Failure is not an option - mitochondrial genome segregation in trypanosomes

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Abstract

Unlike most other model eukaryotes, *Trypanosoma brucei* and its relatives have a single mitochondrion with a single unit mitochondrial genome that is termed kDNA. Replication of the kDNA is coordinated with the cell cycle. During binary mitochondrial fission and prior to cytokinesis, the replicated kDNA has to be faithfully segregated to the daughter organelles. This process depends on the tripartite attachment complex (TAC) that physically links the kDNA across the two mitochondrial membranes with the basal body of the flagellum. Thus, the TAC couples segregation of the replicated kDNA with segregation of the basal bodies of the old and the new flagellum. In this review, we provide an overview of the role of TAC in kDNA inheritance in *T. brucei*. We focus on recent advances regarding the molecular composition of the TAC, discuss how the TAC is assembled and how its subunits are targeted to their respective TAC subdomains. Finally, we will contrast the segregation of the single unit kDNA in trypanosomes to mitochondrial genome inheritance in yeast and mammals, both of which have numerous mitochondria that each contain multiple genomes.

Introduction

Mitochondria are a hallmark of eukaryotic cells. They perform many important functions, the most prominent of which is oxidative phosphorylation (Friedman and Nunnari, 2014). The evolutionary origin of mitochondria can be traced back to a single endosymbiotic event between a α-proteobacterium and an archaeal host cell, approximately two billion years ago (Dacks et al., 2016). The endosymbiont subsequently converted into an organelle that is genetically integrated into the physiology of the host cell (Dacks et al., 2016; Gray, 2012; Lane, 2014). Today, more than 95% of all mitochondrial proteins are encoded in the nucleus, synthesized in the cytosol and subsequently imported into the organelle. However, all mitochondria capable of oxidative phosphorylation have retained a genome encoding a small set of proteins, the large majority of which are integral membrane proteins that are essential for oxidative phosphorylation, which underscores the importance of an organellar genome (Bullerwell and Gray, 2004). Consequently, mitochondria not only need their own gene expression system, but also require mechanisms that guarantee that during cytokinesis, each daughter cell receives mitochondria containing intact and complete genomes. Thus, it is a central question of mitochondrial biology how the mitochondrial DNA is replicated and segregated (Gustafsson et al., 2016; Westermann, 2013). In this review, we discuss this problem in the parasitic protozoan *Trypanosoma brucei*. Beginning with the unique machinery that mediates the segregation of the replicated genomes prior to mitochondrial division and cytokinesis (Povelones, 2014), we subsequently compare mitochondrial genome inheritance in *Trypanosoma* with the mechanisms to segregate mitochondria and their genomes in yeast and mammals.

A unique mitochondrial biology

Mitochondrial biogenesis has been studied in detail (Backes and Herrmann, 2017; Friedman and Nunnari, 2014; Nunnari and Suomalainen, 2012; Wiedemann and Pfanner, 2017). However, the vast majority of these studies used a handful of model systems - mainly yeast and mammalian cells - all of which belong to the same eukaryotic supergroup of the Opisthokonta. Thus, the immense diversity of mitochondria after two billion years of divergent evolution is still underappreciated (Gray, 2012; Gray et al., 1999). In order to understand mitochondrial evolution and biology better, we need to include non-Opisthokont eukaryotes in our analyses. Kinetoplastea that belong to the supergroup of the Excavata are a rewarding taxon to consider. They include *T. brucei*, a single-celled parasite that is the causative agent of human African sleeping sickness and further animal diseases (Giordani et al., 2016). Importantly, the *T. brucei* mitochondrion has been investigated in detail and might indeed be the best-studied organelle outside of the Opisthokonta (Harsman and Schneider, 2017; Jensen and Englund, 2012; Mani et al., 2016; Povelones, 2014; Read et al., 2016; Schneider, 2001; Verner et al., 2015).

