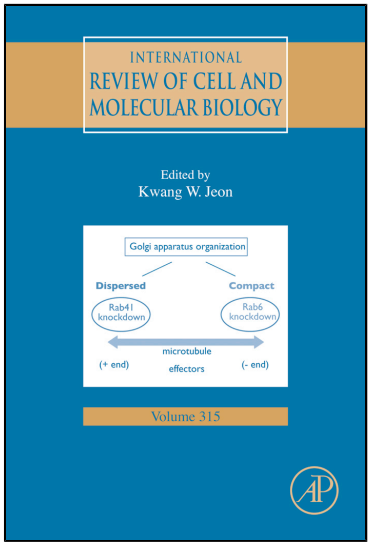

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CHAPTER THREE

Malleable Mitochondrion of *Trypanosoma brucei*

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Contents

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Abstract

The importance of mitochondria for a typical aerobic eukaryotic cell is undeniable, as the list of necessary mitochondrial processes is steadily growing. Here, we summarize the current knowledge of mitochondrial biology of an early-branching parasitic protist, *Trypanosoma brucei*, a causative agent of serious human and cattle diseases. We present a comprehensive survey of its mitochondrial pathways including kinetoplast DNA replication and maintenance, gene expression, protein and metabolite import, major metabolic pathways, Fe-S cluster synthesis, ion homeostasis, organellar dynamics, and other processes. As we describe in this chapter, the single mitochondrion of *T. brucei* is everything but simple and as such rivals mitochondria of multicellular organisms.

1. INTRODUCTION

The importance of mitochondria for a typical eukaryotic cell cannot be exaggerated, as the list of processes in which they are involved is steadily growing (Scheffler, 2007). In this chapter, we demonstrate that this is also true for the mitochondrion of a well-studied parasitic protist, and that in terms of complexity, its organelle matches that of multicellular organisms. It is clear that all extant mitochondria are of singular origin. Although numerous protist lineages harbor organelles such as mitosomes and hydrogenosomes that were thought to have emerged independently of the mitochondrion, the available evidence points to all of them being derived from it (Tachezy, 2008). Some mitosomes were reduced to a mere shadow of the organelle from which they evolved, and currently the only function shared by all known mitochondria and mitochondrion-derived organelles (sometimes also labeled mitochondrion-related organelles (MROs)) is the synthesis of iron–sulfur (Fe-S) clusters (Lill, 2009; Tachezy, 2008).

Out of the six to seven currently recognized eukaryotic supergroups, the exclusively unicellular Excavata brings together protists with a groove-shaped central cytostome (Adl et al., 2012). They carry the most diverse forms of aerobic and anaerobic mitochondria, as well as MROs. However, with more excavates being examined, it appears that there are no strict boundaries defining these categories, but rather a continuum of organelles, ranging from a conventional mitochondrion to a massively reduced MRO (Jedelský et al., 2011). Indeed, excavates arguably evolved higher diversity of their mitochondrial (mt) genomes than all the other eukaryotes combined (Flegontov et al., 2011).

Trypanosoma brucei and related flagellates belong to the excavate kingdom Euglenozoa, the members of which are invariably aerobes. In the most recent taxonomic system, trypanosomes are placed into Kinetoplastea, which along with Euglenida, Symbiontida, and Diplonemea constitute Discicristata, with discoidal mt cristae being their unifying feature (Adl et al., 2012). Among them, only Kinetoplastea, also called kinetoplastids, developed the parasitic lifestyle. Consequently, out of these important and virtually omnipresent protists, kinetoplastids represent the by far best-studied group. All euglenozoans carry a single, typically reticulated mitochondrion that in most species contains unusually high amount of mtDNA. Moreover, their mitochondria are notorious for various other departures from the prototypical organelle, which in this chapter will be described in detail almost exclusively for *T. brucei*.

Because of being the causative agent of African sleeping sickness of humans and livestock, *T. brucei* is the best-studied representative of the euglenozoan protists. Moreover, the nuclear and mt genomes have been sequenced for a number of its strains and subspecies, it can be easily cultured, and is amenable to most methods of forward and reverse genetics. Studies of its mitochondrion contributed substantially to our understanding of widespread and important features of the eukaryotic cell that include RNA editing, fatty acid (FA) biosynthesis, DNA topology, composition of respiratory complexes, and calcium metabolism. Here we present a chapter on the malleable mitochondrion of *T. brucei* with the aim to summarize what is known about the protein component of this fascinating organelle. As will be frequently discussed throughout this chapter, the mitochondrion of this notorious parasite comes in at least three major forms: (1) the fully active and developed one characteristic for the procyclic stage (PCF), a form transmitted by the tsetse fly *Glossina* spp.; (2) the functionally and morphologically repressed form found in the bloodstream form (BSF), which is responsible for the actual disease in vertebrates; (3) the "petite" form of the mitochondrion restricted to what is currently considered a subspecies, *T. brucei evansi*. Strains that lost part of their mt (= kinetoplast; k) DNA are termed dyskinetoplastic, while those which did not retain any kinetoplast DNA (kDNA) are labelled as akinetoplastic (Lai et al., 2008; Schnaufer et al., 2002). Already the combination of these different states, among which the *T. brucei* mitochondrion oscillates, makes it an attractive model organelle for studying processes such as repression and activation of specific mt pathways, and mechanisms as well as consequences of the (gradual) loss of kDNA. Another advantage of studying this mitochondrion rests in the exclusive presence of a single organelle per cell.

Hence, it should be relatively straightforward to study phenotypes ensuing from the disruption of mt division as well as fusion.

Here we present a few examples in which the trypanosomatid mitochondrion has already played a pivotal role in the discoveries of key organellar functions: (1) RNA editing, now known to be widespread in all domains of life, was first described in *T. brucei* (Benne et al., 1986) (Section 2.2.3.); (2) bent helix DNA was for the first time demonstrated in the kDNA of closely related trypanosomatid *Crithidia fasciculata* (Marini et al., 1982); (3) the power of comparative proteomics, taking advantage of the highly diverged respiratory complex I in *T. brucei*, was used to identify some novel subunits in humans (Pagliarini et al., 2008) (Section 4.2.); and (4) the early finding of a putative mt calcium transporter in *Trypanosoma cruzi* (Docampo and Vercesi, 1989a), the causative agent of Chagas disease, was instrumental for recent discoveries of the universally conserved calcium uniporter and associated proteins (Baughman et al., 2011) (Section 3.2).

The comprehensive survey of mt pathways in *T. brucei* attempted below leads to a potentially important conclusion in respect to our view of evolution of this key organelle. Due to their appurtenance to the Excavata, which is arguably the most ancestral of the currently recognized eukaryotic supergroups (Cavalier-Smith, 2010; He et al., 2014), trypanosomes and other kinetoplastid flagellates represent logical candidates for a rather streamlined mitochondrion that has evolved around a limited core, a remnant of the α-proteobacterial endosymbiont. However, their mitochondrion is everything but simple. It was proposed recently that the eukaryotic ancestor that accepted an α-proteobacterial endosymbiont already harboured a membrane-bound organelle (Gray, 2014). Upon fusion of the α-proteobacterium with this organelle, equipped with a number of proteins characteristic for the extant mitochondrion, metabolic complements of both entities would have been brought together, allowing a relatively fast emergence of a pre-mitochondrion that would be quite complex already at a very early stage of its evolution (Gray, 2014). As detailed below, in terms of complexity of its mitochondrion, a miniscule protist such as *T. brucei* indeed rivals that of large and seemingly more complex multicellular animals and plants.

2. MAINTENANCE AND EXPRESSION OF GENETIC INFORMATION

The majority of α-proteobacterial ancestor-derived genes have been transferred from the mt genome to the nuclear genome. Consequently,

the nuclear-encoded mt proteins are synthesized in the cytosol and imported into the organelle. Some genes, however, remain mitochondrial encoded. The overall mtDNA gene composition can differ between species, although in most cases it encodes ribosomal RNAs (rRNAs), transfer RNAs (tRNAs), and messenger RNAs (mRNAs). The mRNAs mainly encode the membrane components of the electron transport chain.

In *T. brucei*, the mtDNA is located at the posterior end of the single mitochondrion and is subsequently referred to as kDNA (Figure 1). The kDNA, a defining structure of the kinetoplastid flagellates, is an extremely complex form of mtDNA. However, unlike in most other eukaryotes, it is only replicated once per cell cycle and is synchronized with nuclear replication and division (Englund, 1978; Woodward and Gull, 1990) (Figure 1). It is composed of two distinct DNA entities known as (kDNA) maxicircles and minicircles, which are organized into an intercatenated network (Liu et al., 2005; Stuart, 1983) (Figure 1). In *T. brucei*, the maxicircles encode two rRNA genes: one small guide RNA (gRNA) gene and 18 protein-coding transcripts (Clement et al., 2004; Lukeš et al., 2005). Of these, six contain open reading frames (so-called never-edited mRNAs), whereas 12 are in a pre-edited form, and have to be posttranscriptionally decoded by a process known as RNA editing (Section 2.2.3). Furthermore, alternative editing has been shown to increase to a limited extent transcript diversity (Ochsenreiter et al., 2008). The maxicircles are homologous to the mtDNA of other eukaryotes, are approximately 23 kb long, and are present in 30–50 copies per cell. Originally it was hypothesized that all maxicircles were identical, however variable regions were identified in *Leishmania major* (Flegontov et al., 2009), although this has not yet been investigated in *T. brucei*. In contrast to the maxicircles, there are several thousand minicircles per kinetoplast, each approximately 1 kb long. They exist as a highly diverse and heterogeneous population, although their origin of replication is conserved (Ntambi and Englund, 1985). The minicircle-encode gRNAs are essential for editing. Unlike mtDNA in humans and yeast, the kDNA does not encode any tRNAs, thus all tRNAs must be imported from the cytosol (Section 2.2.5).

The presence of a protein-coding genome, although very small, requires the maintenance of a functional DNA expression machinery to perform the tasks of transcription, posttranscriptional processing, and translation in the mitochondria.

2.1 kDNA Replication and Maintenance

It is believed that from a noncatenated form present in extant bodonids (Lukeš et al., 2002), kDNA evolved into a single catenated network to

maintain all minicircle classes and ensure their faithful replication (Borst, 1991). The complexity of kDNA replication and maintenance requires a greater repertoire of proteins; to date, approximately 30 proteins have been characterized, although the final number is believed to be more than 150 (Jensen and Englund, 2012). Among the proteins involved in kDNA replication are well-conserved enzymes that include ligases (Sinha et al., 2006, 2004), topoisomerases (Bakshi and Shapiro, 2004; Lindsay et al., 2008; Melendy et al., 1988; Scocca and Shapiro, 2008; Wang et al., 2000), and primases (Hines and Ray, 2011, 2010). Moreover, kDNA contains a staggering six helicases (Liu et al., 2009a) and seven DNA polymerases (Klingbeil et al., 2002; Rajão et al., 2009; Saxowsky et al., 2003), which are unrelated to those found in the mitochondria of other eukaryotes. In addition to these typical replication enzymes, there are numerous proteins unique for *T. brucei*, such as universal minicircle sequence-binding protein (UMSBP) (Tzfati et al., 1992), p38 (Liu et al., 2006), p93 (Li et al., 2007), and hypothetical proteins (Beck et al., 2013). The packaging and structural integrity of kDNA is facilitated by a set of histone-like KAP proteins 1–4 (Avliyakulov et al., 2004).

Replication of the kDNA is preceded by duplication of the basal body (Figure 1). This organelle, together with the tripartite attachment complex (TAC), is responsible for the separation of daughter kDNA networks and ensures their correct positioning within the mitochondrion (Gluenz et al., 2011; Robinson and Gull, 1991). The TAC is a structure composed of filaments that physically connect the basal body to the kDNA through the mt membrane (Figure 1). It is composed of unilateral filaments in the mt matrix, the differentiated membrane, and the exclusion zone filaments in the cytosol (Ogbadoyi et al., 2003). So far, four TAC proteins have been characterized: p166 (Zhao et al., 2008), Mab22 (Bonhivers et al., 2008), AEP-1 (Ochsenreiter et al., 2008), and TAC40 (Schnarwiler et al., 2014); all of them are essential for segregation of daughter kDNAs. The AEP-1 is kDNA-encoded by the same gene as cox3, although these two products are obtained by alternative editing (Ochsenreiter et al., 2008).

Replication of kDNA minicircles involves UMSBP and p38 protein, which recognize and bind the origin of replication, while the roles of p93 and numerous other associated proteins remain unknown. The minicircles are released from the network into the kinetoflagellar zone by the activity of topoisomerase II. Replication of free minicircles is initiated by Pif1 helicase (Liu et al., 2010) and Pri2 primase (Hines and Ray, 2011), followed by Pol1B polymerase which synthesizes both the leading and lagging strands

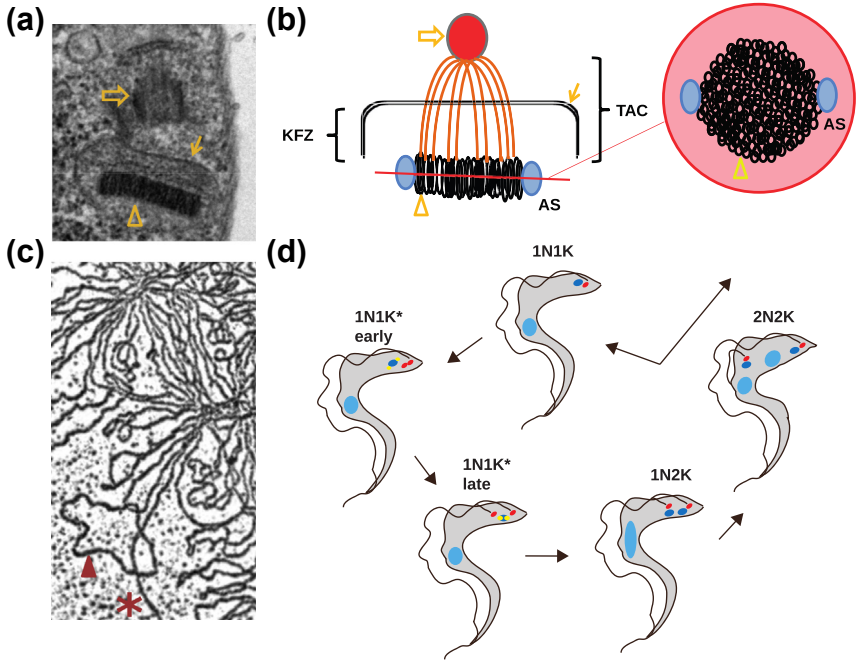

Figure 1 ***The structure and replication of kDNA.*** (a) Transmission electron microscopy of kDNA in *Trypanosoma brucei* (arrowhead, kDNA; simple arrow, mitochondrial double-membrane; open arrow, basal body). (b) Schematic view of the kDNA network and physical connection to the basal body; cross-section of the kDNA is shown in the right (arrows as in Figure 1a). (c) Transmission electron microscopy of kDNA network in *Trypanosoma avium* (asterisk, maxicircle; full arrowhead, minicircle). (d) The cell cycle of *T. brucei* highlighting the replication of its DNA. In the culture, cells predominantly have one nucleus (light blue (pale gray in print versions)) and one kDNA (dark blue (light gray in print versions)), referred to as 1N1K. Other stages during the cell cycle can be distinguished as follows: 1N1K* early, division starts with the basal body (red (gray in print versions)) duplication, followed by the kDNA replication. Newly synthesized kDNA minicircles containing nicks and gaps (yellow (white in print versions)), are reattached to the network at the antipodal sites; 1N1K* late, the migration of basal bodies in opposite directions forces kDNA division, evident as a bilobed structure with two connected daughter networks. The majority of minicircles within the network contains gaps; 1N2K—the two daughter kDNA networks separate, the remaining nicks and gaps are sealed, and the nucleus undergoes S phase; 2N2K—the nucleus divides and the cell is ready for cytokinesis, thus completing the cell cycle. Abbreviations: KFZ, kinetoflagellar zone; TAC, tripartite attachment complex; AS, antipodal sites.

(Bruhn et al., 2010). Topoisomerase IA resolves the theta structure of replicating minicircles (Scocca and Shapiro, 2008), and Pif5 helicase removes primers from Okazaki fragments (Liu et al., 2009b). Prior to the reattachment of minicircles back into the network by topoisomerase II, gap filling is initiated by ligase kβ and polymerase β (Saxowsky et al., 2003; Sinha et al., 2004); however, some nicks and gaps are retained as markers of newly replicated minicircles (Figure 1). The remaining gaps are filled by ligase kα and polymerase β-PAK within the fully replicated daughter networks (Saxowsky et al., 2003; Sinha et al., 2006), although this may occur only after the separation of daughter networks. The minicircles are separated by the reattachment to the opposing poles of the kDNA, thus creating two daughter minicircle networks, which are connected by maxicircle threads (Gluenz et al., 2011); complete separation is achieved after decatenation of the maxicircles.

How kDNA maxicircles replicate is largely unknown, likely due to their low copy number and the fact that their replication occurs within the kDNA network. There is only one known protein, Pif2 helicase, which affects exclusively maxicircle replication (Liu et al., 2009a). In addition, primase Pri1, and polymerases Pol1C and Pol1D, may play primary roles in maxicircle duplication (Hines and Ray, 2010; Jensen and Englund, 2012).

