tim13 and three subunits of Tim8. A related protein, Tim12, associates stably with the TIM22 complex to assist docking of substrate laden Tim9–Tim10 complexes. Components of each complex are labelled by number: the SAM complex subunit ‘37’ refers to ‘Sam37’, ‘35’ refers to ‘Sam35’, the TOM complex subunit ‘70’ refers to ‘Tom70’, and so on. The broken arrow reflects that for a few proteins targeted to the TIM23 complex, stop-transfer signals will see these proteins released into the intermembrane space. Most proteins targeted to the TIM23 complex will be translocated into the matrix.

nas [18], and proteins from *Trichomonas* are targeted to mitochondria in trypanosomes [11]. These short targeting sequences can be used to direct proteins to mitochondria in yeast [11,15], which demonstrates that short targeting sequences contain ‘core’ information within the longer sequences that naturally occur on yeast and human proteins.

Analysis of common determinants has defined a motif of conserved features in short mitochondrial targeting sequences [15]. It seems that six to eight residues that contain a particular combination of serine, leucine and basic residues represent the conserved features of the short targeting sequence and, owing to their functional equivalence with long targeting sequences, of mitochondrial targeting sequences in general. This is consistent with deletion mutagenesis of long mitochondrial targeting sequences in yeast, which shows that ~12 residues including serines, leucines and basic residues encompass the minimal, ‘core’ mitochondrial targeting sequence necessary and sufficient to send a protein to mitochondria [19]. This raises a question, requiring experimental assess-

ment, as to why so many mitochondrial proteins in humans (and in fungi and plants) have such long targeting sequences.

The protein import machinery in *Trypanosoma brucei*

The genomes of *T. brucei* and two other trypanosomatids, *Trypanosoma cruzi* and *Leishmania major*, have been completely sequenced and a few highly-conserved components of their mitochondrial protein import machinery annotated [16,20,21]. A recent analysis, using advanced statistical models for sequence comparison has added further to our knowledge of the machinery [2]. In some cases it might be that potential homologs in *T. brucei* have been missed because our current sequence searching tools are not powerful enough, and the sequences being sought are too far diverged. This could explain why a protein translocase in the outer mitochondrial membrane (TOM complex) is yet to be identified in the trypanosomatids, given the limited sequence similarity found in subunits of the TOM complex. Alternatively, it remains possible that there is no TOM complex and a different type of

protein translocase now functions in the outer membrane of *T. brucei*. Further experimentation is required to distinguish between these possibilities. By contrast, the components of the inner membrane transport and assembly machinery tend to be highly conserved across species, even in protists such as *Plasmodium*, *Giardia*, the diatoms and trypanosomatids [2]. Several aspects stand out in which the *T. brucei* import pathway might be simpler than that found in human mitochondria (Figure 3), and in which experimental work on *T. brucei* might have broad implications for understanding mitochondrial protein import in general.

A single inner membrane translocase

The Tim17-Tim22-Tim23 family of proteins (PFAM: PF02466) [22] are a set of highly-conserved proteins found in animals, plants, fungi and protists that range from *Plasmodium* to *Trichomonas* [2,23,24]. In all cases, the protein has four predicted transmembrane domains with specific amino acid signature motifs [23]. In yeast and in humans, two members of the family (Tim23 and Tim17) combine to form the translocation channel of the TIM23 complex to which the presequence-associated motor complex docks (Figure 2). This complex enables insertion of simple, monotopic membrane proteins into the inner membrane, and translocation of other protein substrates into

the matrix [25–27]. Tim22, a third member of the family and the key component of the TIM22 complex, receives membrane protein substrates of more complex topology, which includes metabolite carrier proteins, from the tiny TIM chaperones and inserts and assembles these substrate proteins into the inner membrane [26–28].

In *T. brucei*, the protein encoded by the gene *Tb11.01.4870* is the only member of the Tim17-Tim22-Tim23 family of proteins. Depletion of this protein by RNAi results in mitochondrial defects and cell growth arrest [29], consistent with an essential function in mitochondrial protein import. But is this protein functionally equivalent to the human Tim17, Tim22 or Tim23?

Trypanosoma brucei has at least twenty-five metabolite carrier proteins that would normally require a TIM22 complex for their assembly into the inner membrane [30]. That a functional equivalent to the TIM22 complex in human mitochondria does exist in trypanosomes is evidenced by work in which a carrier protein from human mitochondria was expressed in transfected *Leishmania* and shown to be correctly assembled into the functional form in the inner membrane of the mitochondrion [31]. But if *Tb11.01.4870* does provide the function of a TIM22 complex, what provides for protein translocation into the matrix?