The mitochondrial genome of *T. brucei*

Unlike mammals and yeast that have a large number of constantly dividing and fusing mitochondria per cell, *T. brucei* only has a single mitochondrion (Tyler et al., 2001). Moreover, unlike in virtually all other eukaryotes, it contains a single unit mitochondrial genome that is called kinetoplast DNA (kDNA). It localizes to a specific region in the organelle: opposite to the basal body of the single flagellum (Povelones, 2014). The kDNA organization is very unusual, since it consists of a network of two genetic elements, the topologically interlocked maxi- and minicircles forming a disc-like structure (Jensen and Englund, 2012) (Fig. 1). The 23kb maxicircles are present in about 25 copies and encode for two mitochondrial ribosomal RNAs and 18 proteins that are subunits of the oxidative phosphorylation complexes, except for the mitochondrial small ribosomal sub-unit protein eS12 (Rps12) and four proteins of unknown function (Shapiro and Englund, 1995). Twelve of the protein-coding genes represent cryptogenes, whose primary transcripts have to be edited by multiple uridine insertions and/or deletions in order to become translatable mRNAs (Hajduk and Ochsenreiter, 2010; Read et al., 2016; Simpson et al., 2003; Stuart et al., 2005). In addition to the maxicircles, the kDNA network contains about 5000 minicircles: they are 1kb in size, heterogeneous in sequence and code for the guide RNAs that mediate numerous RNA editing events (Hajduk and Ochsenreiter, 2010; Read et al., 2016; Simpson et al., 2003; Stuart et al., 2005). Thus, 80-90% of the kDNA mass comes from the minicircles. Another salient feature of the kDNA network is the complete lack of tRNA genes that indicates that all trypanosomal mitochondrial tRNAs have to be imported from the cytosol (Alfonzo and Söll, 2009; Schneider, 2011).

How such an intricate network of two intercalated genetic elements replicates has attracted a lot of interest. In contrast to mitochondrial genomes of other eukaryotes, the single unit nature of the trypanosomal kDNA necessitates that its replication is coordinated with the nuclear cell cycle (Box 1). The segregation of the replicated kDNA discs is completed before the onset of mitosis (Fig. 1 and Box 1). It depends on a unique physical linkage that connects the kDNA disc across the mitochondrial IM and the outer membrane (OM) with the basal body of the flagellum. It is this linkage that couples the segregation of the old and the new flagellum to the segregation of the replicated kDNA (Robinson and Gull, 1991). The structure making up this connection is the focus of this review, as much progress has been made recently regarding its composition, function and assembly.

The tripartite attachment complex and its subdomains

Over a century ago, Muriel Robertson had observed linkage between the trypanosomal kDNA and the flagellum. She suggested that the blepharoplast (meaning the basal body) and the kinetonucleus (kDNA) are connected and that basal bodies “very clearly and constantly play the part of the centrosomes” in the division of the kDNA (Robertson, 1913). The first detailed morphological analysis of the kDNA basal body connection was then an elegant electron microscopy (EM) study in *T. brucei*; it revealed the subdomains of the structure that was named tripartite attachment complex (TAC) (Ogbadoyi et al., 2003) (Fig. 2). The exclusion zone filaments (EZFs) are 5-10 nm wide

electron-dense filaments that create a region in the cytoplasm that is depleted of ribosomes. They range from the proximal end of the basal body to the mitochondrial OM. At the distal end, the EZFs connect to so-called differentiated mitochondrial membranes (DM), which in this area lack cristae, seem resistant to detergent and are more closely apposed than in other regions of the mitochondrion (Ogbadoyi et al., 2003). In addition, a tightly packed filamentous mass called unilateral filaments (ULFs) extends from the differentiated mitochondrial IM to one side of the kDNA disc (Gluenz et al., 2007). The ULFs can be further subdivided into the kDNA-proximal domain that contains basic proteins and DNA, whereas the domain close to the IM likely contains more acidic proteins and seems free of DNA (Gluenz et al., 2007).

Aside from connecting the basal body to the kDNA, the TAC is also responsible for the positioning of the posterior region of the mitochondrial organelle (Hoffmann et al., 2018; Jakob et al., 2016). Furthermore, the partial overlap of ULFs and KFZ suggests an interaction between the mitochondrial replication machinery and the TAC in *T. brucei*. Indeed, we recently showed that the localization of the minicircle replication factor 172 (MiRF172), which is required for reattachment of the minicircles to the kDNA, partially depends on the TAC (Amodeo et al., 2018). Thus, the TAC functions in mitochondrial genome segregation, as well as positioning of the organelle and may also be contribute to kDNA replication. In the following, we describe all known TAC subunits, starting with the ones that localize to the EZF (Table 1, which also contains orthologues of the TAC subunits in other Kinetoplastea) (Fig. 2).

Composition of the TAC

TAC subunits: EZF

The conserved TBCC domain-containing protein 1 (TBCCD1) is the trypanosomal member of the tubulin-binding cofactor C protein family. It localizes to the anterior of the cell body, the Golgi-associated bilobe structure and to the region of the basal body (Andre et al., 2013). Its ablation causes a disorganization of the bilobe structure and an accumulation of cells that either lack or have overreplicated kDNA (Andre et al., 2013). The latter phenotype is typical for a deficient TAC and suggests that TBCCD1 is a structural subunit of the EZF subregion of the TAC. The kinetoplastid-specific protein p197 was initially identified in a proteomics screen for new bilobe proteins (Zhou et al., 2010) and subsequently characterized as part of the TAC (Gheiratmand et al., 2013). Of all known TAC components, it is closest to the base of the flagellum and depletion of p197 leads to kDNA missegregation and mislocalization of all other TAC proteins. However, despite its proximity to the basal body, depletion of p197 did not change its structure (Hoffmann et al., 2018). The component of the EZF that is the most proximal to the OM is the peripheral kinetoplastid-specific OM protein TAC65 (Käser et al., 2016) (Fig. 2). Additionally, there are two monoclonal antibodies that stain the EZF of the TAC: BBA4 detects an unknown antigen lining the basal body, which is dependent on the