The replication of kDNA occurs before nuclear S phase and is strictly regulated (Figure 1). Expression levels of replicating enzymes such as topoisomerase II or primase 1 fluctuate during the cell cycle (Hines and Ray, 2010; Pasion et al., 1994). Protein stability is regulated by the HslVU protease, which controls Pif2 helicase levels as a negative regulator of maxicircle replication (Li et al., 2008), although the target protein involved in minicircle replication is currently unknown. Furthermore, the subcellular localization of Pol1D changes throughout the cell cycle (Concepción-Acevedo et al., 2012). Finally, changes in the activity of replication proteins provide an additional level of regulation. For example, UMSBP oligomerizes under oxidative conditions, and is unable to bind DNA, although this process is reverted under reducing conditions (Onn et al., 2004).

The kDNA is confined to kinetoplastid flagellates and represents a highly complex and regulated mtDNA, replicates once per cell cycle, and has specific localization within the single mitochondrion. Indeed, the composition of the kDNA network is remarkable, as it is composed of two distinct entities, contains a plethora of enzymes and proteins, several of which are unique for trypanosomes and related flagellates.

2.2 Mitochondrial RNA Metabolism

2.2.1 Transcription

The transcription of the human mt genome is relatively well studied, with described promoter regions and transcription factors (Mercer et al., 2011). In contrast, the identity of these elements remains poorly understood in trypanosomes, despite decades of research. Maxicircle transcription starts approximately 1.2 kb upstream of the 12S rRNA gene on the major strand, while the origin of transcription on the minor strand remains to be determined (Michelotti et al., 1992). The transcription start sites have been mapped in a very small subset of kDNA minicircles (Pollard et al., 1990), and while this information has not revealed the identification of bona fide minicircle promoters, both strands seem to be transcribed (Aphasizheva and Aphasizhev, 2010). A single mt RNA polymerase (mtRNAP) is responsible for transcription of both kDNA classes (Grams et al., 2002; Hashimi et al., 2009), and in both cases, the resulting polycistronic transcripts have to be endonucleolytically processed to release monocistrons required for expression.

2.2.2 RNA processing

Mitochondrial RNA processing of polycistronic transcripts in *T. brucei* is an intricate process consisting of numerous steps. Taken together, more than 100 proteins, and counting, are needed for the expression of 18 mitochondrial-encoded proteins (Lukeš et al., 2011). Essentially four maturation steps are required prior to translation of the mRNA: (1) endonucleolytic cleavage of the polycistronic transcript; (2) addition of short 3′ poly A tails; (3) U-insertion/deletion RNA editing (absent in the case of never-edited mRNAs); and (4) addition of long 3′ poly A and U tails to fully edited mRNAs. The processing of individual types of RNAs is summarized in Figure 2.

The maturation of mt mRNAs in *T. brucei* requires endo- and exonuclease activities to cleave polycistronic RNAs into monocistrons (Koslowsky and Yahampath, 1997). The identity of the enzymes providing these activities remains unknown. However, the RNA editing core complex (RECC), also referred as the 20S editosome, contains three RNAse III-type endonucleases and two 3′-5′ U-specific exonucleases (Carnes et al., 2008; Ernst et al., 2009). While it has been established that these enzymes are involved in U-insertion/deletion RNA editing, it is possible that they function in other processing steps, such as cleavage of the polycistronic maxicircle RNAs.

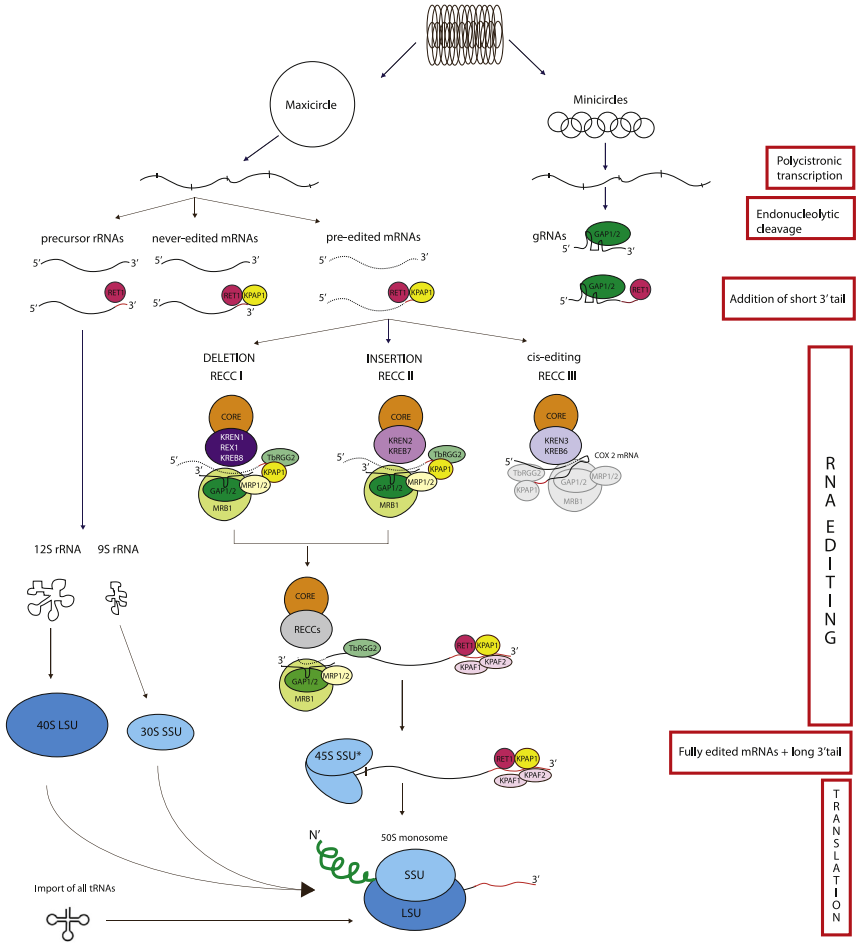

Figure 2 ***Mitochondrial gene expression and translation.*** The flow of information from the kDNA (top) to translation of mature mRNA (bottom) is depicted. Major steps in this process are labeled on the right. The kDNA is composed of dozens of maxicircles (left), which encode rRNA and protein-coding genes, and thousands of minicircles (right), encoding gRNA genes. All RNA molecules are transcribed polycistronically, with monocistrons being subsequently released from these long RNAs by endonucleolytic cleavage. rRNAs obtain short 3′ U tails (red line (light gray line in print versions)) via RET1 (dark pink (gray in print versions)). The 12S rRNA and 9S are incorporated into the 40S LSU (dark blue (light gray in print versions)) and 30S SSU (light blue (pale gray in print versions)) of the mitochondrial ribosome. Monocistronic gRNA molecules are stabilized by the GAP1/2 complex (dark green (dark gray in print versions)) and obtain a short 3′ U tail via RET1. Never-edited and preedited mRNAs are equipped with a short 3′ tail via KPAP1 (bright yellow (pale gray in print versions)) and presumably also RET1. Preedited mRNAs undergo RNA editing performed by one of three RECC isoforms (RECC I–III). RECCs are composed of an invariant protein core (orange (light gray in print

The *T. brucei* minicircle encodes from two to five gRNAs (Pollard et al., 1990) transcribed in a polycistronic fashion, which subsequently undergo endonucleolytic processing. An RNAse III-type endonuclease, called mt RNA precursor-processing endonuclease 1 (mRPN1), that is responsible for the cleavage of these minicircle-derived precursors has been recently identified (Madina et al., 2011). As is typical for this class of proteins, it acts as a dimer to cleave its substrate RNA into individual gRNAs. Antisense gRNA molecules have been suggested to play a role in guiding proper endonucleolytic cleavage of the proper gRNA transcripts (Aphasizheva and Aphasizhev, 2010).

The next step after endonucleolytic cleavage is the posttranscriptional addition of short 3′ extensions to the various mt RNA species. For example, pre-rRNAs obtain short 3′ U tails by an enzyme called RNA-editing terminal uridylyl transferase 1 (RET1) (Aphasizhev et al., 2003), before their incorporation into the ribosome (Section 2.3) (Aphasizheva and Aphasizhev, 2010). Pre-gRNAs are first stabilized via association with a heterotetramer of gRNA-associated proteins 1 and 2 (GAP1/GAP2), also known as the gRNA-binding complex (Hashimi et al., 2009; Weng et al., 2008) and then are appended with a short 3′ U tail by RET1 (Aphasizheva and Aphasizhev, 2010). How this processing is coordinated with endonucleolytic cleavage by mRNP1 is still not understood. In the case of pre-mRNAs, the processing of never-edited mRNAs requires only the addition of 20–25 nt short 3' (U/A/AU) tail, which is later elongated to 120–250 nt long 3' (A/U) tail, marking the transcript ready for translation (Etheridge

versions)), as well as of a module that recognizes the gRNA:mRNA duplex that marks either the deletion subcomplex (KREN1, REX1, and KREPB8) (dark purple (dark gray in print versions)) and the insertion subcomplex (KREN2 and KREB7) (medium purple (gray in print versions)), or the cox2 mRNA, which carries a *cis*-gRNA element (KREN3 and KREB6) (light purple (pale gray in print versions)). The gRNA:mRNA association is promoted by the MRP1/2 complex (light yellow (pale gray in print versions)). Core subunits of the MRB1 complex (light green (pale gray in print versions)) and the TbRGG2 subcomplex (medium green (light gray in print versions)) aid the processing of mRNAs that need *trans*-gRNAs, and are most likely not involved in cox2 editing (depicted by faded gray shapes). During the editing process, mRNAs obtain long 3′ tails via KPAP1 and RET1, with the participation of the PPR proteins KPAF1 and KPAF2 (pink (pale gray in print versions)). The fully edited transcripts are believed to associate with the 45S SSU*, which associates with the 30S SSU (light blue (pale gray in print versions)), an interaction that may facilitate the assembly of the latter with the 40S LSU of the mitochondrial ribosome. Translation is performed by the ribosome assembled on the mRNA, with the imported tRNAs participating in the elongation of the polypeptide.

et al., 2008). The processing of pre-edited RNAs that have to undergo RNA editing to decrypt a translatable sequence also undergoes short and long 3'-tail additions as in the case of never-edited mRNAs. However, the latter processing step is intertwined with RNA editing (Section 2.2.3).

The polyadenylation complex plays a critical role in the 3′ tailing process in mRNA. The kinetoplastid poly(A) polymerase 1 (KPAP1) is involved in the synthesis of both short and long 3′ tails (Etheridge et al., 2008). RET1 has been experimentally proved to be involved in the generation of long 3′ tails as well (Aphasizheva and Aphasizhev, 2010; Ryan and Read, 2005). Pentatricopeptide repeat (PPR) proteins called kinetoplast polyadenylation/uridylation factors 1 and 2 (KPAF1 and KPAF2) coordinate long 3′ A/U tail synthesis (Aphasizheva et al., 2011). The 3′ poly A/U tails play an important role in the regulation of RNA stability and diverse effects on these mRNAs at various stages of processing. The pre-edited, partially edited, and fully edited transcripts are stabilized by the addition of a short 3′ tail (Etheridge et al., 2008; Kao and Read, 2007, 2005), although only never-edited and fully edited mRNAs bearing long 3′ A/U tails are translated (Aphasizheva et al., 2011). These long-tailed mRNAs interact with the small ribosomal subunit (SSU) (Section 2.3). After assembly of the SSU and large ribosomal subunit (LSU), tRNAs are recruited to the mRNA and protein synthesis can begin.

2.2.3 RNA editing

RNA editing refers to any posttranscriptional processing step introducing changes in a transcript sequence relative to the corresponding gene, thus changing the information content of the RNA, except for splicing and terminal processing (Gott and Emeson, 2000). This process occurs throughout eukaryotes in different forms, such as the substitution adenosine-to-inosine editing that is prevalent in mammals. Yet RNA editing was originally discovered as four U residues inserted posttranscriptionally into cytochrome *c* oxidase subunit 2 (cox2) mRNA of *T. brucei* (Benne et al., 1986). Transcripts of several maxicircle genes were revealed to undergo more extensive pan-editing, in which hundreds of Us are inserted and tens of Us are deleted (Feagin et al., 1988). In general, RNA editing in trypanosomes generates open reading frames in edited mRNAs that serve as templates for translation. The role of this process remains poorly defined, although some transcripts are differentially edited between the BSF and PCF, suggesting its additional regulatory role in controlling the expression of maxicircle genes (Feagin and Stuart, 1988; Souza et al., 1992). The limited phylogenetic distribution of

U-insertion/deletion editing indicates that it evolved after the kinetoplastid clade of the Excavata branched off the eukaryotic tree.

U-insertion/deletion editing has been recapitulated at a single editing site with synthetic mRNA, gRNA, and crude mt extract, providing all necessary cofactors (Seiwert and Stuart, 1994). An editing site on a pre-edited mRNA is defined by the so-called anchor domain of a gRNA, 8–12 nt of sequence on the 5′-end that anneals to its cognate transcript. This hybridization between gRNA and mRNA also employs noncanonical G:U base pairs. The mRNA editing site starts at the first base pair mismatch within the RNA duplex, which also defines the beginning of the information domain of the gRNA. It is this part of the gRNA that actually specifies U-insertions and deletions in a small region of mRNA until this molecule is complementary with the information domain. The 3'-oligo(U) tail that is adjacent to the gRNA information domain has been proposed to interact with the downstream purine-rich sequence of the mRNA that remains to be edited (McManus et al., 2000).

In the case of pan-edited mRNAs, several gRNAs are needed for their editing, which proceeds in the 3′ to 5′ direction (Maslov and Simpson, 1992). This polarity eliminates the co-occurrence of editing and translation. There is a higher number of gRNAs encoded in the minicircles than is required for the decoding of all mRNAs, as gRNAs with slightly varied sequences seem to be able to decode part of an edited mRNA (Koslowsky et al., 2014). Thus, a large and redundant population of gRNAs is encoded by minicircle kDNA. The cox2 transcript was the first example of RNA editing, although it does not utilize the aforementioned gRNAs for its sole four U-insertions. Its editing is mediated by a *cis*-acting gRNA-like element located in its 3′ untranslated region, making it a unique substrate for the editing machinery that performs the enzymatic steps of this process (Golden and Hajduk, 2005).

Numerous protein complexes coordinating the highly complex editing process have been described. The well-studied heterotetrameric complex consisting of mt RNA-binding proteins 1 and 2 (MRP1 and MRP2) (Schumacher et al., 2006) has been proposed to act in gRNA–mRNA annealing, a necessary association for the initiation of editing (Müller et al., 2001; Zíková et al., 2008a). This complex has an electropositive face that facilitates the nonspecific binding of RNAs via their negatively charged sugar-phosphate backbone, exposing their base moieties to potential hybridizing transcripts (Schumacher et al., 2006).

The multiprotein complex, called RECC or the 20S editosome, provides the core enzymatic activities needed to achieve a single round of RNA editing (Panigrahi et al., 2001; Seiwert and Stuart, 1994), and also contains proteins with just a structural role. There are at least three RECC isoforms bearing different endonucleases, each partnered with a unique protein that selectively cleaves U-insertion (by RNA-editing endonuclease 2 (REN2)), deletion (REN1), and cox2 mRNA (REN3) editing sites (Carnes et al., 2011, 2008). After cleavage of an mRNA at an insertion site into 5′- and 3′-cleavage products, Us are added to the 3′-end of the former by terminal uridylyl transferase 2 (RET2), a less processive enzyme than RET1 (Ernst et al., 2003). When a deletion site is cleaved by REN1, excess Us on the 5′-cleavage fragment are removed by RNA-editing exonuclease 1 (REX1) (Carnes et al., 2012; Ernst et al., 2009). Interestingly, REX1 exclusively associates with REN1 in this RECC isoform (Carnes et al., 2011). Once the appropriate number of Us has been added or deleted from the 5′-cleavage fragment as dictated by the gRNA, the two mRNA fragments are resealed by RNA-editing ligase 1 (REL1) to complete a single round of editing. All RECC isoforms also contain less-dominant REL2 and RNA-editing exonuclease 2 (REX2), whose roles in the process remain unclear (Carnes et al., 2012; Ernst et al., 2009; Gao and Simpson, 2003).

The mt RNA-binding complex 1 (MRB1) has emerged as another key player in RNA editing. Thirty one proteins have been found in various preparations of MRB1, many of which bear motifs or domains that have been associated with RNA binding and processing, giving the complex its name. In addition, several of these proteins have known protein–protein interaction motives, further evidence that MRB1 represents a macromolecular assembly (Etheridge et al., 2008; Hashimi et al., 2008; Hernandez et al., 2010; Panigrahi et al., 2008; Weng et al., 2008). The architecture of this complex appears to be made up of a core of six proteins that is present in all MRB1 purifications (Ammerman et al., 2012). It contains the aforementioned GAP1/2 heterotetramer that binds and stabilizes gRNAs (Hashimi et al., 2009; Weng et al., 2008), MRB3010, and MRB11870, which are involved in early steps of RNA editing (Ammerman et al., 2013, 2011), and MRB8620 and MRB5390, whose role in the process remains undefined.