The strong sequence conservation between all Tim17-Tim22-Tim23 family members makes it unlikely that two

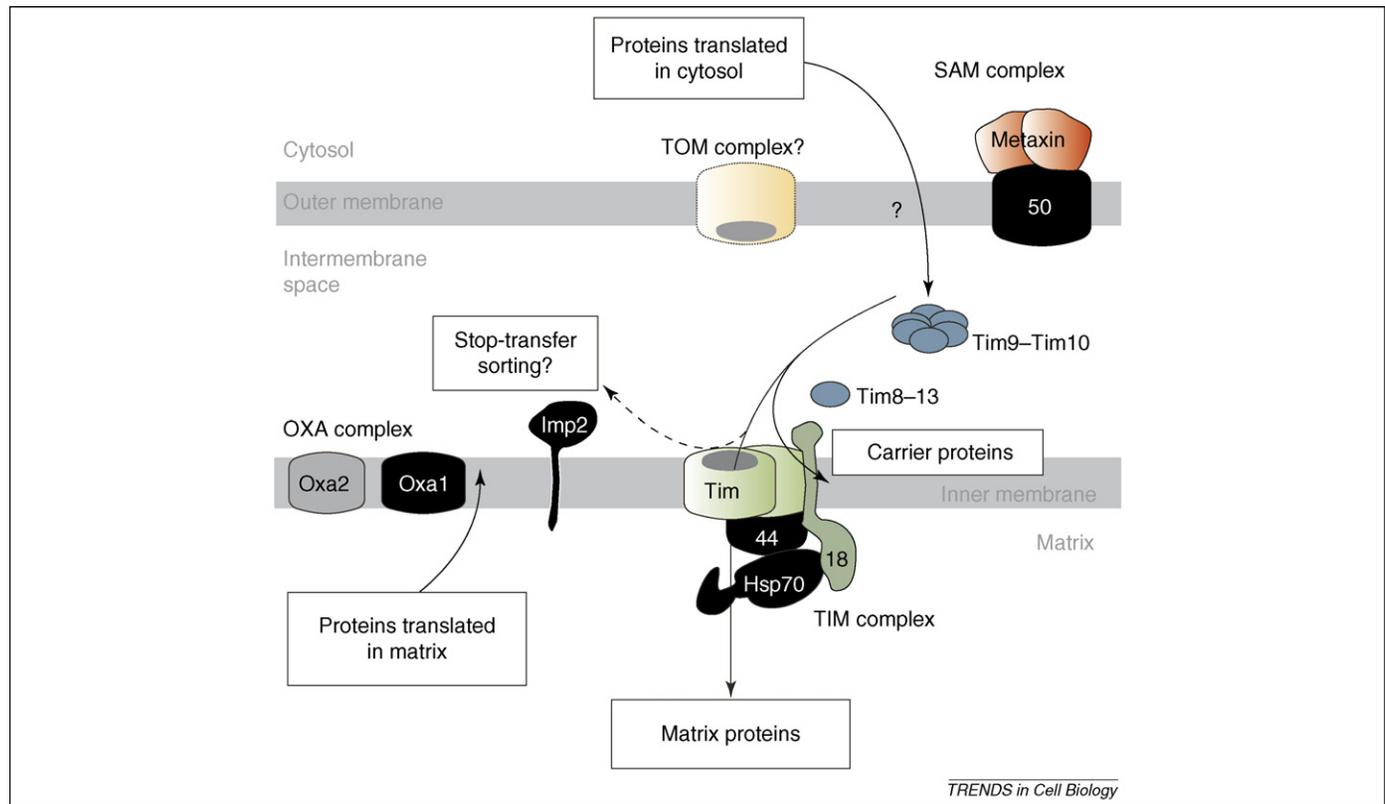


Figure 3. A model for protein import into the mitochondrion of *T. brucei*. The proteins found by sequence analysis are mapped out by comparison with the complexes found in mitochondria of yeast and humans. The core component of the SAM complex is Sam50, and *T. brucei* has a clear homolog (*Tb927.3.4380*), as well as several proteins that share sequence similarity to the peripheral (metaxin) subunits. Although candidate subunits exist for a translocase of the outer mitochondrial membrane (TOM) complex, their sequence signatures are weak and experimental work is required to determine by which route proteins cross the outer membrane of the *T. brucei* mitochondrion. As discussed in the text, *T. brucei* has small TIM chaperones that would assist its carrier proteins (and other substrates) across the intermembrane space. *Tb11.01.4870* is a Tim protein that would form an inner membrane translocase, and a PAM module would assist protein translocation into the matrix. IMP and OXA complexes to assist the assembly and processing of proteins encoded on the mitochondrial DNA that need to be transported from the matrix. Arrows show the protein transport pathways to and through the inner membrane, with the broken arrow indicating that *T. brucei* might or might not be capable of ‘stop-transfer’ sorting of proteins [32]. Components of each complex are labelled by number, as in Figure 2.