presence of p197. However, loss of BBA4 localization does not lead to any obvious changes in the basal body structure (Hoffmann et al., 2018). The second antibody, Mab22, detects an unknown antigen in the EZFs whose localization also depends on p197 (Bonhivers et al., 2008) (Hoffmann et al., 2018) (Fig. 2).

TAC subunits: DM

Four TAC subunits are known to localize to the DM subdomain of the TAC; they are integral mitochondrial OM proteins and specific for Kinetoplastea. Three of these proteins, TAC60, TAC40 and TAC42 form a complex (Käser et al., 2017). TAC60 has two transmembrane domains and its N- and C-termini face the cytosol. Its C-terminal domain shares homology with bacterial tRNA/rRNA methyltransferases, but is not required for TAC60 function (Käser et al., 2017). TAC40 belongs to the voltage-dependent anion-selective channel (VDAC)-like protein family (Schnarwiler et al., 2014), whereas TAC42 defines a novel class of kinetoplastid-specific mitochondrial β–barrel proteins (Käser et al., 2017). The fourth integral mitochondrial OM protein is the kinetoplastid-specific protein peripheral archaic translocase of the OM 36 (pATOM36) (Pusnik et al., 2012). Remarkably, pATOM36 is not only essential for TAC function, but also for the biogenesis of a subset of mitochondrial OM proteins (Käser et al., 2016). In fact, experiments in yeast and *T. brucei* revealed that pATOM36 is a functional analogue of the yeast mitochondrial inner-membrane import machinery (MIM) complex, which consists of Mim1 and Mim2 (Vitali et al., 2018). In line with its dual function, pATOM36 localizes to the DM subdomain of the TAC, as well as all over the OM (Käser et al., 2016). Thus, pATOM36 integrates mitochondrial protein import with mitochondrial DNA inheritance. Interestingly, the cytosol-facing C-terminal part of pATOM36 is dispensable for its TAC function, but required for mitochondrial OM protein biogenesis. Furthermore, pATOM36 is closely associated with the EZF-protein TAC65 (Käser et al., 2016). Ablation of EZF TAC subunits and ablation of the OM protein to which the EZFs connect to should increase the distance between the OM and the basal body, and in absence of either pATOM36 or p197 such an increase is indeed observed. In contrast, when the ULF subunit TAC102 (Hoffmann et al., 2018; Käser et al., 2016; Trikin et al., 2016) was ablated, the distance remained unchanged. This suggests that the EZFs - possibly through TAC65 - link to pATOM36 (Fig. 2).

It is evident from the architecture of the TAC that it must contain at least one subunit that is an integral IM protein that connects to a TAC subunit in the OM, as well as to the ULFs in the matrix. However, no such subunit has been found yet. The best candidate for an IM TAC subunit is p166, the first molecular component of the TAC to be discovered (Zhao et al., 2008). p166 is an acidic (pI 5.2) protein with a predicted transmembrane domain at its C-terminus. However, this has not been experimentally verified and this domain is not required for TAC localization of p166 (Zhao et al.,