This core interacts with the TbRGG2 subcomplex, which is named after a residing RNA-binding protein (Ammerman et al., 2012). TbRGG2 has an N-terminal region with an annealing activity, and a C-terminal part, which

confers double-stranded RNA unwinding activity (Foda et al., 2012). This protein interacts with MRB8180 as well as two novel RNA-binding proteins, MRB8170 and MRB4160, in a mutually exclusive manner (Ammerman et al., 2012; Kafková et al., 2012). The downregulation of TbRGG2, MRB8170, and MRB4160 leads to a preferential decrease in pan-editing (Fisk et al., 2008; Kafková et al., 2012), which along with the biochemical properties of TbRGG2 seems to indicate that this subcomplex mediates multiround editing (Hashimi et al., 2013b).

The MRB complex also associates with RNA-editing helicase 2 (REH2), which has been found to interact with RECC in an RNA-dependent fashion (Hernandez et al., 2010). This protein has double-stranded RNA unwinding activity and appears to play a role in dislodging gRNA from an edited mRNA. Another RNA helicase dubbed REH1, which does not interact with MRB1 but has been found to associate with RECC, has been proposed to play a role in this process, too (Li et al., 2011; Missel et al., 1997). MRB1 subunits interact with the PPR protein KPAF1, which is involved in the addition of long 3'-tails on mRNAs (Ammerman et al., 2010). Due to this association with proteins in other mt RNA processing steps, MRB1 may be involved in integrating the RNA-editing process with the general mt RNA metabolism from transcription to translation (Hashimi et al., 2013b).

2.2.4 RNA turnover

RNA maturation pathways are not the only elements in the regulation of expression of mt-encoded genes. Directed RNA degradation also contributes to this process by controlling the abundance of a given RNA, and also serves in quality control, as aberrant transcripts are eliminated. As previously discussed, various RNAs are appended with 3′-extensions that affect their stability (Section 2.2.2). Several proteins involved in RNA turnover have been characterized. A degradosome-like complex (containing TbSUV and TbDSS-1) has been described in the *T. brucei* mitochondrion, the first detection of this complex outside of the yeast *Saccharomyces cerevisiae*, where it has 3′ to 5′ exoribonuclease and RNA helicase activities (Mattiacio and Read, 2009). Indeed, the *T. brucei* ortholog TbDSS-1 appears to affect the stability of a subset of mt mRNAs (Penschow et al., 2004) and process the 3′-end of 12 S rRNA (Mattiacio and Read, 2008). However, it still remains to be seen whether this endonuclease is truly involved in the bulk turnover of mt RNAs, indirectly or in conjunction with other factors.

Another mt 3′ to 5′ exonuclease bearing an RNase D domain has been shown to preferentially act on poly(U) (Zimmer et al., 2011). Consistent with this finding, the enzyme trims 3′-oligo(U) tails of small gRNAs. The biological relevance of this activity is still a mystery, although it seems that the enzyme does not act on bulk gRNA turnover, a process that would presumably also be beneficial in maintaining this pool of RNA species. Furthermore, the 3′-oligo(U) tails are not essential for the stability of gRNAs (Aphasizheva and Aphasizhev, 2010).

2.2.5 Mitochondrial tRNA import and modifications

In contrast to protein-coding genes, mitochondria generally encode all structural RNAs (rRNAs and tRNAs) that are needed for organellar translation (Adams and Palmer, 2003). However, the loss of mt-encoded tRNA genes apparently occurred multiple times during eukaryotic evolution. In such cases, the corresponding nuclear-encoded tRNAs have to be imported into the mitochondrion to sustain organellar translation (Rubio and Hopper, 2011). Most, perhaps even all, organisms are able to import tRNAs from the cytosol into mitochondria regardless of whether a complete set of tRNAs is encoded by the organellar genome or not (Rubio et al., 2008). The most extreme situation evolved in two groups of parasitic protozoa, namely the apicomplexan and kinetoplastid parasites, as both groups lost their full complement of mt tRNA genes, and as a consequence must import them from the cytosol (Esseiva et al., 2004; Hancock and Hajduk, 1990; Tan et al., 2002).

A number of studies have investigated necessary factors or mechanisms that perform and control tRNA import. In general, there are two tRNA import pathways. One utilizes the protein import pathway, requiring the mt membrane potential for tRNA translocation. The other process, which is present in *T. brucei*, is not dependent on the protein import pathway and does not require mt membrane potential (Paris et al., 2009). The only common feature for both import pathways is a need for ATP. Despite laudable efforts, both mechanisms remain poorly understood.

Transfer RNA molecules are of course crucial for protein synthesis. A typical tRNA does not represent a nude structure as it carries a high number of modified nucleotides. Over 100 naturally occurring chemical modifications have been described in tRNAs, with each tRNA molecule containing an average of 12 of them (Grosjean, 2009), and it is hypothesized that these modifications carry more information than tRNA genes themselves (Björk et al., 2001). The comprehensive distribution and roles of tRNA

modifications are based on different structural and physical properties, although there are modification "hot spots." These include three residues in the anticodon loop that have a critical function in accurate codon selection and the prevention of translational frame shifting (Gustilo et al., 2008). Additionally, the structural modifications located in the core of the tRNA molecule influence its stability and half-life (Motorin and Helm, 2010).

The role of the tRNA modifications is particularly relevant in kinetoplastids, as in contrast to other eukaryotes, they mostly regulate gene expression at the posttranscriptional level (Daniels et al., 2010). It has been suggested that tRNA modifications can be used by the cell as determinants or antideterminants for tRNA import, as they potentially provide a fine-tuning mechanism for marking or unmarking of tRNAs subjected to organellar import, which does not seem available in their relatively inflexible sequence context (Grosjean, 2005). The role of tRNA modifications in relation to mt tRNA import was investigated in *Leishmania* and *Trypanosoma*. It has been proposed that compartment-specific thio-modifications (2-thiouridine; s^2U) in the anticodon loop of the $tRNA^{Glu}$ and $tRNA^{Gln}$ may represent negative determinants for tRNA import in *Leishmania tarentolae* (Kaneko et al., 2003). Cytosol-specific localization of this modification was used as evidence supporting this scenario. Moreover, it has been demonstrated that the mt import of natively purified thio-modified $tRNA^{Glu}$ is less efficient than that of its in vitro-transcribed counterpart, which lacks the thio-modification (Kaneko et al., 2003).

Later, the same type of mechanism was analyzed in *T. brucei* (Paris et al., 2009). This RNAi functional study was inspired by the observation that thiolation in yeast depends on components of the Fe-S cluster biosynthesis pathway (Section 4.5.2). However, the ablation of cysteine desulfurase (Nfs), a key component of this pathway (Lill, 2009), did not alter the levels of the (non) thio-modified trypanosomal $tRNA^{Glu}$ and $tRNA^{Gln}$ after the import into the *T. brucei* mitochondrion. Additionally, the same extent of in vitro import was achieved with native thio-modified $tRNA^{Glu}$ and its chemically dethiolated counterpart (Paris et al., 2009). This result is in full agreement with a recent study explaining the mt enrichment of the nonthiolated $tRNA^{Glu}$ by postimport removal of the modification rather than selective import of nonthiolated tRNAs; however, no protein responsible for this predicted dethiolation activity has been identified (Bruske et al., 2009).

Due to the potential role on tRNA distribution, the synthesis of modified methylated guanosine at position 37 (m1G37) was studied in *T. brucei*

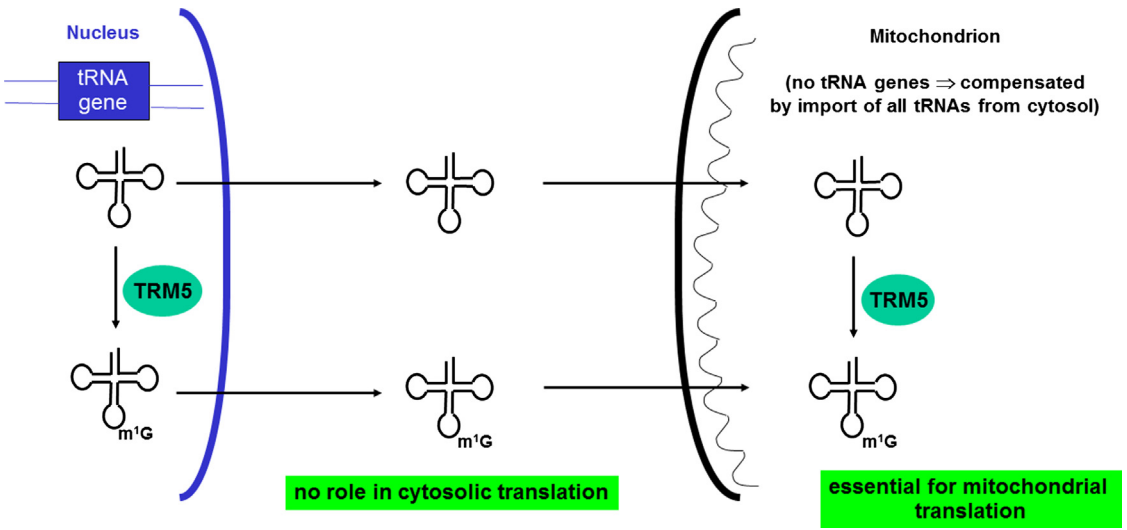

Figure 3 ***Mitochondrial tRNAs.*** The TbTRM5 protein is responsible for the formation of 1-methylguanosine (m1G) at position 37 of several tRNAs. The mitochondrial tRNA import machinery does not differentiate between methylated and unmethylated tRNAs. High levels of unmethylated tRNAs were detected in the mitochondrion, thus TRM5 has dual localization to prevent defects in mitochondrial translation.

(Paris et al., 2013) (Figure 3). However, the *T. brucei* import system does not appear to discriminate between tRNAs fully methylated and unmethylated at G37, resulting in significant amounts of unmethylated tRNA in the mitochondrion. The RNAi-mediated ablation of TRM5, a protein responsible for this modification, led to its disappearance. This resulted in significant consequences for mt but not for cytosolic translation, suggesting mt TRM5 is required for maturation of unmethylated tRNAs that reach the organelle. This study also revealed an unexpected lack of import selectivity between some fully modified and some potentially defective tRNA species (Paris et al., 2013).

In trypanosomatids, single tryptophanyl-tRNA ($tRNA^{Trp}$) undergoes extensive mitochondrion-specific C to U editing in the anticodon, which allows decoding the predominant mt tryptophan codons (Alfonzo et al., 1999). Besides RNA editing, the imported $tRNA^{Trp}$ is subject to unusual thiolation at position 33, the only known modification at this nucleotide in any tRNA. Furthermore, it was shown that thiolation of $tRNA^{Trp}$ serves as a negative determinant for C to U editing, providing a possible regulatory link for maintaining ratios of unedited versus edited tRNAs, with a still enigmatic role in the *T. brucei* life cycle (Wohlgamuth-Benedum et al., 2009).

The genome of *T. brucei* encodes 24 genes of the canonical aminoacyl-tRNA synthetase (aaRS) homologs covering the key amino acids, almost all of which are encoded by single-copy genes. Similar to tRNAs, trypanosomatids do not encode any aaRS genes in their kDNA and fully depend on import from the cytosol to maintain mt translation. This import mechanism has been experimentally demonstrated for GluRS and GlnRS (Rinehart et al., 2004). Notable exceptions among the single-copy aaRS are tRNA synthetases for tryptophan, lysine, and aspartate, which are encoded by two genes. Most likely, one of these gene products is responsible for the aminoacylation in the cytosol, and the other in the mitochondrion. Additionally, there are two genes encoding alpha and beta subunits of PheRSs, presumably functioning as a heterodimeric complex in both compartments. Recent data demonstrated that a number of aaRSs are essential for *T. brucei* (Kalidas et al., 2014). Its aaRSs are organized within a multiple aminoacyl-tRNA synthetase (MARS) complex, which contains at least six aaRS enzymes, and three additional non-aaRS proteins (Cestari et al., 2013). The MARS complex enhances tRNA-aminoacylation efficiency, which is in part dependent on a MARS complex-associated protein, mitochondrial carrier protein (MCP2), which binds tRNAs (Cestari et al., 2013). The dual localization of many individual MARS components indicates that the

existence of MARS in the *T. brucei* mitochondrion is plausible, although evidence is lacking.

In a broader context, a number of neuromuscular degenerative and metabolic diseases are caused by mutations in mt tRNA genes. As these genes are absent from the *T. brucei* kDNA, this protist represents an ideal model system for studying mechanisms of mt tRNA import, leading to insights that could one day be of therapeutic use.

2.3 Translation and Ribosomes

As described in the previous section, the mt translation in *T. brucei* relies on imported nuclear-encoded tRNAs. The prokaryotic-like translation system retained by the mitochondria must function exclusively with the eukaryotic-type tRNAs. In general, a functional mt translation requires a formylated initiator $tRNA^{Met}$, prokaryotic-like translation factors, and mt ribosomes. In *T. brucei*, only one nuclear-encoded elongator $tRNA^{Met}$ is used for both translation initiation and elongation (Cristodero et al., 2010). To initiate translation, a fraction of the mt-imported $tRNA^{Met}$ becomes formylated by an atypical methionyl-$tRNA^{Met}$ formyltransferase (MTF). Next, the formylated $tRNA^{Met}$ is recognized by an mt translation initiation factor 2 (mtIF2) and interacts with the organellar ribosomes. In addition to MTF and mtIF2, four other translation factors were identified in the genome of *T. brucei* based on sequence homology to their bacterial counterparts. Elongation factors Tu, Ts, and G, along with the release factor 1 are expressed, imported into the mitochondrion and essential for PCF (Cristodero et al., 2013; Charrière et al., 2005). An interesting deviation was described for elongation factor Tu, which contains a trypanosomatid-specific subdomain that might represent a specific adaptation, allowing its binding to the structurally unique mt ribosome (Cristodero et al., 2013).

Mitochondrial ribosomes are an indispensable component of all mtDNA-containing eukaryotes as they translate mt-derived mRNA into functional protein. Because of their origin, typical mt ribosomes share similar features, structure, and composition with the prokaryotic ribosome, which is a ribonucleoprotein complex consisting of small (30S SSU) and large (50S LSU) ribosomal subunits. The 30S SSU contains 21 proteins and 16S rRNA. The catalytic 30S SSU contains aminoacyl, peptidyl, and exit RNA-binding sites, and is also responsible for keeping mRNA in the correct open reading frame. The essential function of 50S LSU lays in binding peptide in the peptidyl transferase center and structural roles in the integrity and stability of the ribosome (Ban et al., 2000; Clemons et al., 1999).

Trypanosome mt ribosomes display several unique properties in comparison to their bacterial counterparts; the fully assembled ribosome (monosome) is much smaller, sedimenting at 50S (Maslov et al., 2006). The 30S SSU particle contains 9S rRNA (610 nt), while the 40S LSU carries 12S rRNA (1173 nt) with both rRNAs encoded by the kDNA maxicircle (de la Cruz et al., 1985a,b; Eperon et al., 1983). Interestingly, these rRNAs belong to the smallest known rRNAs molecules. A comparison of *T. brucei* 9S rRNA to its 16S rRNA bacterial homolog revealed similarities, but also identified large gaps in the former molecule (de la Cruz et al., 1985a). A similar comparison of the *T. brucei* 12S rRNA and its bacterial 23S rRNA revealed the highly conserved regions, as well as the loss of some typical domains and stem-loop regions (Eperon et al., 1983). Importantly, a stem-loop region responsible for binding chloramphenicol, a potent inhibitor of prokaryotic translation, is extensively altered and thus *T. brucei* mt translation is chloramphenicol-insensitive. The extremely reduced 12S and 9S rRNAs indicate essential regions for the function of the mt ribosomes. Moreover, existence of these relatively protein-rich mt ribosomes supports a hypothesis that some of the rRNA structures can be replaced by proteins (Maslov et al., 2007, 2006; Zíková et al., 2008b).

Mitochondrial ribosomes were characterized by several studies demonstrating the composition, organization, and arrangement of their SSU and LSU (Maslov et al., 2006; Sharma et al., 2003; Scheinman et al., 1993). Electron microscopy of the *L. tarentolae* 50S monosomes suggested several important structural features: (1) the overall structure is very porous; (2) the SSU and LSU are held together only by 9 intersubunit bridges (consisting of protein–protein, RNA–RNA, and protein–RNA interactions) in contrast to 13 bridges in eubacterial ribosomes and 15 in mammalian mt ribosomes; (3) an intersubunit space, which is involved in interaction with translation factors and tRNA, has a unique topology. Furthermore, trypanosomatid-specific proteins form major portions of the mRNA channel, the tRNA passage, and the polypeptide exit tunnel, implying significantly different mechanisms to recruit mRNA, bind tRNA, and release the nascent polypeptide. Despite the specific structural differences and strikingly small rRNAs, the overall morphology of the trypanosomatid mt ribosome is remarkably similar to its bacterial counterpart, suggesting the existence of strong functional and structural constraints on this early diverged mt ribosome (Sharma et al., 2009, 2003).