further members remain to be discovered in *T. brucei*. There are some sequence features that distinguish the Tim17s from the Tim22s and from the Tim23s and that therefore allow phylogenetic analysis, but *Tb11.01.4870* sits distinct from all three families in phylogenetic trees (Bursac and Lithgow, unpublished data). This situation is mirrored by another unrelated group of eukaryotes, the microsporidians, which also have only one predicted Tim17-Tim22-Tim23 protein in the genome [17]. This common scenario in diverse organisms suggests that a single TIM complex is capable of protein translocation into the matrix and the assembly of proteins into the inner membrane. If it is true that a single ‘jack-of-all-trades’ translocase fulfils all inner membrane translocation requirements in these organisms, it is plausible that the first eukaryotes also required only a single TIM protein.

The presequence-associated motor for the ‘TIM23 complex’

The driving force for protein translocation across the mitochondrial inner membrane is provided by the presequence-associated motor (PAM) [27,32]. This PAM docks as a module onto the TIM23 complex, through interactions with the Tim17 subunit. The core subunits of the motor: Tim44, Pam18 and the mitochondrial Hsp70, are found ubiquitously in eukaryotes [2,18,32]. In yeast and in humans, several additional subunits are found as part of the PAM, including Pam16, Pam17 and Mdj2. Although it is not yet clear whether *T. brucei* has all of these additional subunits, the core Pam18, Tim44 and mHsp70 subunits of the PAM are encoded in the *T. brucei* genome. The docking of this PAM module to the *T. brucei* TIM complex would activate it to allow protein translocation into the matrix, analogous to the function of the TIM23 complex in yeast and humans, driven by the ATP dependent action of mHsp70 (Figure 3). The PAM complex is a hallmark of the TIM23 complex, and *Tb11.01.4870* might therefore provide the Tim17 and Tim23 function to this TIM complex in *T. brucei*. But there is also a hallmark of a TIM22 complex, the small TIM chaperones, and these too are found in *T. brucei*.

Tiny TIMs for the ‘TIM22 complex’

Humans express five tiny TIMs that form three distinct heteromeric complexes for delivery of carrier proteins across the intermembrane space to the TIM22 complex (Figure 2). In *T. brucei*, *Tb927.7.2200* is a homolog of Tim9 and *Tb927.3.1600* is a homolog of Tim10 [29]; by analogy to the human protein import pathway, these two proteins would form a Tim9–Tim10 hexamer for delivering carrier protein substrates to the TIM complex in the inner membrane [33]. The third gene, *Tb11.02.3065*, encodes a ~10 kDa protein that has sequence features of both the Tim8 and Tim13 proteins from humans, and might represent an ancestral form that, through gene duplication and modification, gave rise to the genes that now encode Tim8 and Tim13 in humans and other organisms [29]. Whether *Tb11.02.3065* functions as a distinct chaperone free in the intermembrane space or docked onto the TIM complex in the inner membrane remains to be determined.

Bacterial type protein translocase(s) and peptidase(s)

Two components of the protein import machinery in the inner mitochondrial membrane were inherited from the ancestral proteobacterium. The OXA complex that assists assembly of at least some of the inner membrane proteins that have multiple α -helical transmembrane segments is derived from the bacterial protein YidC [34]. The inner membrane-associated protease (IMP) that processes signal sequences from proteins inserted into the inner membrane is derived from the bacterial signal peptidase [35]. A core subunit of these complexes is present in *T. brucei*.

In humans, the hetero-oligomeric IMP complex cleaves targeting sequences from proteins transported by both the TIM23 and OXA complex, respectively [35]. In fungi, animals and plants, two catalytically active subunits, Imp1 and Imp2, provide an ability to process a range of targeting sequences and enable the ‘stop-transfer’ targeting of proteins into the inner membrane; whereby a substrate protein is targeted to the TIM23 complex, but additional targeting information signals for a ‘stop’ to translocation and for a lateral ‘transfer’ from the translocase into the inner membrane [36]. The entire targeting sequence, including the stop-transfer signal, is then processed from the substrate by the IMP complex. A motif in the catalytic region of the peptidase distinguishes Imp1 and Imp2 [36]; *T. brucei* has just a single protease subunit (*Tb10.70.2630*) with the sequence characteristics of Imp2 (Figure 3).