2008). Furthermore, it is unknown whether it is required for TAC function. Thus, it remains unclear whether p166 indeed is an IM TAC subunit or whether it is a part of the ULFs.
Another possible candidate for an IM TAC subunit is alternatively edited protein 1 (AEP-1) (Ochsenreiter et al., 2008). AEP-1 is unusual in that it originates from an alternatively edited mRNA that is derived from the primary transcript of the cytochrome oxidase subunit 3 (Cox3) cryptogene encoded on the maxicircle DNA. AEP-1 has four predicted transmembrane domains and localizes between the basal body and the kDNA without overlapping with either of the two structures (Ochsenreiter et al., 2008). Nuclear expression and mitochondrial targeting of the soluble domain of AEP-1 results in a transient growth arrest. Moreover, consistent with a TAC function of AEP-1, an increase in cells that lack kDNA or have two kDNAs was observed. Interestingly, the recombinant soluble domain of AEP-1 can bind to DNA (Ochsenreiter et al., 2008). Thus, whereas it is conceivable that AEP-1 alone attaches kDNA to the mitochondrial IM, it is very unlikely since trypanosomes lacking maxicircles - and therefore AEP-1, such as many *T. evansi* isolates - are not impaired in kDNA segregation (Schnaufer et al., 2002). Moreover, AEP-1 is difficult to study, because it is mitochondrially encoded and evidence that the protein is present in the predicted form is still lacking. In summary, whereas four OM TAC subunits have been characterized recently, the identity of the postulated IM TAC subunit(s) remain(s) elusive.
Whereas nothing is known about the lipid composition of the DM subdomain of the TAC, there is evidence that it might be important for TAC function: the bilayer in the DM region - unlike the rest of the mitochondrial membranes - is at least in part resistant to extraction by non-ionic detergent. Moreover, conditional knock-down of the mitochondrial acyl carrier protein (ACP) in the bloodstream form of *T. brucei* that caused changes in the cellular phospholipid composition also resulted in defects of the segregation of the replicated kDNA (Clayton et al., 2011). This suggests that changing the lipid composition of the mitochondrial membranes in a yet unknown way may affect TAC structure or assembly and thus interfere with kDNA segregation.

TAC subunits: ULF

The soluble kinetoplastid-specific TAC102 is an ULF protein and the TAC component most proximal to the kDNA currently known. Based on super-resolution microscopy, TAC102 does not directly interact with the kDNA (Hoffmann et al., 2016; Hoffmann et al., 2018). Its localization and basic pI of 9.5 supports the view that the ULF subregion closest to the kDNA is dominated by basic proteins. TAC102 migrates in a complex that is distinct from the much larger complexes in which p166, TAC40 and TAC60 reside (Hoffmann et al., 2018), which further suggests that TAC102 is not in contact with the DM. Another component of the ULF is α–KDE2 that localizes to the entire mitochondrion and is also recovered in isolated flagella that are still attached to the kDNA, where it localizes to the antipodal sites of the kDNA disc (Sykes and Hajduk, 2013). Ablation of the protein in bloodstream forms causes a growth arrest and accumulation of cells either lacking kDNAs or

containing two kDNAs discs. This suggests that α−KDE2 is involved in kDNA segregation, but not in its replication. Thus, α−KDE2 likely has a dual function as structural TAC subunit and as an enzyme of the TCA (Sykes and Hajduk, 2013).

TAC assembly

During kinetoplast replication, the newly developing TAC assembles in a hierarchical order from the base of the flagellum towards the kDNA (Hoffmann et al., 2018). Depletion of basal body-proximal TAC components like p197 leads to loss of the localization of all currently known TAC proteins, whereas depletion of a basal body distal protein like TAC102 does not affect the localization of the remaining TAC components (Fig. 3). Interestingly, although the depletion of p197 leads to destruction of the overall TAC, the individual TAC proteins are not degraded; it thus seems likely that assembly into subcomplexes protects them from proteolysis, as has been shown for p166 and TAC60 (Hoffmann et al., 2018). Mitochondrial genome missegregation is the common phenotype in all TAC protein depletion experiments. Interestingly, the missegregation is not random; rather, kDNA is always retained at the old basal body, whereas the new basal body only keeps a small fraction of the kDNA, or lacks it altogether (Schnarwiler et al., 2014; Trikin et al., 2016; Zhao et al., 2008). This suggests that - once established - the TAC is a stable structure that has no significant turnover during the cell division cycle. Furthermore, the non-random missegregation also suggests that the TAC is assembled *de novo*, rather than in a semi-conservative way, where a random missegregation phenotype would be expected. Support for the *de novo* assembly mechanism also comes from experiments in which p197 was depleted for >15 generations in the γL262P cell line that survives without mitochondrial genome (Dean et al., 2013; Hoffmann et al., 2018). In these cells, the components of the TAC are either absent or, in the case of TAC102, mislocalized inside the mitochondrion (Hoffmann et al., 2018). If subsequently p197 is re-expressed, the TAC seems to form *de novo* without the requirement of a template. An alternative explanation would be that tiny amounts of the TAC that are not sufficient to retain the kDNA remain after depletion of p197 and then serve as template for the re-establishment of a new TAC. However, this model would not explain the specific connection to the old basal body that leads to the non-random missegregation phenotype (Hoffmann et al., 2018).

It is currently unknown what controls the assembly of the TAC from the basal body. An interesting candidate for this would be polo-like kinase (PLK) that localizes to the basal bodies during initiation of the cell cycle and is required for basal body segregation in procyclic-form trypanosomes (Hammarton, 2007). Furthermore, p197, the TAC component that is closest to the basal body, is phosphorylated by PLK (McAllaster et al., 2015) and the involvement of PLKs in basal body or centriole biogenesis is widely conserved in biology. However, there is no direct experimental evidence that the PLK is required for TAC biogenesis. Also, it is currently unknown whether the EZF

of the TAC are directly attached to the basal body, or whether they originate from the material that surrounds the basal body. In summary, the results discussed above strongly support the model that the TAC is assembled *de novo* in a hierarchical way, starting with the subunits that are most proximal to the basal body (Fig. 3).