Another unique feature of trypanosomatid mt ribosomes is the presence of a distinctive subcomplex forming an asymmetrical bilobed structure (Maslov

et al., 2007; Sharma et al., 2009). The 45S SSU* complex comprises, in addition to the classical SSU proteins, 29 hypothetical proteins of unknown function. Some of these additional proteins contain a PPR or a tetratricopeptide repeat (TPR) motif (Maslov et al., 2006), and thus may play a role in RNA stability, expression, and regulation. The RNA-binding PPR and TPR proteins are abundant in plants; however, they have also been extensively studied in trypanosomatids, which contain an unusually high number of them compared to other eukaryotes (Pusnik and Schneider, 2012; Pusnik et al., 2007). The precise role of this 45S SSU* moiety remains elusive, although it is speculated that this complex is involved in recognition of mature mRNAs, and assembles the SSU and LSU moieties, forming a functional monomer. Furthermore, the 45S SSU* subcomplex may prevent the uncontrolled re-association of the free LSU with a free SSU, thus regulating translation of specific transcripts (Ridlon et al., 2013).

3. MITOCHONDRIAL IMPORT

As a membrane-bound organelle, the mitochondrion requires protein machinery to allow the sorting and flux of components that integrate protein content, metabolism and replication processes inside the organelle. As discussed below, these arrays of proteins facilitate transport of nuclear-encoded proteins, as well as cofactors, ions, and metabolites between the cytosol and the mitochondrion.

3.1 Protein Import and Processing

The majority of mt proteins are synthesized in the cytosol and then translocated into the mitochondria. This process is evolutionarily conserved among all extant eukaryotes. The translocation process is undertaken by the mt translocation machinery, an array of proteins located in the mt outer membrane (OM), the intermembrane space (IMS), the mt inner membrane (IM), and the mt matrix (Hildenbeutel et al., 2008; Koehler, 2000).

A majority of mitochondria-targeted proteins bear an N-terminal targeting signal that is recognized by the translocase of the outer mt membrane complex (TOM). Proteins with this targeting signal are commonly known as mt precursor proteins or preproteins. After entering the TOM complex, preproteins follow different import pathways depending on the final submitochondrial space they are targeted into (Kutik et al., 2007; Schatz and Dobberstein, 1996). The TOM complex has been widely studied in

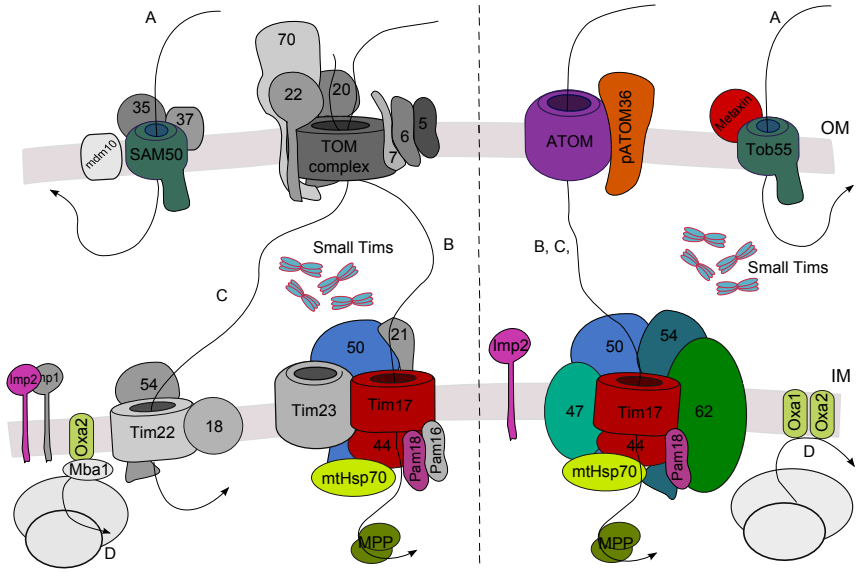

Figure 4 ***Mitochondrial translocation machinery.*** Comparative schematic representation of the translocation machinery in Opisthokonta (left) and *Trypanosoma brucei* (right). The components shown in gray represent those absent in the *T. brucei* genome. Homologs present in both groups have the same color. (A) Outer mitochondrial membrane proteins import pathway. (B) Tim23-mediated matrix proteins translocation. (C) Tim22-mediated mitochondrial carrier proteins inner membrane insertion. (D) Mitochondrial-encoded membrane proteins insertion pathway. Abbreviations: ATOM, archaic translocase of the outer membrane; IM, mitochondrial inner membrane; Imp, inner membrane peptidase; MPP, mitochondrial processing peptidase; mtHSP70, mitochondrial heat shock protein 70; Mba1, multicopy bypass of AFG3; OM, mitochondrial outer membrane; Oxa, oxidase assembly mutant 1; PAM, presequence-associated motor protein; pATOM36, archaic 36 kDa translocase of the outer membrane; SAM, sorting and assembly machinery; TIM, translocase of the inner membrane; TOM, translocase of the outer membrane; Tob55, topogenesis of β-barrel protein.

opisthokonts and fungi, where it was subject to a certain degree of diversification. In particular, based on in silico studies, the TOM complex seems to have highly diverged between the opisthokonts and excavates, to which trypanosomes belong (Eckers et al., 2013). A comparison of the divergent import machineries is shown in Figure 4.

The composition of the TOM complex in the opisthokonts involves two main subcomplexes: Tom20 and Tom70 (with additional Tom22 in yeast), which bind the preprotein and then release it into the β-barrel channel Tom40 (Hill et al., 1998; Kutik et al., 2008). In trypanosomes, the TOM complex subunits have been found neither by sequence

homology nor by phylogenetic analyses. However, a recent study described an archaic translocase of the outer membrane (ATOM) that might perform the activity of the TOM complex (Pusnik et al., 2011). ATOM, a β-barrel protein upregulated in PCF *T. brucei*, is involved in the translocation of proteins from the cytosol through the OM; it is not evolutionarily related to the TOM machinery of the opisthokonts, but instead appears to have originated from a bacterial ortholog of the Omp85-like protein, YtfM (Pusnik et al., 2011; Urbaniak et al., 2012). While some researchers failed to identify the evolutionary relationship between ATOM and Omp85, claiming that ATOM is a divergent ortholog of TOM40 (Žárský et al., 2012), others found a very divergent, different homolog of Tom40 for *T. cruzi* (Eckers et al., 2013). This gene also appears to have a homolog in the genome of *T. brucei* that does not correspond to ATOM. However, a recent study investigated the electrophysiological characteristics of ATOM in vitro, its capability to perform single channel conductance and its gating behaviour, characterized by short and infrequent events (Harsman et al., 2012). These results have established that the activity of ATOM is far from that of its putative TOM40 homolog and is more similar to the activity observed in Toc75 from plastids, which also share a common ancestry with Omp85-like proteins from proteobacteria.

Proteomic analysis of the OM fraction of PCF *T. brucei* confirmed the existence of another component of the TOM machinery: a peripheral subunit of the archaic translocase, pATOM36 (Hildenbeutel et al., 2008; Koehler, 2000; Pusnik et al., 2012). pATOM36 appears to function as a receptor for preproteins prior to translocation through ATOM, a role that has been designated to Tom20 in the opisthokonts, which is absent in trypanosomes and other excavates. However, unlike Tom20, pATOM36 does not exhibit an α-helical or a β-barrel structure, as do all the other components of the TOM complex (Pusnik et al., 2012).

After translocation by the TOM machinery, the preprotein may follow different routes depending on its final destination. For β-barrel and α-helical proteins targeted to the OM, a machinery known as SAM (sorting and assembly machinery) is necessary (Krimmer et al., 2001; Kutik et al., 2007; Paschen et al., 2003; Schatz and Dobberstein, 1996). *Trypanosoma brucei* bears a homolog of SAM50, termed Tob55 (topogenesis of β-barrel protein) which displays high homology to the Omp85 family of proteins of α-proteobacteria (Eckers et al., 2013; Sharma et al., 2010). In plants and mammalian cells, a protein called metaxin is involved in the machinery for the assembly of OM proteins (Hill et al., 1998; Kutik et al., 2008; Schneider

et al., 2008). Despite the fact that metaxin is present in the *T. brucei* genome and is considered an integral part of the Tob55 (or SAM) complex, its involvement in the mt translocation machinery remains to be elucidated.

In contrast to proteins of the OM, the proteins of the IM, IMS, and the matrix are received by the translocase of the inner membrane complex (TIM). At this point, a bifurcation in the pathway presents two options: the Tim23 pathway for subsequent translocation into the mt matrix and IM-insertion via the stop-transfer pathway (Emtage and Jensen, 1993; Pusnik et al., 2011), or the Tim22 route, for the insertion of N-terminal, presequence-devoid MCPs into the IM (Pusnik et al., 2011; Sirrenberg et al., 1996; Urbaniak et al., 2012). The Tim23 complex is composed of two parts: (1) a membrane-anchored part, composed of Tim17, 21, 23, and 50; and (2) a mostly soluble, motor part (Mokranjac et al., 2009; Ryan et al., 1994; Yamamoto et al., 2002; Žárský et al., 2012). Of the core subunits for the membrane-anchored portion of the TIM complex, only Tim17 and Tim50 have orthologs in *T. brucei* (Duncan et al., 2013; Eckers et al., 2013; Singha et al., 2012). However, a novel array of proteins (Tim 47, 54, and 62) has been described as forming components of the Tim machinery in *T. brucei* (Singha et al., 2012). This is in fact one of the most salient features of the mt translocation machinery in this parasite: its lack of homology to the components of the TIM machinery in the opisthokonts, ascribing the combined function of Tim17/Tim22/Tim23 to the trypanosome Tim17.

The soluble motor portion of the Tim23 complex is composed of Mge1, mtHsp70, Tim44, Tim16/Pam16, Tim14/Pam18, Pam17 (Gaume et al., 1998; Harsman et al., 2012; Mokranjac et al., 2006, 2003). Protein substrates are tethered into the mt matrix by the presequence-associated motor proteins. The core components of this part of the complex (Tim44, Pam18, Mge1, and mtHsp70) are all found in the *T. brucei* genome (Schneider et al., 2008). Mge1, a nucleotide exchange factor, binds mtHsp70 to release proteins out of the channel into the matrix, in a membrane potential-driven process (Koehler, 2004). The preprotein is tethered out of the channel by this complex and is sequentially processed by the mt processing peptidase (MPP). Tim23 also processes those presequence-bearing transmembrane proteins that are translocated by stop-transfer and insertion of the transmembrane fraction of the protein, with the N-terminus of the protein facing the mt matrix for the subsequent processing by the MPP (Hildenbeutel et al., 2008).

Insertion of an MCP into the IM requires the Tim22 complex. This complex, formed by transmembrane proteins Tim22, 54, and 18, mediates the insertion of carriers that bear non-N-terminal sorting signals. Associated with the Tim22 complex are the small Tim proteins (Tim8, 9, and 12), an array of approximately 10 kDa chaperones involved in the tethering of carriers in the IMS (Baker et al., 2009; Paschen et al., 2000; Sirrenberg et al., 1998). These proteins work as hexamers that bind the hydrophobic segments of the carrier before it is taken by the Tim22 complex, which inserts it into the membrane. The fact that there is no homolog of Tim22 in *T. brucei* suggests that Tim17/Tim22/Tim23 may have a redundant role for MCP translocation and insertion. Despite the absence of Tim22, these small Tims are present in the genome of *T. brucei* (Tim9, Tim10, and Tim8-Tim13). Their involvement has been proved by their role in the insertion of the ATP/ADP carrier AAC into the IM (Gentle et al., 2007; Schneider et al., 2008).

Small Tim proteins also participate in a fourth translocation mechanism: that of cysteine (SH)-rich proteins directed to the IMS through the disulfide relay system (Herrmann and Riemer, 2012). After translocation through the TOM complex, SH-rich proteins are transferred to the Mia40-Erv1 complex by small Tim hexamers. The oxidoreductase Mia40 (mt IMS import and assembly pathway of 40 kDa) and the sulfyhydryl oxidase Erv1 (essential for respiratory growth and viability 1) constitute the core of the disulfide relay system. *Trypanosoma brucei* lacks Mia40 but carries a homolog of Erv1, which may perform the activity of the Mia40/Erv1 complex on its own as the sole representative of the core for the disulfide relay system (Basu et al., 2013).

A group of proteins that also requires insertion into the IM are those encoded by the mt genome. Once translated, they are imbedded in the membrane by a mechanism orchestrated by Oxa1 (oxidase assembly mutant 1). In yeast, this α-helical transmembrane protein is nuclear-encoded and it collaborates with Tim23 for the insertion in the membrane of the Atp6, Atp8, and Atp9 subunits of the F_oF_1-ATPase, cytochrome *b* of the bc_1 complex, and subunits of cytochrome *c* oxidase (Altamura et al., 1996; Bonnefoy et al., 2009; Hell et al., 1997). The C-terminus of Oxa1 ushers mt ribosomes for the tethering of hydrophobic mt translation products into the IM (Keil et al., 2012). Despite the phenotypic features of Oxa1 mutants in yeast, it has been observed that there is a basal level of Oxa1 substrates still present and active in the cells, suggesting the presence of an Oxa1-independent route for the translocation of matrix-to-membrane hydrophobic proteins

(Hildenbeutel et al., 2008). The genome of *T. brucei* codes for homologs of Oxa1 and Oxa2, with Oxa1 being upregulated in the PCF (Schneider et al., 2008; Urbaniak et al., 2012).

The mt translocation machinery of *T. brucei* has apparently undergone a simplification process in which several proteins have combined the role of various others. This evolutionary process, despite greatly reducing the complexity of the machinery, has not changed the capability of its mitochondrion to translocate mt proteins from other organisms, neither are trypanosomal proteins exempt from integration into the mitochondria of the opisthokonts. For this reason, it has been postulated that despite the great divergence of individual components of the machineries, the translocation pathways have remained conserved and adapted the mt apparatus accordingly (Eckers et al., 2012).

As stated above, most mt proteins directed to the matrix bear N-terminal presequences that are recognized by the mt translocation machinery. Once in the matrix, the presequence is cleaved by MPP and the protein may be folded into its proper active form. A canonical MPP is a heterodimeric protein composed of two highly conserved subunits: α-MPP acts as a recognition particle for the cleavage substrate, while the β-MPP subunit performs the cleavage (Adamec et al., 2002; Brown et al., 2007; Šmíd et al., 2008). MPP has a specificity for certain substrate sequences and both subunits are required for the cleavage (Gavel and von Heijne, 1990; Saavedra-Alanis et al., 1994). *Trypanosoma brucei* bears highly conserved homologs of the α-MPP and β-MPP subunits, known to be upregulated in BSF (Desy et al., 2012; Mach et al., 2013; Urbaniak et al., 2012).

Following the cleavage by MPP, some presequences are further processed by the mt intermediate peptidase (MIP or Oct1 in yeast) (Isaya et al., 1992, 1991; Kalousek et al., 1992). A highly conserved MIP is present in *T. brucei*. It has been proposed that the cleavage by MIP stabilizes substrates as the presence of certain N-terminal amino acid residues may render the protein unstable (Mogk et al., 2007; Schmidt et al., 2010; Vögtle et al., 2011). Another processing peptidase of the mt matrix, Icp55 cleaves of a single amino acid after the proteolytic activity of MPP (Naamati et al., 2009). Its deletion in yeast has deleterious effects on mt respiration (Stames and O'Toole, 2013), as some of its substrates are the δ and γ subunits of the F_1-ATPase moiety, as well as Atp11, a chaperone for the assembly of the same moiety of the complex (Vögtle et al., 2009). The *T. brucei* genome contains at least three homologs of Icp55. Cysteine-rich proteins targeted to the IMS are proteolytically cleaved by the inner membrane-associated

peptidase (IMP), which in mammals is associated with the Mia40/Erv1 complex (Burri et al., 2005). Furthermore, some proteins to be inserted into the membrane by Tim23 by stop-transfer are substrates of this protease. A putative homolog of one of the two IMP subunits is present in the *T. brucei* genome (Schneider et al., 2008).

In a fashion similar to the translocation machinery, the processing peptidases have adapted their mechanism of action to the repertoire of proteins that require processing, in order to not just fold and insert them into the active mt pool, but also to ensure half-life stability. Overall, these enzymes remained highly conserved both in sequence and activity, despite the divergence of the translocation machinery and the evolutionary distance between the opisthokonts and excavates, which confers great differences between their mt proteomes.

3.2 Transport of Metabolites

As previously discussed, the OM and IM contain machinery dedicated to facilitate the transport of nuclear-encoded proteins to the mt matrix. As mitochondria are impermeable to many metabolites and ions, special mechanisms have evolved to transport them between the cytosol and the mt matrix. The voltage-dependent anion channel (VDAC), conserved in all known mitochondria, is the main mediator of low-molecular weight compounds across the OM (Colombini, 2004). An ortholog of VDAC was characterized in *T. brucei*, although the authors report evidence of an additional OM transporter, which is yet to be elucidated (Pusnik et al., 2009). Proteomic analysis indicated that VDAC was upregulated in the PCF (Gunasekera et al., 2012; Urbaniak et al., 2012). A diverse group of structurally related membrane-bound proteins, clustered into the mitochondrial carrier family, is responsible for transporting ions and metabolites across the IM to the mt matrix (Aquila et al., 1987). An array of 24 MCPs was annotated in an inventory for *T. brucei* (Colasante et al., 2009). Homologs were identified by either sequence or phylogenetic analysis and listed among others as ATP, ATP/ADP, ATP-Mg/Pi, phosphate, oxoglutarate/dicarboxylate, folate, CoA, ornithine, carnitine/acyl-carnitine, S-AdoMet, pyrimidine, and iron. The analysis failed to find any homologs for the uncoupling protein. Moreover, unlike metazoans, *T. brucei* bears a homolog for a GTP/GDP carrier, a member of the mt carrier family to date only described in yeast (Vozza et al., 2004). The majority of *T. brucei* MCPs have not been characterized, with the exception of MCP6 (Colasante

et al., 2006), MCP5, and MCP15 (Peña-Diaz et al., 2012). MCP6 has a homology to eukaryotic ATP-Mg/Pi and ATP/ADP carriers, but lacks the canonical motifs that identify them. Transport assays in bacteria revealed that MCP6 is neither an ATP-Mg/Pi nor an ATP/ADP carrier (Colasante et al., 2006). However, abnormalities in cell morphology and defective kDNA division suggest a possible role in the transport of alternative nucleotides. The ATP/ADP carriers are responsible for the translocation of ATP and ADP across the IM; in trypanosomes, this function is performed by MCP5 (Peña-Diaz et al., 2012). Therefore, this carrier provides an important link between the ATP-consuming reactions in the cytosol and oxidative phosphorylation, an ATP generating process in the mitochondrion (Section 4.1).