Embedded in the inner membrane of bacteria is a chaperone called YidC, which assists membrane insertion of proteins that range from phage coat proteins to subunits of the respiratory chain. Homologous proteins, Oxa1 and Oxa2, perform functionally conserved roles in mitochondria [34]. Previous phylogenetic analysis had suggested that Oxa2 was derived by recent gene duplication events in fungi and animals, with Oxa2 having a more specialist function [37]. However, the gene *Tb11.02.4020* encodes the *T. brucei* Oxa1, and *Tb09.211.1580* encodes what appears to be a homolog of Oxa2. Whether a single duplication event of the YidC type gene took place very early in evolution (to give rise to Oxa1 and Oxa2), or later duplication events gave rise to Oxa1 and Oxa2 independently (in different lineages of eukaryotes such as humans and the protists like *T. brucei*) is unclear and requires more detailed study.

What the simple pathway in *T. brucei* tells us about ourselves

One needs to be wary of claiming that an organism displays ‘primitive’ features because loss of features, which might be selected for by a parasitic life style, could mimic primitiveness. In microsporidians, the short presequences, a single TIM complex and absence of Imp1 are clearly instances of secondarily derived characters [17]. However, in the case of the mitochondrial protein import pathway in *T. brucei* and in other eukaryotes that diverged early in evolution, the short presequences and the single inner membrane translocase might well reflect the primitive condition, even if they have since become sophisticated in their own right. Figure 4 outlines a scenario that would