Targeting of TAC proteins

Each TAC subunit needs to be correctly targeted and integrated into the corresponding TAC subregion. In the case of DM and ULF proteins, this requires mitochondrial import followed by lateral sorting to the single-unit TAC in non-dividing cells. For the potential DM component p166, a canonical N-terminal presequence has been described; however, the ULF component TAC102 does not contain such a signal, but requires a region in the C-terminus for proper mitochondrial localization (Trikin et al., 2016). Recently, the biogenesis pathway for TAC40, TAC42 and TAC60 - three TAC subunits of the DM region - has been elucidated (Käser et al., 2017). As expected for β–barrel proteins, TAC40 and TAC42 depend on the main protein translocase of the OM (ATOM) (Mani et al., 2015), as well as on the sorting and assembly machinery (SAM) to reach their destination (Sharma et al., 2010). Targeting to the latter is mediated by C-terminal conserved β–barrel signals (Kutik et al., 2008) that also mediate OM insertion of the proteins in the heterologous yeast system. The case is different for TAC60 that has two transmembrane domains, and its N- and C-terminus face the cytosol: it contains separate mitochondrial and TAC-targeting sequences (Kaser et al., 2017). For TAC60, the segment comprising the intermembrane-space-exposed loop and the more C-terminal transmembrane domain are required for targeting of the protein to the mitochondrial OM (Kaser et al., 2017). Localization to the TAC requires an additional 26aa-long region, which essentially consists of the first transmembrane domain (Käser et al., 2017). However, this targeting signal is not conserved in other TAC subunits, and it is not known how this signal might function. Based on the hierarchical assembly model of the TAC presented above (Fig. 3), TAC subunits of DMs might diffuse within the OM and IM until they interact with the already assembled EZFs that "touch" the mitochondrial OM. This would stop further lateral diffusion of the protein and allow the next member of the DM region to interact with the now fixed integral membrane subunit. Sorting of dually localized TAC subunits to their destinations, such as the OM protein pATOM36 (Käser et al., 2016) and the matrix-localized α–KDE2 (Sykes and Hajduk, 2013), represents a further challenge. It is unclear how identical proteins can end up in two distinct places. Furthermore, it is also not known whether TAC assembly requires chaperones, as it is the case for the formation of some respiratory complexes (Mimaki et al., 2012).

Binary fission of the trypanosomal mitochondrion

Prior to cytokinesis, the single trypanosomal mitochondrion is divided in two, whereby each of the two replicated and segregated kDNA discs end up in one of the two daughter organelles (Jakob et al., 2016). As in other eukaryotes, the process is mediated by a dynamin-like protein (DLP) (Chanez et al., 2006; Morgan et al., 2004) that is encoded by two genes that give rise to proteins, which are more than 97% identical (Benz et al., 2017). DLP is the only member of the dynamin protein family in trypanosomes. Besides its function in mitochondrial fission, it is also required for endocytosis (Chanez et al., 2006). Moreover, ablation of DLP does not only block mitochondrial fission, but also cytokinesis, resulting in the accumulation of cells with two nuclei and two segregated kDNA networks, but only a single mitochondrion (Chanez et al., 2006). This cell cycle phenotype is linked to the mitochondrial fission function of DLP, as the ablation of clathrin, which is essential for endocytosis, does not result in a cytokinesis defect (Chanez et al., 2006). In summary, these results suggest that in trypanosomes, unlike in other eukaryotes, mitochondrial fission might serve as a checkpoint for cytokinesis.

Comparison to other eukaryotes and bacteria

When comparing mitochondrial DNA segregation in trypanosomes with the corresponding processes in other eukaryotes and bacteria, the systems show striking differences. The number of mitochondria in yeast and mammals is highly variable and each organelle contains multiple genomes that are termed nucleoids (Bogenhagen, 2012; Friedman and Nunnari, 2014; Gustafsson et al., 2016; Labbe et al., 2014). The mitochondrial genome of trypanosomes, however, is a single unit that is reminiscent of bacteria, most of which have a single chromosome only.