Carboxy acids such as pyruvate and malate participate in several mt processes. Pyruvate is a key metabolite for oxidative phosphorylation via the Krebs cycle and for the generation of acetate, which is exported for cytosolic FA biosynthesis. A specific mt pyruvate carrier (MPC) in mitochondria isolated from rat liver cells had been defined biochemically (Halestrap, 1975), although the gene(s) responsible remained elusive until three MPCs were identified in humans, *Drosophila melanogaster* and *S. cerevisiae* (Bricker et al., 2012; Herzig et al., 2012). MPC1 and MPC2/MPC3 are very small proteins of 12–16 kDa, which form an ~150 kDa complex embedded in the IM, likely containing additional proteins. In both life cycle stages of *T. brucei*, pyruvate can be further metabolized in the mitochondrion, and while the majority of pyruvate is excreted from BSF, low levels are required for cytosolic FA synthesis (Mazet et al., 2013). A hypothetical protein with homology to MPC1 and an MPC protein with homology to MPC2/MPC3 may be involved in pyruvate transport, although neither of these have been characterized experimentally. Malate is responsible for transferring electrons from the cytosol to the mitochondrion, as the IM is impermeable to NADH, the primary reducing equivalent of the electron transport chain. Consequently, NADH is "transported" into the mt matrix via the malate-aspartate shuttle, which includes the oxoglutarate-malate and glutamate-aspartate antiporters (LaNoue and Williamson, 1971). The *T. brucei* MCP12 shows homology to the human oxoglutarate/dicarboxylic acid antiporter, and is upregulated in PCF (Colasante et al., 2009; Urbaniak et al., 2012; Veitch et al., 2010), while no homolog for the glutamate-aspartate antiporter has been identified to date.

3.3 Transport of Ions

It was established in the early 1960s that mammalian mitochondria have the ability to uptake calcium cations (Ca^{2+}) in a manner that relies on the inner membrane potential generated by the electron transport chain and sensitive to the ruthenium red dye (Deluca and Engstrom, 1961). Consistent with the role of Ca^{2+} as a potent secondary messenger in various cell signaling pathways (Clapham, 2007), an increase of the cation in the matrix directly stimulates the Krebs cycle enzymes isocitrate dehydrogenase and 2-oxoglutarate dehydrogenase and indirectly the pyruvate dehydrogenase (PDH) complex (Hansford, 1994). This process in turn increases the $NADH/NAD^+$ ratio, which boosts oxidative phosphorylation. In addition to being a target of Ca^{2+} stimulation, mitochondria play a role in shaping the spatiotemporal distribution and levels of the cation in the cytosol (Rizzuto et al., 2012). Mitochondrial calcium uptake can occur within microdomains of high Ca^{2+} concentration at sites in close proximity to the endoplasmic reticulum or plasma membrane (Rizzuto et al., 1998, 1993). Yet, the concentration of matrix Ca^{2+} must be carefully regulated as a high load sensitizes the opening of the permeability transition pore, which commits a cell to the mitochondria-dependent apoptosis (Haworth and Hunter, 1979; Szalai et al., 1999).

Strangely, this mt Ca^{2+} uptake mechanism is not present in yeast (Carafoli and Lehninger, 1971), even though these belong to the opisthokonts. Even more surprisingly, mitochondria isolated from *T. cruzi* did demonstrate this activity in exactly the same manner as the mammalian organelle (Docampo and Vercesi, 1989a,b). A series of follow-up studies established that this biochemical property is conserved throughout trypanosomatids (Docampo and Lukeš, 2012). Of particular interest was the observation that mitochondria isolated from BSF retained this activity, despite not having the energy-producing Krebs cycle or classical electron transport chain (Vercesi et al., 1992).

Studies on mt Ca^{2+} uptake in kinetoplastids became vital almost a quarter of a century later in identifying the protein components underlying this activity. Of the 1098 mouse proteins making up the MitoCarta (Pagliarini et al., 2008), only a handful fit the criteria for proteins putatively involved in mt calcium uptake: (1) inner membrane localization; (2) expression in most mammalian tissues; (3) orthologs present in vertebrates and kinetoplastids, but not yeast. This procedure led to the discovery of two proteins that were part of calcium uptake mechanism conserved between vertebrate and

trypanosome mitochondria. The mitochondrial calcium uptake protein 1 (MICU1) is an EF-hand-containing protein with a single transmembrane domain (Perocchi et al., 2010). Later studies in mammals have demonstrated that MICU1 regulates the opening of the bona fide calcium channel by sensing Ca^{2+} concentration in the IMS, which presumably reflects the situation in the cytosol (Csordás et al., 2013; Mallilankaraman et al., 2012). The actual pore is formed by oligomers of the mitochondrial calcium uniporter (MCU) protein (Baughman et al., 2011; De Stefani et al., 2011; Raffaello et al., 2013).

Studies of the MCU ortholog in PCF and BSF showed that mt calcium uptake is essential for viability (Huang et al., 2013). The authors concluded that as in mammalian mitochondria, matrix calcium stimulates energy metabolism in PCF as the MCU-depleted trypanosomes suffered from a higher intracellular AMP/ATP ratio and an upregulation of autophagy markers, a common cell response to starvation. The enzymes that are regulated by matrix calcium in mammalian cells are also present in PCF, albeit separated into different metabolic fluxes (Section 4.1). The function of mt calcium uptake is not so straightforward in the BSF, as the mitochondrion switches to an ATP consumer. However, PDH remains present in BSF to convert pyruvate from glycolysis to acetyl-CoA, which is then used for essential FA synthesis via acetate or directly by the FAS II pathway (Mazet et al., 2013; Stephens et al., 2007). As this protein complex is stimulated by matrix Ca^{2+} in mammals, perhaps it is as well in trypanosomes, giving MCU at least one raison d'etre in this stage (Huang et al., 2013). While mt calcium uptake mediated by MCU appears to shape cytosolic Ca^{2+} in *T. brucei* (Xiong et al., 1997), a possible interplay between the *T. brucei* mitochondrion and endoplasmic reticulum remains mysterious (Docampo and Lukeš, 2012; Vercesi et al., 1993).

In contrast to the tightly regulated Ca^{2+}, potassium (K^+) is the most abundant cation in the cell (Haddy et al., 2006; Rodríguez-Navarro, 2000). In the initial proposition of chemiosmotic theory, the danger that oxidative phosphorylation would pose in terms of cation sequestration in the negatively charged matrix was recognized (Mitchell, 2011). Several protein channels whose molecular identity remains unknown are believed to exist that mediate the entry of K^+ into the matrix across the inner membrane (Szewczyk et al., 2009). Evidence for their existence is based on the sensitivity of mammalian inner membrane potassium conductance to ATP, Ca^{2+}, and compounds such as glibenclamide (Inoue et al., 1991; Paucek et al., 1992). Indeed, mitoplasts isolated

from *T. cruzi* also exhibited such properties, suggesting this phenomenon is conserved throughout a wide range of eukaryotes (Costa and Krieger, 2009).

A mechanism to alleviate matrix monovalent cation overloading as driven in an electroneutral fashion by the proton motive force generated by oxidative phosphorylation was also described as part of the chemiosmotic theory (Mitchell, 2011). A protein called Leucine zipper EF-hand-containing protein 1 (Letm1) was shown to be a component of K^+/H^+ exchange (KHE), as its gene knockout in *S. cerevisiae* caused massive mt swelling that was ameliorated by the ionophore nigericin, which enables KHE chemically (Nowikovsky et al., 2004). This protein has orthologs across eukaryotes, including *T. brucei* (Hashimi et al., 2013a). As in yeast and humans (Dimmer et al., 2008), downregulation of Letm1 initiated mt swelling in both life cycle stages, although it is reversed by nigericin treatment. Interestingly, the swelling was also observed in the petite mutant *T. b. evansi*, which does not possess the ΔpH component of the mt proton motive force (Hashimi et al., 2013a). An alternative hypothesis was proposed in which Letm1 is a mt Ca^{2+}/H^+ exchanger that may regulate KHE (Jiang et al., 2009; Tsai et al., 2014), although this idea remains controversial (Nowikovsky and Bernardi, 2014).

While much of this chapter is devoted to differences between the mt proteomes of *T. brucei* and better-studied model systems such as mammalian cells and yeast, this section also underscores how similarities between diverse eukaryotic clades can further increase our understanding of the biology of the organelle. The regulation of K^+ in the mt matrix by the highly conserved Letm1 and the putative proteins(s) comprising an influx channel for the cation may be a mechanism to modulate mt volume (Nowikovsky and Bernardi, 2014; Nowikovsky et al., 2004), suggesting an ancient origin of this mechanism. Furthermore, a comparative approach of mt proteomes of kinetoplastids and the opisthokont vertebrates and yeast led to the discovery of the proteins responsible for mt Ca^{2+} uptake.

4. MITOCHONDRIAL METABOLISM

Import of proteins and metabolites feeds mt metabolism. Due to the presence of carbohydrate metabolism coupled to oxidative phosphorylation, the mitochondrion represents a powerhouse of the cell. However,

mt metabolism is not limited to these two processes; the organelle also contributes to the cellular metabolism and physiology by production of iron-containing cofactors (heme and Fe-S clusters), biosynthesis of FAs, and metabolism of amino acids. Those features are discussed in the upcoming chapter.

4.1 Carbohydrate Metabolism—Krebs Cycle

The carboxy acids transported from the cytosol are utilized in carbohydrate metabolism by Krebs cycle. A classical Krebs cycle in an aerobic mitochondrion is a central wheel of energy metabolism of the cell (Figure 5). Generally, pyruvate, as an end product of glycolysis, is imported into the mitochondrion and converted to acetyl coenzyme A (AcCoA) by a PDH complex. Next, AcCoA condenses with oxaloacetate to citrate, releasing CoA. Citrate is then metabolized to oxaloacetate in a set of reactions involving three NAD- and one flavin adenine dinucleotide (FAD)-mediated dehydrogenations, two decarboxylations, and substrate phosphorylation (Figure 5). Total yield of this set of reactions is one molecule of high-energy phosphate (GTP), two molecules of CO_2, and four molecules of reduced cofactors (Owen et al., 2002). Apart from the action of PDH, AcCoA is also produced from threonine in a two-step reaction that involves threonine dehydrogenase and AcCoA:glycine C-acetyltransferase; glycine is produced as a by-product (Bringaud et al., 2006). In *T. brucei*, enzymes of tricarboxylic acid cycle are expressed and their enzymatic activities are detectable in PCF (van Hellemond et al., 2005). In the highly reduced BSF mitochondrion, these enzymes appear to be absent together with the cytochrome-containing respiratory chain complexes (Tielens and van Hellemond, 2009).

The most striking difference between trypanosomes and other eukaryotes is the molecule entering the Krebs cycle. Instead of AcCoA, the *T. brucei* Krebs cycle is mainly fed by 2-oxoglutarate originating from a degradation of proline and glutamine, as proline dehydrogenase catalyzes conversion of proline to glutamate-γ-semialdehyde that is subsequently converted to glutamate by pyrroline-5-carboxylate dehydrogenase. This can also be produced from glutamine by the action of L-glutamine deaminase (Bringaud et al., 2006). Glutamate is converted to 2-oxoglutarate by either the action of glutamate dehydrogenase with concomitant production of NADH or that of aspartate aminotransferase (van Weelden et al., 2005). 2-Oxoglutarate is oxidized to succinate, which is subsequently excreted. Alternatively, the Krebs cycle can be fed by malate, which is converted to

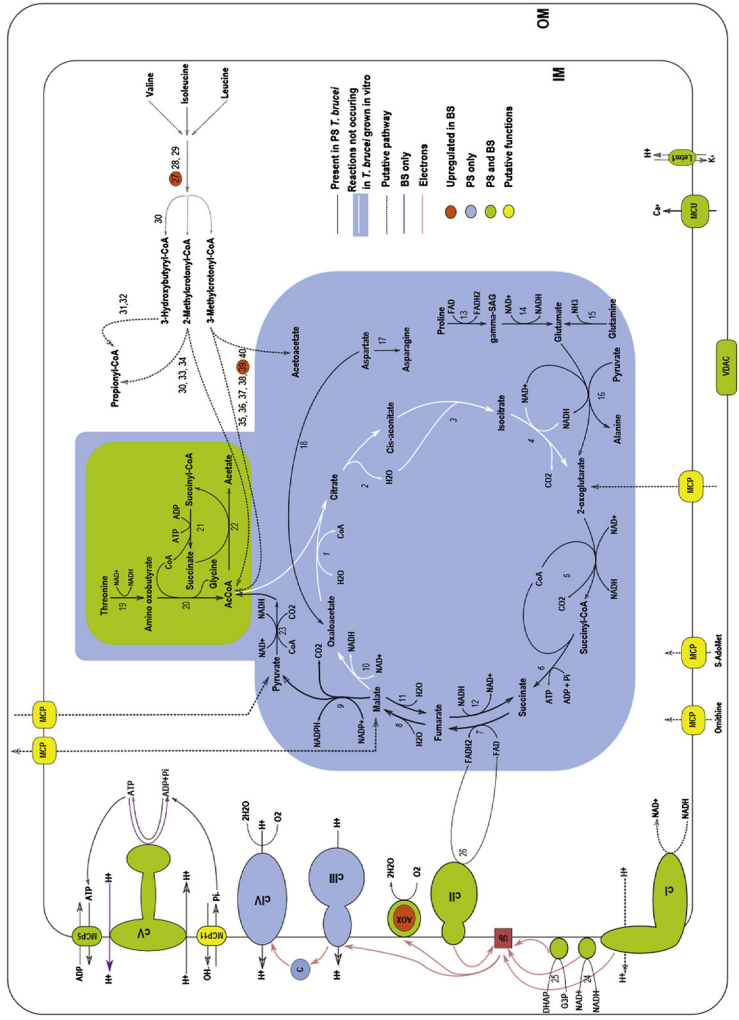

Figure 5 ***Mitochondrial energy metabolism.*** Schematic representation of mitochondrial energy metabolism and transport in *Trypanosoma brucei*. Black lines depict pathways found in *T. brucei*; white lines depict pathways found in mammalian cells; purple lines (dark gray lines in print versions) depict reactions present only in the bloodstream form stage (BSF) of *T. brucei*; pink lines (light gray lines in print versions) depict transit of electrons in the respiratory chain. Pathways with a green (pale gray in print versions) background represent those present in both procyclic form stage (PCF) and BSF trypanosomes. Pathways with blue (gray in print versions) background are present in BSF only; orange (dark gray in print versions) represents pathways upregulated in BSF; yellow (pale gray in print versions) background and dotted lines indicate putative proteins whose activity has not been experimentally verified, but their homologs are annotated in the *T. brucei* genome or have been found by proteomic analyses. Enzymes: (1) citrate synthase; (2) and (3) aconitase; (4) isocitrate dehydrogenase; (5) α-ketoglutarate dehydrogenase (2-oxoglutarate); (6) succinyl-CoA synthetase; (7) succinate dehydrogenase/complex II; (8) fumarase; (9) malic enzyme; (10) malate dehydrogenase; (11) fumarase; (12) fumarate reductase; (13) L-proline dehydrogenase; (14) pyrroline-5-carboxylase; (15) L-glutamine deaminase; (16) glutamate dehydrogenase; (17) asparagine synthetase; (18) aspartate aminotransferase; (19) L-threonine dehydrogenase; (20) AcCoA:glycine C-acetyltransferase; (21) succinyl CoA synthetase; (22) acetate:succinate CoA transferase; (23) pyruvate dehydrogenase; (24) alternative NADH:ubiquinone oxidoreductase (rotenone-insensitive); (25) glycerol-3-phosphate dehydrogenase; (26) succinate dehydrogenase; (27) branched-chain aminotransferase; (28) branched-chain keto acid dehydrogenase; (29) and acyl-CoA dehydrogenase; (30) enoyl CoA hydratase; (31) hydroxyisobutyryl CoA hydrolase; (32) 3-hydroxybutyrate dehydrogenase; (33) 3-hydroxyacyl CoA dehydrogenase; (34) acetyl CoA acyltransferase; (35) 2-oxovalerate dehydrogenase; (36) isovaleryl CoA dehydrogenase; (37) methylcrotonyl CoA carboxylase; (38) methylglutaconyl CoA hydratase; (39) hydroxymethyl glutaryl CoA synthase; (40) hydroxymethyl glutaryl CoA lyase. Abbreviations: AcCoA, acetyl Coenzyme A; AOX, alternative oxidase; C, cytochrome C; cI, cII, cIII, cIV, and cV, respiratory chain complexes; DHAP, dihydroxyacetone phosphate; G3P, glycerol 3-phosphate; Letm1, leucine zipper EF-hand-containing transmembrane protein 1; MCP, mitochondrial carrier protein; MCU, mitochondrial calcium uniporter; Pi, inorganic phosphate; OM, mitochondrial outer membrane; S-AdoMet, S-adenosylmethionine; Ub, ubiquinone; VDAC, voltage-dependent anion channel.

fumarate by a mt fumarase, followed by a reduction of fumarate to succinate by soluble fumarate reductase (Bringaud et al., 2006; Coustou et al., 2005). In this setup, the Krebs cycle is not operating as a cycle (van Hellemond et al., 2005). The fate of AcCoA produced by either PDH or metabolism of threonine, leucine, or isoleucine rests in its conversion to an acetate by the activity of either AcCoA thioesterase (ACH) or acetate:succinate-CoA transferase (ASCT). The ASCT produces succinyl-CoA that is further converted to succinate by a succinyl-CoA synthetase with a concomitant production of ATP instead of GTP thus contributing to the cellular energy supply (Rivière et al., 2004; Van Hellemond et al., 1998), while the former acetate-producing enzyme, ACH, is not coupled to energy production (Millerioux et al., 2012). AcCoA is diverted from the Krebs cycle toward acetate production, most likely due to kinetics of some of its enzyme(s) (van Hellemond et al., 2005).