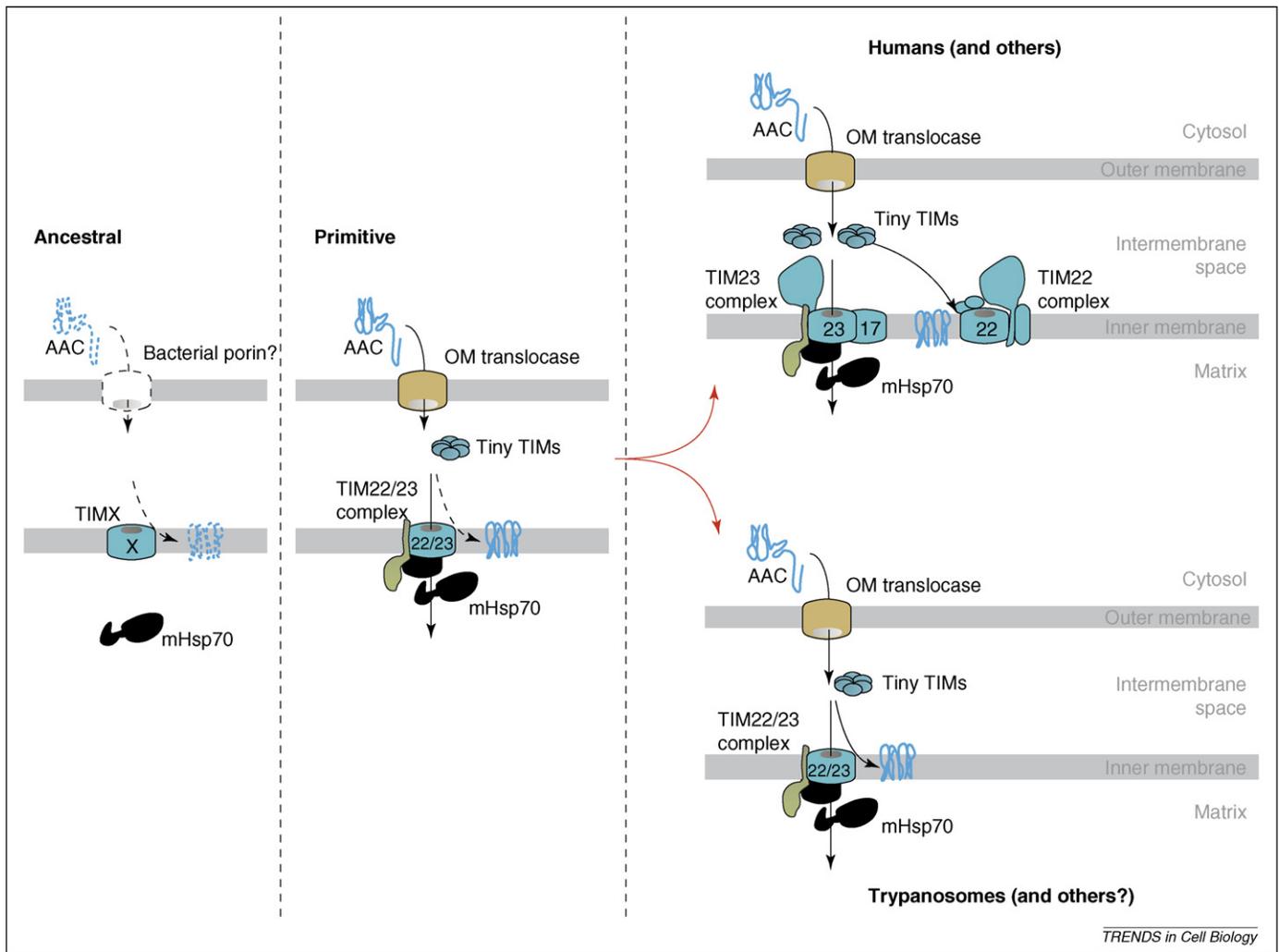


Figure 4. Evolution model for the TIM translocases in humans (and other organisms including animals and fungi) and trypanosomes (including other trypanosomatids and perhaps also other groups of eukaryotes). In an initial condition, ‘import’ of the ADP/ATP carrier (AAC) protein into the inner membrane of a bacterial endosymbiont was inefficient and perhaps mediated by pre-existing bacterial proteins. The inner membrane component assisting assembly of the AAC is referred to as TimX, which might be related to LivH-type amino acid transporters [23]. With effective delivery of ATP to the cytosol being a driving force, changes in TimX to make it a better translocase would be highly selectable. In humans, protein translocation into the matrix and insertion into the inner membrane were accomplished by duplication events with specialization (a partial neofunctionalization) of Tim22, Tim23 and Tim17. By contrast, we suggest organisms like trypanosomes allowed selective changes to a single Tim protein, which makes it effective at forming both a translocase for protein translocation into matrix and protein assembly into the inner membrane. The broken arrows indicate the relatively poor efficiency of protein translocation in the ancestral condition. Abbreviation: OM, outer membrane.

explain the evolution of the import machinery in the mitochondrial inner membrane.

It remains a matter of discussion as to how and why mitochondria were initially acquired [38,39], but the ongoing driver for evolution of mitochondria would be the generation of ATP by mitochondria and its supply to the host cytosol [39]. This export would require insertion of a novel and primitive ADP/ATP carrier (AAC) into the inner membrane of the ‘protomitochondrion’. Any bacterial protein that could, even inefficiently, catalyze this insertion reaction would provide a basis on which natural selection could act to produce the first ‘mitochondrial’ protein translocase. This inefficient targeting of an AAC is represented by broken arrows in Figure 4 (Ancestral). Mutations that improve the activity of that translocase would be readily selected for, with the increased access to ATP (from increased efficiency inserting AAC) being a huge prize for natural selection. We suggest that a primitive protein, TimX, fulfilled this role and thereby serves as the founding member of the Tim23-Tim17-Tim22 protein

family. It is possible that TimX was derived from a bacterial LivH-type amino acid translocase [23], although this remains to be established.

With a developing need to translocate some proteins through into the mitochondrial matrix, organisms (such as those that gave rise to animals and fungi) made use of gene duplication and gradual specialization to provide two distinct TIM complexes: the ‘TIM22 complex’ with the core protein Tim22 drifting towards a more efficient membrane protein assembler; and the ‘TIM23 complex’ where the core protein Tim23, absolved of AAC insertion duties, drifted in sequence to become a better protein translocator. A second duplication would provide the subunit Tim17, to further enhance the function of this TIM23 complex.

We argue, however, that this progressive gene duplication strategy was not the only way forward and that in other organisms, like those ancestral to the trypanosomes, the single TIM complex has been specifically modified to perfect both its membrane protein assembly and protein translocation capabilities.

Humans now have longer mitochondrial targeting sequences and distinct protein translocases, which are composed of three different members of the Tim23-Tim17-Tim22 family of proteins, in the mitochondrial inner membrane. The development of longer targeting sequences allowed for an increased pulling force to enable unfolding of folded domains [40] and to sort some proteins into the intermembrane space by a stop-transfer mechanism [32], where processing the various stop-transfer substrates relies on a heteromeric IMP complex. *Trypanosoma brucei* might have been denied these developments and, consequently, this would constrain the protein substrates able to be imported into the mitochondrion. However, if the single TIM complex in *T. brucei* has become a 'jack-of-all-trades', effective at both protein translocation across the inner membrane and at protein insertion into the inner membrane, understanding how it works promises to provide a unique insight into the protein translocases of all mitochondria.

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