Bacterial segregation compared to trypanosomes

The textbook view is that the bacterial chromosome is attached to the cell membrane and this is important since expansion of the membrane segment between two attachment sites by cell growth mediates the segregation of the replicated genomes (Jacob and Brenner, 1963; Toro and Shapiro, 2010). This attachment seems analogous to the situation in trypanosome mitochondria, where the single unit kDNA is attached to the IM through the ULF of the TAC. However, in trypanosomes, the actual force that is required for mitochondrial genome segregation is provided from the outside of the organelle by a still unknown system that segregates the basal bodies of the two flagella and is mediated by microtubules (Robinson and Gull, 1991). Additionally, in most bacteria, the genome is not permanently attached to the cell membrane and DNA segregation is actively achieved by cell-internal segregation machineries that might not necessarily be attached to the cell membrane (Toro and Shapiro, 2010). Thus, the filaments of the trypanosomal TAC that link the kDNA to the IM do not represent an ancestral trait inherited from the bacterial endosymbiont, since the TAC is a permanent structure and connects the kDNA to a segregation system (basal body) that is on the outside of the organelle. Nevertheless, there is evidence that two of the TAC subunits - TAC40 and

TAC42 - originate from the endosymbiontic ancestor of mitochondria, as they are β-barrel membrane proteins whose occurence is restricted to the OM of bacteria and endosymbiontic organelles (Ulrich and Rapaport, 2015; Webb et al., 2012). Furthermore, it is peculiar that in some α-proteobacteria, the chromosomes are anchored at the poles of the cell and attached to the membrane just where the flagellum of these bacteria resides (Berge and Viollier, 2018). - Thus, whereas the genome segregation systems in bacteria and trypanosomal mitochondria appear superficially similar, the two systems likely have different evolutionary roots.

Yeast and mammals compared to trypanosomes

In the yeast *Saccharomyces cerevisiae* and in mammals, the nucleoids in mitochondria show a punctate intra-mitochondrial distribution, and in mammals they contain a single-copy mitochondrial genome (Brown et al., 2011; Kukat et al., 2011). In these systems, inheritance of mitochondria and their genome is thought to be mainly stochastic, although some active segregation mechanisms may contribute to the process. (Labbe et al., 2014). In contrast to trypanosomes, the mitochondria of yeast and mammals constantly divide and fuse. However, the positioning of division sites is not random: it occurs at mitochondrial regions that are both adjacent to a subpopulation of replicating nucleoids and in contact with the ER (Meeusen and Nunnari, 2003; Murley et al., 2013). In yeast, contact sites of ER and mitochondria are formed by the ER mitochondria encounter structure (ERMES), a protein complex that consists of mitochondrial distribution and morphology protein 10 (Mdm10), Mdm12, Mdm34 and maintenance of mitochondrial morphology protein 1 (Mmm1) (Kornmann et al., 2009). Thus, the ER marks future mitochondrial division sites and might facilitate constriction of the organelle to allow subsequent mitochondrial fission by the dynamin-like protein (Lewis et al., 2016). At least a fraction of nucleoids is associated with the mitochondrial IM in both yeast and mammals, but it has been difficult to determine the molecular basis of this interaction (Labbe et al., 2014). The best mammalian candidate that might link nucleoids to the IM membrane is an ATPase family AAA domain-containing protein 3 (ATAD3). It is enriched at ER-mitochondria contact sites and seems to extend as a single polypeptide across both the mitochondrial IM and the OM (Baudier, 2018). However, it is unclear whether ATAD3 directly binds to DNA. Instead, it has been suggested that nucleoids may bind to a cholesterol-rich platform that is found at mitochondria-ER contact sites, and that formation of such a platform might be influenced by ATAD3 (Gerhold et al., 2015). Interaction of nucleoids with a specialized membrane region is also supported by their association with the IM protein prohibitin that has been implicated in the formation of protein and/or lipid scaffolds (Osman et al., 2009). In yeast, ERMES might be part of a larger complex that spans the IM and the OM and that connects nucleoids with the ER to control mitochondrial DNA segregation (Boldogh et al., 2003). Mitochondrial IM proteins that could mediate this process are unknown. Candidates include the two related IM proteins Mdm31 and Mdm32, since their deletion leads to loss of mitochondrial DNA and

is synthetic lethal in combination with loss of genes encoding ERMES subunits (Dimmer et al., 2005). There is ample evidence that mitochondria in yeast and mammals are associated with cytoskeletal structures (Boldogh et al., 2003; Labbe et al., 2014); however, it is not clear whether this interaction preferentially occurs close to nucleoids. Moreover, even if this is the case, it remains to be established whether there is a direct physical linkage that connects the mitochondrial DNA to cytoskeletal elements. Thus, at present, the trypanosomal TAC is the only example of a permanent physical linkage between the mitochondrial DNA and elements of the cytoskeleton, in this case the basal body of the flagellum.