Searching the available proteomic (Emmer et al., 2011; Niemann et al., 2013; Panigrahi et al., 2009) and stable isotope labeling by amino acids in cell culture (SILAC) sources (Butter et al., 2013; Gunasekera et al., 2012; Urbaniak et al., 2012) revealed the presence of all enzymes required for conversion of pyruvate by the Krebs cycle, confirming previous observations (Tielens and van Hellemond, 2009). Several genes encoding subunits of the Krebs cycle enzymes are present in multiple copies. Dihydrolipoamid dehydrogenase (DHLDH), a component of 2-oxo-acid dehydrogenases (e.g., PDH, oxoglutarate dehydrogenase [2-OD] or glycine-cleavage system), is present in five copies, while malate dehydrogenase, isocitrate dehydrogenase, a subunit of 2-oxoglutarate dehydrogenase component 1 (2-ODC1), and E2 subunit of PDH are all present in two copies. Some of the metabolites generated by DHLDH, 2-ODC1, and PDH-E2 are utilized in more than one classical biochemical pathway; therefore, a higher cellular demand for them may reflect the gene duplication events observed.

Based on the SILAC data, most proteins are upregulated in PCF (Butter et al., 2013; Gunasekera et al., 2012; Urbaniak et al., 2012). These data are, however, conflicting for two proteins involved in carbohydrate metabolism, namely one paralog of DHLDH- and NADP-dependent isocitrate dehydrogenase, and were found to be upregulated only by Urbaniak et al. (2012) and Gunasekera et al. (2012), respectively. The conflicting observations might stem from variances in methodology or strains used for respective studies. Nevertheless, the proteomic data confirm the global observation that carbohydrate metabolism is suppressed in the reduced BSF mitochondrion.

4.2 Oxidative Phosphorylation

Excess of reduced cofactors produced in the mitochondrion or transported therein by shuttle reactions is regenerated by the respiratory chain, a series of enzymatic complexes that harness energy of passing electrons for other beneficial purposes. A textbook respiratory chain of an aerobic eukaryote consists of four multi-subunit complexes, labeled complexes I through IV. Complexes I–III are linked by hydrophobic membrane carrier ubiquinone, and complexes III and IV are connected via a mobile soluble heme-containing protein cytochrome *c* (cyt *c*), located in the IMS. Complex I oxidizes NADH to NAD, while complex II catalyzes FAD-dependent dehydrogenation of succinate to fumarate. In this classical setup, complexes I and II are electron entry points, complex III is an electron transmitter, and complex IV sinks electrons to oxygen, producing water. Energy of passing electrons is coupled to a proton translocation across the IM. This proton gradient is subsequently used for transport of various molecules (Section 3) and ATP synthesis by complex V. Together, the respiratory chain complexes coupled to ATP synthases form the process known as oxidative phosphorylation.

Complex I (NADH:ubiquinone oxidoreductase) is the largest protein complex catalyzing the oxidation of NADH. While a bacterial homolog is composed of 14 subunits (Price and Driessen, 2010), its eukaryotic counterpart constitutes 7–12 prokaryotic-type core subunits encoded by the mt genome (Duarte and Tomás, 2014), and up to 30 novel eukaryotic subunits (Gabaldón et al., 2005). Electrons enter the complex through a covalently bound flavin mononucleotide, and then travel via seven Fe-S clusters to ubiquinone (Sazanov, 2007). Energy of this process is sufficient to translocate four protons across the IM (Janssen et al., 2006). Complex II (succinate:ubiquinone oxidoreductase), an integral part of the Krebs cycle, is the smallest complex, typically composed of four nuclear-encoded subunits (Rutter et al., 2010). It possesses FAD, three different Fe-S clusters, and heme *b* bound to subunits SDHC and SDHD in *Escherichia coli* as well as in mammals (Maklashina and Cecchini, 2010). Electrons originating from succinate dehydrogenation are passed to ubiquinone via a covalently bound FAD cofactor. However in this case, energy of the passing electrons is not sufficient to translocate any protons (Ackrell, 2000), hence this complex does not directly contribute to the proton gradient.

Ubiquinone is reduced to ubiquinol, which in turn is subsequently reoxidized by complex III (ubiquinol:cytochrome *c* oxidoreductase). This is done by the so-called Q-cycle whose energy is again harnessed for proton

translocation. The electrons are donated to the soluble cyt *c* located in the IMS (Cramer et al., 2011). In yeast and mammals, complex III is composed of 10 and 11 subunits, respectively, out of which 3 proteins contain redox cofactors (Bénit et al., 2009; Zara et al., 2009). Those are the Fe-S cluster-bearing Rieske protein and cytochromes *b* and c_1 containing two different classes of heme (Zara et al., 2009). Bacterial complex III contains only these three redox subunits (Schütz et al., 2000). In eukaryotes, the only mt-encoded subunit of this complex is cytochrome *b*. From the reduced cyt *c*, the electrons flow through complex IV (cytochrome *c*:oxygen oxidoreductase) to oxygen, the final electron acceptor. Electrons initially flow from cyt *c* to the copper center containing the cox2 subunit, and then are passed to cox1 via heme *a* and a binuclear center composed of heme a_3 and another copper center (Fontanesi et al., 2008). This passage is again linked to the proton translocation (Kaila et al., 2010). The bacterial enzyme constitutes of only the redox subunits cox1, cox2, and cox3, while its eukaryotic homolog is more intricate, as it is composed of these mt-encoded subunits, as well as additional nuclear-encoded ones. In yeast and mammalian complexes, there are a total of 11 and 13 subunits, respectively (Fontanesi et al., 2008).

Finally, complex V (F_oF_1-ATP synthase/ATPase) constitutes an elaborate proton pore allowing for a backflow of protons to matrix down the concentration gradient. Energy of this proton flow spins the central stalk of the F_1 subcomplex, which in turn changes conformation of the F_1 catalytic subunits to enable synthesis of ATP form Pi and ADP (Weber and Senior, 2003). In *E. coli,* the F_oF_1-ATP synthase is composed of five different subunits constituting the soluble F_1-moiety, and three subunits giving rise to the membrane-bound F_o-part. The yeast and human complex V consist of 17 and 15 subunits, respectively, with newly acquired subunits primarily forming the F_o-moiety (Devenish et al., 2008; Weber and Senior, 2003).

The *T. brucei* oxidative phosphorylation pathway differs from the classical eukaryotic one as it contains at least three additional enzymes which are capable of passing electrons to and from ubiquinone. These include an mt glycerol-3-phosphate dehydrogenase (G3PDH) and an alternative rotenone-insensitive NADH dehydrogenase (NDH2) that transfer electrons from glycerol-3-phosphate and NADH to ubiquinone, respectively. G3PDH catalyzes dehydrogenation of glycerol-3-phosphate, giving rise to dihydroxyacetone phosphate (Guerra et al., 2006; Škodová et al., 2013), while NDH2 is essentially a single-protein counterpart of complex I (Fang and Beattie, 2003). The reduced ubiquinol can then be reoxidized

by a single subunit trypanosome-specific alternative oxidase (TAO) that passes the electrons to oxygen. While none of these enzymes is able to directly contribute to the proton gradient, the two former ones can participate via an increase in the electron flow through ubiquinol and complex III.

The respiratory chain differs dramatically between the two life cycle stages of *T. brucei*, with BSF containing a reduced mitochondrion, having the respiratory chain downgraded to the alternative G3PDH-TAO pathway responsible for the reoxidization of the glycolytic glycerol-3-phosphate (Clarkson et al., 1989). In contrast, the PCF trypanosomes express a conventional respiratory pathway coupled with ATP synthesis, yet the alternative pathways are also active. The respiratory complexes I through IV perform the same functions as in higher eukaryotes, however, dramatically differ from them in terms of their composition. Although specific rotenone-sensitive activity was initially associated with complex I (Beattie et al., 1994), the use of very high concentrations of this specific inhibitor was later criticized, eventually questioning the very presence of complex I in *T. brucei* (Hernandez and Turrens, 1998). Moreover, the discovery of NDH2 (Fang and Beattie, 2003) and studies showing the loss of complex I upon a prolonged in vitro cultivation of other trypanosomatids (Sloof et al., 1994; Speijer et al., 1997) further weakened its importance in this flagellate. Depletion of complex I by RNAi in either PCF or BSF showed limited, if any, effect on the mitochondrion and cellular fitness (Surve et al., 2012; Verner et al., 2011), being in good correlation with the observation in *T. cruzi*, where partial deletion of kDNA encoding complex I subunits had no influence on cell viability (Carranza et al., 2009). Nevertheless, the conserved subunits of complex I have been identified in the genome of *T. brucei* and were subsequently detected by proteomic approaches (Butter et al., 2013; Gunasekera et al., 2012; Panigrahi et al., 2009, 2008; Urbaniak et al., 2012). Indeed, up to 50 proteins can be identified as either complex I subunits or proteins involved in its assembly, based on either the KEGG database (Kanehisa et al., 2014) or a previous study (Acestor et al., 2011). Out of these 50 proteins, 16 are currently annotated as hypothetical proteins, 3 of them are homologs of human complex I, and 2 additional proteins (adrenodoxin precursor and NI2M subunit) are expressed, yet have not been detected by proteomic approaches. All identified proteins of complex I are either upregulated in PCF (24 proteins) or nonregulated (5 proteins) (Butter et al., 2013; Gunasekera et al., 2012; Urbaniak et al., 2012).

The *T. brucei* complex II was shown to be nonessential for PCF grown in a glucose-rich environment, although it was essential when amino acids

were the principle carbon source (Coustou et al., 2008). In contrast to a typical 4-subunit complex, the *T. brucei* and *T. cruzi* enzymes are composed of up to 9 and 14 subunits, respectively (Acestor et al., 2011; Morales et al., 2009). All complex II subunits were also identified in proteomic studies (Acestor et al., 2011; Gunasekera et al., 2012; Panigrahi et al., 2009; Urbaniak et al., 2012), and most were upregulated in PCF (Butter et al., 2013).

The proton-pumping complexes III and IV were shown to be essential for parasite viability (Gnipová et al., 2012; Horváth et al., 2005). With its six identified nuclear-encoded subunits (Acestor et al., 2011), complex III constitutes a transition state between the prokaryotic- and eukaryotic-like complexes. Two proteins represent the core of the complex (Rieske Fe-S protein and cytochrome c_1), two subunits are diverged components of the protein import machinery (α- and β-subunits of MPP), while the remaining proteins are annotated as hypothetical, and are all upregulated in PCF (Butter et al., 2013; Gunasekera et al., 2012; Urbaniak et al., 2012). In contrast, complex IV was shown to be more diverged, containing at least 19 subunits (Zíková et al., 2008c), all being upregulated in PCF (Butter et al., 2013; Gunasekera et al., 2012; Urbaniak et al., 2012).

As mentioned earlier, BSF has a limited set of respiratory proteins present in its simple tubular mitochondrion. G3PDH activity was shown to be coupled to TAO (Clarkson et al., 1989) while cytochrome-containing enzymes are not expressed (Tielens and van Hellemond, 2009). Surprisingly, complexes I and II are physically present, although their functions remain mysterious (Mazet et al., 2013; Surve et al., 2012). Although its function in BSF is unknown, NDH2 is present throughout the life cycle of *T. brucei* (Urbaniak et al., 2012), hence it is most likely active in this stage too.

The F_oF_1-ATP synthase/ATPase represents another fascinating example of the unique *T. brucei* oxidative phosphorylation pathway, as in PCF and BSF it has dramatically different functions. While in PCF this complex possesses the conventional role in ATP synthesis, in BSF it maintains the mt membrane potential by its reverse ATP hydrolytic function coupled to the proton translocation, as the proton-pumping complexes III and IV are missing in this stage (Schnaufer et al., 2005). This derived F_oF_1-ATP synthase consists of the well-conserved F_1-moiety composed of subunits α, β, γ, δ, ε and trypanosome-specific subunit p18, and the less-characterized F_o pore and peripheral stalk where only subunits c, a, and OSCP were identified at the gene or protein level (Zíková et al., 2009). Nevertheless, the complex contains up to 14 kinetoplastid-specific subunits that lack homology to any of

the previously described subunits. Importantly, F_oF_1-ATP synthase/ATPase is essential in both life cycle stages (Schnaufer et al., 2005; Zíková et al., 2009).

4.3 Fatty Acid Biosynthesis

In addition to oxidative phosphorylation, the metabolism of FAs is considered as a principal source of energy for the cell. However, their role is not limited to energy metabolism, as they are known to contribute to the following processes: (1) serve as secondary metabolites and eukaryotic-like messenger molecules; (2) play an important role in the integrity and dynamics of cellular membranes; (3) are the building blocks of some coenzymes; (4) provide an environment for vitamin solubility; (5) participate in the regulation of cell metabolism and physiological functions. Therefore, FA biosynthesis belongs to the basic metabolic repertoire of each cell (Schweizer and Hofmann, 2004). FA synthase, a crucial enzyme for their de novo FA synthesis, can be divided into two classes: eukaryotic-type I and

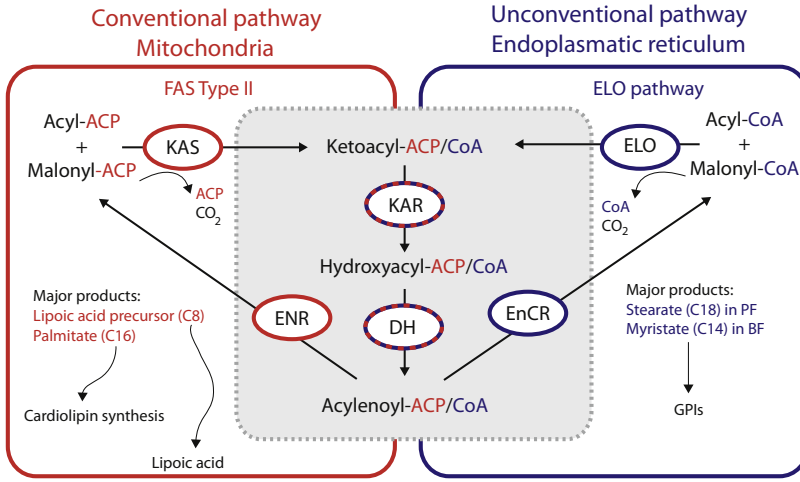

Figure 6 ***Fatty acid biosynthesis.*** In *Trypanosoma brucei*, fatty acids are synthesized by two pathways: the mitochondrial type II fatty acid synthesis (FAS type II) (red panel (light gray panel in print versions)), and an unconventional method performed by elongases in the endoplasmic reticulum (ELO pathway) (blue panel (dark gray panel in print versions)). In the FAS II pathway, the Acyl-ACP and malonyl-ACP are used as substrates by KAS, whereas in the ELO pathway, Acyl-CoA and malonyl-CoA are used as substrates by ELO. Abbreviations: ACP, acyl carrier protein; CoA, Coenzyme A; DH, β-hydroxyacyl-ACP/CoA dehydrase; GPI, glycosylphosphatidyl inositol; ELO, elongase; EnCR, enoyl-CoA reductase; ENR, *trans*-2-enoyl-ACP/CoA reductase; KAR, β-ketoacyl-ACP/CoA reductase; KAS, enzyme β-ketoacyl-ACP synthase.

prokaryotic-type II. Although they catalyze similar biochemical reactions, they differ dramatically in structure and subcellular localization.

Type I synthase is a multifunctional enzyme with multiple catalytic activities in separate domains (Paul et al., 2001; Smith, 1994) that is found in the cytosol of mammalian and fungal cells, and has not been identified in the *T. brucei* genome. Type II synthase is a multi-subunit complex (White et al., 2005) found in bacteria, plants, and eukaryotic organelles of prokaryotic origin (mitochondria, plastids). As trypanosomes encode a mt type II system, it was initially hypothesized that FAS II is responsible for the production of their bulk FA (Lee et al., 2006).