Conclusions and perspectives

Whereas the TAC has been characterized morphologically for many years, its composition has essentially been a black box. Owing to work in the past few years the situation has changed quite dramatically. At least seven dedicated and essential TAC subunits have been discovered and characterized (Table 1). Three further TAC subunits that are localized to the TAC, as well as to other subcellular regions and that have a dual or even multiple functions, are also known (Table 1). Moreover, we begin to understand the overarching principle of TAC assembly (Fig. 3) and have started to analyze the biogenesis pathways of some of its subunits.

The TAC is unique to trypanosome and the requirement for such a hardwired linkage becomes apparent from the biology of the parasite: it cannot afford to lose a single-unit mitochondrial genome. A stochastic distribution of replicated genomes would lead to segregation failures and is therefore not an option; an active segregation mechanism is required. This is achieved by the TAC, which links kDNA segregation to the segregation of another essential single unit organelle, the flagellum. Consequently, the plane of division of the single mitochondrion must be positioned between the segregated kDNA discs. As other systems, trypanosomes likely have contact sites between the ER and the mitochondrion; importantly, they have proteins that show sequence similarities to the ERMES subunits Mdm12 and Mdm34, even if these proteins are not involved in the formation of ER-mitochondria contact sites (Schnarwiler et al., 2014). Thus, the molecular nature of such contact sites in trypanosomes is presently unknown. Moreover, there is no evidence that ER-mitochondria interactions in *T. brucei* are restricted to the kDNA region. In fact, this region has been extensively analyzed by EM and no contact sites between the mitochondrion and the ER were found (Lacomble et al., 2009). This suggests that in *T. brucei*, the ER is not involved in positioning of the division plane for mitochondrial fission.

Four different essential TAC subunits have been characterized in the OM alone (Table 1). If the TAC has a purely structural function, a single OM subunit linking to the ULF on the outside and to the IM on the inside could be sufficient. Thus, the extraordinary complexity of the TAC that is being revealed right now remains unexplained. More in depth studies of the TAC and its subunits are therefore

required and it is possible that they will reveal connections to other cellular functions we cannot yet anticipate.

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The authors declare no competing or financial interests.

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Figure legends

Fig. 1: The TAC during the cell cycle. (I) The TAC (dotted grey lines) connects the kDNA (blue) across the mitochondrial membranes (magenta and in the TAC region yellow) to the basal body of the flagellum (grey). (II) In the G0/G1 stage, the old basal body (grey) is connected to a one-unit kDNA disc and has a probasal body (pro-bb, green) attached. (III) During kDNA replication, the old basal body maintains the connection to the kDNA and the new basal body is formed and eventually rotates (orange arrow) around the old basal body during maturation (IV). (V) After maturation, the new basal body connects to the kDNA disc and the new flagellum starts to grow. (I) During segregation of the two replicated kDNA discs, the *nabelschnur* structure appears (dark blue). It represents the replicated maxicircles that are subsequently divided between the two discs. The red dotted line represents the position of mitochondrial fission.

Fig. 2: Overview of the TAC. Top left, a trypanosome cell with its single mitochondrion (magenta) the flagellum (red) and the nuclear and mitochondrial DNA (blue). Top right, enlargement of the TAC region depicting the mature basal body (grey) connected through the TAC (green shading) to the kDNA (blue). The differentiated mitochondrial membranes are shown in yellow. The pro basal body is depicted in green. Bottom, enlargement of the TAC (green shading) with its components (solid line, precise position on the basal body kDNA axis is known; dashed line, precise position unknown). Connecting lines indicate protein complexes. EZF, exclusion zone filaments; DM, differentiated membranes; ULF, unilateral filaments; MOM, mitochondrial outer membrane; MIM, mitochondrial inner membrane. Some components like TbTBCCD1, α-KDE2 and pATOM36 have multiple locations that are indicated. *, it remains unclear if p166 is a membrane protein or not.

Fig. 3: Elucidation of the hierarchical TAC model. (A-C) Scenarios where one TAC component from either the exclusion zone filaments (EZF), the differentiated membranes (DM) or the unilateral filaments (ULF) is depleted by RNAi. (A) A ULF component of the TAC is depleted (red X) and the resulting phenotype - overreplicated kDNA attached to the old basal body and no kDNA at the new basal body - is shown. (B) and (C) focus on the new basal body. (B) Depletion of an OM component of the TAC leads to the same phenotype as in (A) plus an increased distance of the basal body to the OM due to detachment and mislocalization of the kDNA proximal TAC components in the IM and the ULF. (C) Depletion of a basal body proximal TAC component leads to the phenotype described in (B) plus a mislocalization of EZF and OM TAC components.
OM, mitochondrial outer membrane; IM, mitochondrial inner membrane; bb, basal body. Colored ellipsis represent TAC components; green shading depicts the overall TAC structure. Red X indicates the depletion of a TAC component by RNAi. kDNA is in blue, the mitochondrial membranes in

magenta and yellow. The old basal body and axoneme in grey and the new basal body and axoneme in green.