The general type II FA synthesis system comprises four main steps (Figure 6). In the first step, the growing acyl chain is linked to acyl carrier protein (ACP) and condensed with malonyl ACP by β-ketoacyl-ACP synthase (KAS), generating β-ketoacyl-ACP. Next, this product is converted to β-hydroxyacyl-ACP by β-ketoacyl-ACP reductase (KAR). The penultimate step involves dehydration of β-hydroxyacyl-ACP by β-hydroxyacyl-ACP dehydrase, resulting in the formation of enoyl-ACP, which in the final step is further reduced by *trans*-2-enoyl-ACP reductase (ENR). The final products of mt FA synthesis are octanoate (C8), the precursor of lipoic acid (LA), and palmitate (C16). Almost all components of the mt FA II machinery are encoded in the *T. brucei* genome. The ACP protein was identified based on homology to ACP1 from *Arabidopsis thaliana* and yeast (Stephens et al., 2007), whereas the KAS and KAR genes were identified from the yeast homologs CEM1 and OAR1, respectively (Stephens et al., 2007). Two additional KAR genes are related to members of the short-chain dehydrogenase family and contain predicted mt targeting signals. Two homologs of the yeast 2-enoyl thioester reductase (ETR1) (Torkko et al., 2001) are labeled mENR1 and mENR2 (Stephens et al., 2007). The presence of multiple isoforms of KAR and ENR is unusual for eukaryotes, suggesting that some of them may not contribute to FA synthesis (Stephens et al., 2007). No homolog for β-hydroxyacyl-ACP dehydrase was identified, which may be caused by the high divergence of this protein (van Weelden et al., 2005). The above-identified FAS II components were localized to the PCF mitochondrion (Stephens et al., 2007). Another system involved in the de novo FA biosynthesis in trypanosomes is the elongase system, which includes microsomal elongases (ELO), enzymes commonly used to extend preexisting FA chains (Lee et al., 2006).

Disruption of the ACP and KAS enzymes of the FA type II system results in the reduction of the LA levels (Stephens et al., 2007). LA serves as cofactor

in numerous biochemical reactions, including those of PDH and α-ketoglutarate dehydrogenase (Cronan et al., 2005). Therefore in its absence, the activity of PDH is compromised; the production of acetyl-CoA from pyruvate is hampered. Trypanosomal ELOs and FA synthesis type II are considered to catalyze, with one exception, the same reaction by using a functionally homologous set of enzymes. In the ELO pathway, malonyl-CoA is used as carbon donor, meanwhile in the FA biosynthesis this role is undertaken by malonyl-ACP (Lee et al., 2007, 2006; Morita, 2000). Therefore, acetyl-CoA availability is a key contributor of each pathway, and thus establishes a clear connection between them. In *T. brucei*, the ELO pathway contributes to 90% of FA biosynthesis, as CoA is obtained from threonine catabolism, a metabolic pathway not dependent on LA (Stephens et al., 2007). The ELO pathway is primarily used by BSF, which requires substantial levels of myristate for incorporation into the variant surface glycoprotein (Paul et al., 2001), although no phenotype was observed when ELO was knocked out (Lee et al., 2006). In contrast, this pathway is essential in PCF, as RNAi-mediated ablation of enoyl-CoA reductase resulted in cell growth arrest, although the addition of stearate, the main FA produced by this life cycle stage, reversed this phenotype (Lee et al., 2006). The regulation of the ELO pathway is dependent on the levels of lipids present in external environment. It is fivefold upregulated in PCF (Cronan, 2006; Lee et al., 2006), although the molecular mechanism of FA sensing is currently unknown.

In summary, trypanosomes represent the first example of single-celled organism to modulate microsomal elongases for the production of the vast majority of FA, with a minor contribution of the conventional method via FA synthase.

4.4 Metabolism of Amino Acids

Unlike other metabolic pathways, such as glucose breakdown for energy production, the metabolism of amino acids in *T. brucei* has not been completely elucidated. Neglect in these pathways owes its origin to the fact that these parasites are unable to incorporate carbons originated from most amino acids into their overall energy pool (with the exception of carbohydrate metabolism; see below) (Lamour et al., 2005). Gluconeogenesis, a known pathway for incorporation of carbons not derived from glucose into the cell's energy pool, does not happen in these parasites as it does in mammalian cells; therefore this flux does not include any contribution from amino acids (Allmann et al., 2013).

The only amino acid metabolic pathway that is complete in *T. brucei* as in other eukaryotes is that of the breakdown of branched-chain amino acids, such as valine, leucine, and isoleucine (Figure 5) (Opperdoes and Coombs, 2007). In mammalian cells, the enzymatic machinery for the catabolism of these amino acids is distributed between the cytosol and the mitochondrion. All of them share the initial three enzymatic steps catalyzed by branched-chain aminotransferase, branched-chain keto acid dehydrogenase, and acyl-CoA dehydrogenase (Harper et al., 1984). In mammalian cells, the first step occurs in the cytosol, whereas the other two occur in the mitochondrion. In *T. brucei*, two branched-chain aminotransferases have been found in the mitochondrion, with one of them being upregulated in BSF (Gunasekera et al., 2012; Niemann et al., 2013; Panigrahi et al., 2009; Zhang et al., 2010). The branched-chain keto acid dehydrogenase is a complex composed of multiple enzymes, also containing dihydrolipoamide dehydrogenase, which is present in at least five copies in the genome, and functions within the PDH complex, as well as in the degradation of FAs.

Following acyl-CoA dehydrogenase, the pathway divides into separate branches. The breakdown of leucine produces acetyl-CoA and acetoacetate; acetoacetyl-CoA may be also interconverted to acetoacetate by 3-keto acid-CoA transferase (Harper et al., 1984). All the enzymes for the breakdown of leucine are annotated in the genome of *T. brucei,* and most of them were found to be upregulated in PCF (Urbaniak et al., 2012).

The intermediate for the breakdown of isoleucine, 2-methylcrotonyl-CoA, is metabolised sequentially by three enzymes to propionyl-CoA, which is also the final product of the catabolic pathway for valine. However, the enzyme that catabolizes propionyl-CoA into succinyl-CoA is not present in *T. brucei*. In trypanosomes, aspartate may be converted to asparagine through the activity of an asparagine synthetase and also to oxaloacetate by an aspartate aminotransferase (Ginger et al., 2007). Asparagine synthetase has not been annotated as an mt protein, but at least one proteomic analysis has found it associated with the organelle (Panigrahi et al., 2009). There are several aspartate aminotransferases, annotated as both mitochondrial and cytosolic, however L-asparaginase, the enzyme responsible for the interconversion of asparagine to aspartate is absent from the genome (Ginger et al., 2007).

Previous in silico analysis postulates that trypanosomes lack arginase (Opperdoes and Coombs, 2007). Arginase, or agmatinase, as it is annotated in the genome, is part of the urea cycle, and is responsible for the production of ornithine from arginine. Ornithine is known to enter the mitochondrion

for its conversion to citrulline in the urea cycle. Trypanosomatids in general lack a complete urea cycle, and *T. brucei* does not have a functional arginase, despite its annotation in the genome (Vincent et al., 2012). However, there are two putative mt carriers for ornithine annotated in the same genome (Colasante et al., 2009). In mammalian cells, phenylalanine and tyrosine are metabolized through the same pathway in mammalian cells, with the hydroxylation of phenylalanine to tyrosine for the production of fumarate and acetoacetate. *Trypanosoma brucei* lacks this array of enzymes (six in total), with the exception of aspartate aminotransferase, which is involved in the catabolism of more than one amino acid. Despite this, some studies have reported that transamination of aromatic amino acids plays a role in the regeneration of methionine in the parasite (Berger et al., 1996). Key enzymes involved in the catabolism of various other amino acids are also missing from the *T. brucei* genome, such as those for the breakdown of histidine and tryptophan (Ginger et al., 2007), yet previous studies have detected the formation of tryptophol and indole acetic acid from tryptophan in *T. b. gambiense*-infected rats (Stibbs and Seed, 1975).

Methionine, an essential amino acid for *T. brucei*, is metabolized in the cytosol, and is the precursor of S-adenosylmethionine (S-AdoMet), a metabolite in the synthesis of polyamines (Brosnan and Brosnan, 2006; Brun and Schönenberger, 1979). In mammals, S-AdoMet is also the precursor of homocysteine, which may be remethylated to methionine or converted to cysteine by the *trans*-sulfuration pathway (Brosnan and Brosnan, 2006; Nozaki et al., 2005). S-AdoMet is synthesized in the cytosol, but its transport into mitochondria has been described for several organisms, and *T. brucei* bears a putative MCP for S-AdoMet (Colasante et al., 2009). Products of the reverse *trans*-sulfuration pathway, e.g., cysteine, homocysteine and cystathione, are produced when BSF cells are grown in the presence of labeled methionine (Yarlett and Bacchi, 1988). Cysteine plays a role in glutathione synthesis, a process that also takes place in the cytosol (Brosnan and Brosnan, 2006; Meister and Anderson, 1983). In trypanosomes, glutathione is conjugated with spermidine to form trypanothione, which is involved in the detoxification of reactive oxygen species (Fairlamb et al., 1985).

In contrast to mammalian cells, *T. brucei* is unable to synthesize the majority of amino acids de novo, as the biosynthetic pathways of serine, threonine, methionine, and lysine are absent from the genome. Branched-chain amino acids, which are essential in other organisms, and the aromatic ones, cannot be synthesized by trypanosomes either. For methionine

regeneration, homocysteine methyltransferase, a cytosolic enzyme, is present in two forms: cobalamine-dependent and cobalamine-independent synthases. *Trypanosoma brucei* is unique among eukaryotes in having this redundant trait (Nozaki et al., 2005). The formation of methionine from methylthioadenosine in a methionine-recycling pathway has also been proposed (Berger et al., 1996).

4.5 Metabolism of Cofactors

The mitochondrion is involved in the biosynthesis of several cofactors, namely lipoic acid, molybdenum, heme, and Fe-S clusters. Here, we will give emphasis to the iron-containing cofactors; heme, and Fe-S clusters, which are involved in crucial electrochemical reactions, such as electron-transfer chain in the organelle of *T. brucei*.

4.5.1 Heme metabolism

Heme, the main functional form of iron which is coordinated in the porphyrin ring, has the capability to transfer electrons and bind diatomic gases. Three biologically important forms of heme (types *a*, *b*, and *c*) differ by modifications in the porphyrin ring. The most common type is heme *b* (or protoheme), which is noncovalently bound to the apoprotein, whereas heme *c* is attached via a covalent thioether bond. The tetrapyrrol structure of heme *b* contains two propionate, two vinyl and four methyl side chains. In heme *a*, a methyl side chain is oxidized to a formyl group, and one of the vinyl side chains is replaced by an isoprenoid chain (Moraes et al., 2004). Most eukaryotes are able to synthesize heme *b* from δ-aminolevulinic acid through seven universally conserved enzymatic steps (Panek and O'Brian, 2002). In contrast to prokaryotes, much of the eukaryotic heme-based metabolism is oxygen dependent. Consequently, these oxygen-dependent reactions are dispensable under anaerobiosis and hence, anaerobic species possess relatively few hemoproteins (Kořený et al., 2013). Organisms that obtain energy via oxidative phosphorylation require large amounts of heme, primarily for the respiratory cytochromes. In response to the lowered respiratory activity, the synthesis of many heme-containing enzymes dramatically drops, which is particularly relevant in the life cycle of *T. brucei* (Bringaud et al., 2006).

Trypanosomes belong to a small group of eukaryotes that lacks the heme biosynthetic pathway. As heme auxotrophs, they scavenge heme from external sources through porphyrin membrane transporters (Huynh et al., 2012; Vanhollebeke et al., 2008), although heme import into the mitochondria and other compartments remains an open question. While *T. brucei* and

other trypanosomes require heme for several essential processes including sterol synthesis and FA desaturation, the related plant-pathogenic flagellate *Phytomonas* lacks most hemoproteins and can survive in the absence of heme (Kořený et al., 2012).

The electron transport chain contains heme groups of all types, which are directly involved in electron transfer reactions. The best-known examples are the IMS-situated c-type cytochromes, cyt *c* and cyt c_1 (Section 4.2) with a kinetoplastid-specific form of heme attachment to a single cysteine residue at the heme-binding motif (Allen et al., 2008). On the genomic level, trypanosomes lack a recognizable cyt *c* biogenesis system, which leads to the possibility of a third, highly divergent mt machinery (Allen et al., 2008; Tripodi et al., 2011).

Respiratory complex IV is the only protein complex that needs heme *a* for its activity. It is synthesized from heme *b* by two enzymatic steps involving heme *o* synthase (cox10) and heme *a* synthase (cox15). In eukaryotes, the heme *a* biosynthesis occurs in the mitochondria, with both synthases present in the IM (Barros and Tzagoloff, 2002). Both respective genes have been characterized in *T. cruzi* (Buchensky et al., 2010) and are conserved in other trypanosomatids, including *T. brucei*. Furthermore, the *T. cruzi* homologs were recognized by the yeast mt import machinery, and produced active enzymes restoring the formation of heme *a*. It was suggested that ferredoxin (Fdx) and ferredoxin reductase (FdR) function simultaneously with cox15 in the hydroxylation of heme *o* to form heme *a* (Barros et al., 2001). The participation of Fdx in Fe-S biogenesis (Section 4.5.2) bridges these two iron-dependent cellular processes. Out of two *T. brucei* Fdxs, only one (FdxA) is the essential component of these processes, while the other one is dispensable and its function remains unknown. Moreover, both human homologs of mt Fdxs (hsFdx1, hsFdx2) successfully rescued PCF cells depleted for FdxA, confirming the versatility of these Fdxs in different eukaryotic systems (Changmai et al., 2013).

4.5.2 Fe-S cluster and protein biogenesis

Fe-S clusters are evolutionary ancient inorganic cofactors that participate in numerous essential biological functions that include respiration, amino acid metabolism, and regulation of gene expression (Lill, 2009; Stehling and Lill, 2013). Despite their structural simplicity, the biogenesis of Fe-S clusters is complicated, requiring the active participation of at least 30 proteins (Lill, 2009; Lill et al., 2012). The Fe-S protein biogenesis, located in the mitochondrion and termed Iron-sulfur cluster (ISC) pathway, is a highly

conserved set of proteins, which is retained even in highly reduced mitochondrion-derived organelles, such as mitosomes (Tovar et al., 2003; Maguire and Richards, 2014). Even without any functional mitosomal Fe-S protein, the ISC machinery is retained to support the maturation of the extra-mitosomal Fe-S proteins (Goldberg et al., 2008). Moreover, it is known that yeast mutants lacking mtDNA can grow in fermentable media, due to the nonessentiality of oxidative phosphorylation (Lill and Kispal, 2000), while the ISC machinery remains indispensable for yeast viability under any growth condition (Lill and Kispal, 2000). Indeed, the Fe-S protein biogenesis may be the sole-indispensable mt function, justifying the retention of mitochondria or mitochondrion-derived organelles in all extant eukaryotic cells (Goldberg et al., 2008; Lill et al., 2005; Maguire and Richards, 2014). The mt ISC machinery is responsible for all mitochondrial, cytosolic, and nuclear Fe-S protein maturation. Additionally, the ISC export system, along with the cytosolic Fe-S protein assembly (CIA) machinery, is required for the cytosolic and nuclear Fe-S protein maturation (Roche et al., 2013).

The mt ISC machinery comprises three key biogenesis steps (Mühlenhoff et al., 2003) (Figure 7). First, in a process requiring the cysteine desulfurase module Nfs-Isd11 as the sulfur donor, synthesis of a [2Fe-2S] cluster takes place on the scaffold protein IscU, working as an assembly platform. The precise mechanism by which iron binds to the scaffold IscU (Isu1 in yeast) remains unclear, although association of the iron-binding protein frataxin is apparent (Colin et al., 2013; Stehling and Lill, 2013). A dedicated electron transfer chain is necessary for the reduction of the Nfs (Nfs1 in yeast)-held persulfide to the Fe-S cluster specific sulfide form. It has been proposed that Fdx (Yah1 in yeast) is responsible for this reduction, providing electrons in cooperation with FdR and NADPH (Lange et al., 2000). In the second step, the IscU-bound Fe-S cluster is released, and ensures the transient association with the transfer proteins. This step is aided by a Hsp70 chaperone system involving Hsp70 ATPase Ssq1, the co-chaperone Jac1, and the nucleotide exchange factor Mge1 (Stehling and Lill, 2013). The released Fe-S cluster can be transferred to the apoproteins via the mt monothiol glutaredoxin Grx5 (Mühlenhoff et al., 2003). Recently, an unusual interaction of Grx5 and Ssq1 has been portrayed showing their crucial involvement in all cellular Fe-S protein maturation (Uzarska et al., 2013).

The final step involves several ISC targeting factors that transfer and insert the Fe-S cluster to specific apoproteins. The specific insertion of [4Fe-4S] clusters is served by alternative scaffold proteins Isa1 and Isa2

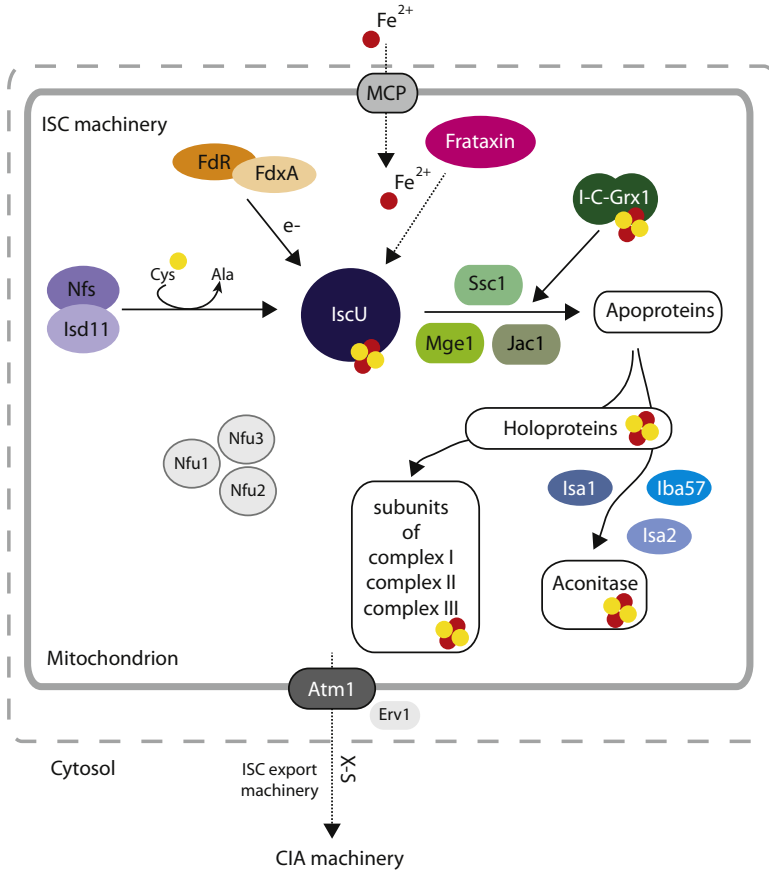

Figure 7 ***Mitochondrial Fe-S cluster assembly.*** The mitochondrion of *Trypanosoma brucei* contains two Fe-S cluster biosynthetic systems: the ISC machinery and ISC export machinery. The ISC machinery functions as the central Fe-S protein biosynthesis apparatus upstream to the ISC export machinery. First, a Fe-S cluster is assembled on the scaffold protein IscU, a step occurring in the cooperation with the cysteine desulfurase (Nfs)-Isd11 module that supplies the sulfur (yellow (white in print versions)). The Nfs-Isd11 module releases sulfur from free cysteine residue converting it to alanine and produces the persulfide intermediate, which is later transferred to IscU. The probable import of ferrous iron (Fe^{2+}) (red (dark gray in print versions)) is carried out by the putative MCP. Frataxin is an essential ISC component but its precise role (dashed arrow) is yet to be defined. Ferredoxin (FdxA), along with its putative partner ferredoxin reductase (FdR), provides electrons. Next, the Fe-S is released from IscU and transferred to the apoproteins via a dedicated chaperone system, consisting of Ssc1, Mge1, and Jac1. The in vitro [2Fe-2S] cluster coordinating monothiol glutaredoxin 1 (1-C-Grx1) is functionally qualified to take part in this step. Aconitase is shown as the example of a holoprotein, which is specifically matured by Isa1, Isa2, and Iba57. Several other Fe-S cluster-requiring holoproteins are subunits of respiratory complexes I, II, and III. The specific roles of three Nfu proteins (Nfu1–3) (gray) in the ISC system are yet to be deciphered. Finally, an unknown sulfur compound (X-S) is exported to the cytosol via the ISC export machinery composed of Atm1 and Erv1. The X-S is later utilized by the cytosolic CIA machinery.

(Ollagnier-de-Choudens et al., 2001; Wu et al., 2002) assisted by Iba57 (Gelling et al., 2008). Several maturation factors showing target protein specificity are involved in this final step, including Ind1, required for the maturation of complex I (Sheftel et al., 2009), and Nfu1, which displays a broader specificity in the maturation of respiratory complexes I and II, and lipoylate synthase (Navarro-Sastre et al., 2011). The contribution of the core ISC members involved in the first two steps are essential for the maturation of extra-mt Fe-S cluster proteins. The mitochondria-produced glutathione-sulfur moiety (Srinivasan et al., 2014) is exported to the cytosol by the dedicated ISC export machinery (Figure 7) composed of Atm1, an ABC transporter found in the IM (Kispal et al., 1999), Erv1, an IMS sulfhydryl oxidase (Lange et al., 2001), and glutathione (Sipos et al., 2002).

The *T. brucei* genome encodes all mt ISC machinery genes. The initial phase includes Nfs, Isd11, and IscU (Paris et al., 2010; Šmíd et al., 2006), which are important for the activity of both mt and cytosolic aconitases. RNAi-mediated ablation of both Nfs and IscU displayed an impact on overall mt metabolism, revealing dramatic increase of pyruvate and a significant drop in ATP production that triggers the PCF mitochondrion to mimic its BSF counterpart. This observation evokes the mt suppression in the BSF, which shifts to glycolysis, producing pyruvate as a key metabolic end product (Šmíd et al., 2006). Recently, dual localization of Nfs in the mitochondrion and nucleolus has been observed, though the function in the nucleolus remains to be established (Kovářová et al., 2014). Additionally, Nfs and Isd11 were shown to be involved in tRNA thiolation (Paris et al., 2010) connecting Fe-S protein maturation and tRNA thio-modification in these flagellates (Section 2.2.5). Moreover, the genome of *T. brucei* also contains gene for selenocysteine lyase (SCL) the product of which is, however, confined to the cytosol and nucleus (Poliak et al., 2010). As the depletion of Nfs but not SCL resulted in a growth phenotype, it was proposed that Nfs could fully complement the function of SCL, while this enzyme could only partially rescue the depletion of Nfs (Poliak et al., 2010).

Another major ISC factor, frataxin, is essential in the PCF, as Fe-S cluster-dependent activities, mt membrane potential, and oxygen consumption were reduced in the RNAi-interfered cells (Long et al., 2008b). Although no changes in mt iron content were observed, it is possible that in trypanosomes frataxin monomers do not form large aggregates as in higher eukaryotes (Adinolfi, 2002; Cook et al., 2006), thus limiting the chances of frataxin to play a role in iron storage (Long et al., 2008b). Frataxins from distantly related organisms, such as human, *Trichomonas vaginalis, A. thaliana,* and

Thalassiosira pseudonana, were expressed in the *T. brucei* frataxin-depleted cells in an attempt to rescue the phenotype. These frataxins were able to functionally replace their *T. brucei* homolog in the PCF cells (Long et al., 2008a,b,c). Moreover, the *T. vaginalis* frataxin, equipped with its genuine hydrogenosome import signal was efficiently imported into the *T. brucei* organelle (Long et al., 2008a). These data indicate the highly conserved nature of these eukaryotic proteins and import mechanisms. Expectedly, *T. brucei* possesses both Fdx and FdR for the necessary electron transfer chain (Section 4.5.1).

The final segment of the ISC system requires chaperones Ssc1, Mge1, and Jac1, all of which are present in PCF. Furthermore, Isa1 and Isa 2, required for efficient transfer of Fe-S clusters to respective apoproteins, are needed for the viability of PCF. Moreover, the expression of both human Isa homologs was able to partially rescue Isa1/2-depleted cells, revealing overlapping functions of both human Isa proteins (Long et al., 2011). As neither Isa1 nor Isa2 are indispensable for BSF, it was concluded that a group of proteins functionally dependent on the incorporation of Fe-S clusters is absent from the highly reduced BSF organelle (Long et al., 2011).

Apart from the above-mentioned emblematic ISC factors, trypanosomes possess some additional candidates involved in the biogenesis of Fe-S cluster-containing proteins. One of them is the *T. brucei*-specific monothiol glutaredoxin 1-C-Grx1, which was shown to bind a Fe-S cluster in vitro using glutathione as a cofactor (Manta et al., 2013b). Moreover, mutation in its active site lowered pathogenicity, providing a novel link between Fe-S cluster metabolism and parasite infectivity (Manta et al., 2013b). Additionally, the kinetoplastid-specific trypanothione, part of the enigmatic redox system (Fairlamb et al., 1985), was verified to be essential for viability and virulence, with an impact on Fe-S cluster metabolism, too (Manta et al., 2013a). There are three different Nfu genes in the *T. brucei* genome that are expected to function as target-specific maturation factors; however, their precise functions are yet to be unravelled (Figure 7). Finally, depletion of mt IM-located ABC transporter Atm1 leads to the downregulation of the cytosolic Fe-S cluster machinery, with a disrupted Fe-S cluster incorporation into the cytosolic proteins (Lukeš and Basu, 2014). Recent advances in the study of Fe-S cluster metabolism in trypanosomes demonstrate the evolutionary conserved arrangement of this essential process, but also identified potentially exciting features, in which the parasite differs from its mammalian host, as targets for further exploration.

5. STRUCTURAL PROTEINS AND FISSION

The mt network is extremely dynamic and its morphology mainly depends on the two opposing processes of fusion and fission, which can be regulated in response to external factors such as environmental cues or nutrient availability (Hoppins, 2014). A family of large self-assembling GTPases, the dynamin-related proteins (DRPs), is at the heart of the mt fusion and fission machineries (van der Bliek et al., 2013). In yeast, the DRP mitochondrial genome maintenance 1 (Mgm1) and the DRP fuzzy onions homolog 1 (Fzo1) are essential for fusion of the IM and OM, respectively, while Ugo1 (for "fusion" in Japanese) acts as a bridging factor and potential regulator between the two (Sesaki and Jensen, 2004) (Figure 8). The main GTPase responsible for mt fission in yeast is dynamin-related protein 1 (Dnm1) which is recruited by soluble adapters Mdv1/Caf4 that target Dnm1 to mitochondria through their interaction with the transmembrane

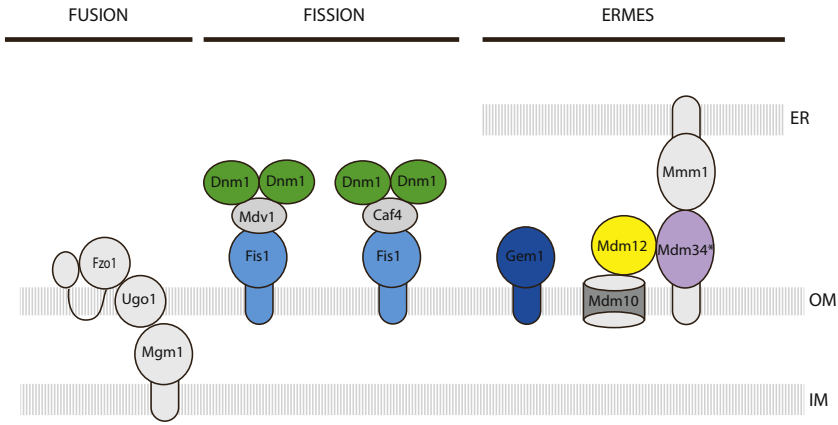

Figure 8 ***Mitochondrial fission and fusion.*** The yeast GTPases Mgm1 and Fzo1 mediate fusion of outer and inner mitochondrial membranes, with OM protein Ugo1 linking the two. Obvious homologs for these proteins are absent in the *Trypanosoma brucei* genome. Dnm1 is the mitochondrial fission effector GTPase in yeast which is recruited by the OM protein Fis1 via adaptors Mdv1 or Caf4. While trypanosome dynamin-like protein (DLP) plays a role in the organellar fission, Fis1 is dispensable and adaptor proteins have not been identified to date. The yeast ERMES complex marks sites of the mitochondrial division. Of the identified homologs in *T. brucei*, Gem1 localizes to the endoplasmic reticulum (ER), while Mdm12 and Mdm34 are cytoplasmic. Involvement of any of these proteins in mitochondrial fission is not apparent (Mdm12 and Mdm34) or has not been investigated (Gem1). (*Of note, *T. brucei* Mdm34 lacks the TM domain); OM, mitochondrial outer membrane; IM, mitochondrial inner membrane.

anchor mt fission protein 1 (Fis1) (Bui and Shaw, 2013). Sites of mt division are generally defined by interactions with the ER, a process that is conserved from yeast to mammals (Klecker et al., 2014). In yeast, these sites are called the ER mitochondrion encounter structure (ERMES); here mitochondria are first constricted in an ER-dependent manner which then allows for Dnm1 recruitment and eventual membrane scission (Friedman et al., 2011). Known ERMES proteins are found in the ER membrane (maintenance of mt morphology 1, Mmm1), soluble in the cytosol (mt distribution and morphology 12, Mdm12) and in the OM (Mdm10 and Mdm34) (Kornmann et al., 2009). GTPase EF-hand protein of mitochondria (Gem1), an OM protein, has been suggested to be a regulatory subunit of the ERMES (Kornmann et al., 2011) (Figure 8).

Trypanosomes belong to a small group of eukaryotes that have a single continuous mitochondrion which only divides once per cell cycle just prior to cytokinesis (Hammarton, 2007). Moreover, mt morphology between the BSF and PCF differs dramatically: While PCF have a highly branched and metabolically active mitochondrion, the BSF organelle is highly reduced, simple, and tubelike in structure and not used for energy generation (Tielens and van Hellemond, 2009). Mitochondrial fusion has not been directly observed in *T. brucei*, however the occurrence of gamete-like parasites capable of cellular (and presumably organellar) fusion was recently noted (Peacock et al., 2014). Further evidence for mt fusion and genetic exchange of mtDNA in vivo was obtained from the study of kDNA inheritance patterns in genetic crosses almost 20 years ago (Gibson et al., 1997). Another indication that the trypanosome fusion machinery is indeed present and active (at least in the PCF stage) stems from the fact that recovery from mt fission induced by expression of mammalian Bax is possible, with mt fragments re-fusing upon withdrawal of the inducing agent (Esseiva et al., 2004). Despite this functional conservation, none of the proteins that are part of the fusion machinery in yeast or mammals are found in the parasite; mt fusion proteins must thus be highly diverged or novel proteins unique to *T. brucei*.

Perhaps not surprisingly, the sole dynamin found in trypanosomes, which is a dynamin-like protein (DLP), has functions in both endocytosis and mt division (Chanez et al., 2006; Morgan et al., 2004). Of the fission adapter proteins, the membrane anchor Fis1 is the only one present in the trypanosome genome. It is expressed in both BSF and PCF (Urbaniak et al., 2012) and localizes to the OM in PCF (Niemann et al., 2013). In contrast to mammals which lack a similar structure, *T. brucei* has several putative subunits of the yeast ERMES complex: Mdm34, Mdm12, and Gem1

(Niemann et al., 2013; Wideman et al., 2013). However, neither Mdm12 nor Mdm34 localizes to the *T. brucei* mitochondrion or mitochondrion-ER contact sites, indicating that a functional ERMES-like complex is most probably not formed in the parasite (Schnarwiler et al., 2014). In the same study, a novel β-barrel protein functionally homologous to Mdm10 was identified; this protein localizes to the TAC and is not essential for mt division but instead required for kDNA segregation (Schnarwiler et al., 2014). Thus far, trypanosome Gem1, the yeast Miro GTPase regulating mt morphology, has not been functionally analyzed but was found to associate with the OM (Niemann et al., 2013).

Overall, despite conservation of parts of the mt fission machinery, the trypanosome must have evolved unique regulatory mechanisms to ensure proper segregation of this single-copy organelle during cytokinesis and to effect morphological changes during its life cycle. Several unique and trypanosome-specific proteins with functions in mt morphology regulation are indeed beginning to emerge, for example, the POMP proteins (present in outer membrane proteome) POMP9, POMP14, and POMP40 (Niemann et al., 2013), as well as TbLOK1 (Loss of kDNA), initially identified in a screen for loss of kDNA as the name implies (Povelones et al., 2013). Silencing of the POMPs caused specific morphological changes to the mitochondrion. While RNAi-mediated ablation of POMP40 creates a BSF-like mitochondrion in PCF cells, ablation of POMP9 and POMP14 causes collapse of the mt network (Niemann et al., 2013). Similarly, depletion of OM protein TbLOK1 in PCF cells results in mt morphology that resembles that of the BSF flagellates (Povelones et al., 2013).

6. CONCLUDING REMARKS

In this chapter we provide a comparison between *T. brucei* and the more established eukaryotic model systems and reveal numerous similarities and differences in mt processes at the DNA, RNA, and protein levels. Trypanosomes contain surprisingly complex machineries for many of the classical biological mt features including energy metabolism via oxidative phosphorylation, RNA editing, Fe-S cluster biogenesis, and mt fission machinery. Apart from highly conserved mechanisms, these processes also contain differences, which is not surprising given that *T. brucei* is one of the earliest known diverging eukaryotes. Consequently, these conserved features between diverse eukaryotic clades, and the presence of a single

mitochondrion make *T. brucei* an attractive model to further enhance our general understanding of both evolution and cellular biology of the mitochondrion. It is also important to explore the differences in the mt proteome and processes between this parasite and mammalian host. An essential aspect of drug development is the ability to target diverse or novel proteins, which include those found in kDNA replication and FA biosynthesis, in addition to a substantial repertoire of kinetoplastid genes that currently have no known function.

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