Text Boxes

Box 1. kDNA replication

kDNA replication initiates prior to nuclear S-phase (Woodward and Gull, 1990) with the topoisomerase-mediated release of minicircles into the kinetoflagellar zone (KFZ), a region between the kDNA disc and the mitochondrial inner membrane (IM) (Drew and Englund, 2001; Jensen and Englund, 2012). Minicircle replication then progresses unidirectional through θ–intermediates. Subsequently, each daughter minicircle moves to two 180°-opposing regions at the kDNA disc that are called antipodal sites; movement occurs by an unknown mechanism (Gluenz et al., 2007; Ryan and Englund, 1989b). Antipodal sites are the location for primer removal and gap repair. Finally, the minicircles are re-attached to the growing kDNA disc (Melendy et al., 1988; Ryan and Englund, 1989a). Maxicircle replication - in contrast to the minicircles - occurs within the network. Maxicircles are likely replicated unidirectionally, as well as through θ–intermediates (Carpenter and Englund, 1995). Once the kDNA is entirely replicated, it adopts a bilobed shape with only maxicircles remaining between the two daughter discs. Further segregation of the replicated kDNAs results in the formation of a filament that connects the two lobes, known as nabelschnur (Gluenz et al., 2011). A topoisomerase activity presumably then releases the maxicircles from the nabelschnur region so that further segregation of the two kDNA discs can proceed. kDNA replication has been discussed in further detail in recent reviews (Jensen and Englund, 2012; Povelones et al., 2013; Verner et al., 2015).

Box2. Criteria to define TAC subunits

TAC components are by definition localized between the kDNA disc and the basal body of the flagellum. This is true in whole cells, as well as in isolated flagella, if they are still connected to the kDNA. However, using conventional immunofluorescence, it is often impossible to determine such a precise localization. As a consequence, it can be difficult to decide whether proteins that co-localize with either the kDNA or the basal body are dedicated TAC subunits, kDNA replication factors or bona fide basal body components, respectively.

Many proteins that are specifically involved in and essential for TAC function can be identified by the fact that their ablation that will selectively interfere with kDNA segregation, but not with its replication. Thus, in their absence, we should see kDNA loss, as well as overreplication of the kDNA disc in the few cells that have retained the mitochondrial genome. In trypanosomes, the flagellum and thus the basal body is not only essential for motility, but also for cytokinesis (Broadhead et al., 2006). With regards to basal body proteins, they can be distinguished from TAC subunits by being essential in an engineered cell line of the bloodstream form of *T.brucei*, γL262P, that does not require the TAC since it can grow in the absence of kDNA (Dean et al., 2013). Whereas the proposed criteria provide an operational definition for TAC subunits they are quite strict and cannot be applied to proteins that

are involved in kDNA replication and at the same time connected to and essential for the formation of the TAC (as it might be the case for MiRF172 (Amodeo et al., 2018)). The same is true for TAC subunits that have a second function that is unrelated to the TAC, such as pATOM36 (Käser et al., 2016) and α-KDE2 (Sykes and Hajduk, 2013).

Table 1: Components of the TAC

Name	MW (kDa)	Protein features	Localization	Essential in PC and BSF	Essential in L262P BSF	Orthologues	Reference
p197	197	- 3 repeats of 174 aa	EZF	PC: yes	No	BA, BS, CF, EM, LM, LS, TC	[1, 3]
TbTBCCD1	59	- tubulin-binding cofactor C protein family - second function in maintenance of bi-lobe structure	EZF	PC: yes	ND	BA, BS, CF, EM, LM, LS, PC, TC	[2]
BBA4	ND	ND	EZF	ND	ND	NA	[3]
Mab22	ND	ND	EZF	ND	ND	NA	[4]
TAC65	65	- complex with pATOM36	EZF	PC: yes	No	BA, BS, CF, EM, LM, LS, PC, TC	[5]
pATOM36	36	- 1 to 3 TMD - C-terminus IMS-exposed - complex with TAC65 -second function in OM protein biogenesis	DM (OM) whole OM	PC: yes BSF: yes	Yes	BA, BS, CF, EM, LM, LS, PC, TC	[5, 6]
TAC40	40	- β–barrel protein (VDAC-like) - complex with TAC42/TAC60	DM (OM)	PC: yes BSF: yes	No	BA, BS, CF, EM, LM, LS, PC, TC	[7, 8]
TAC42	42	- β–barrel protein - complex with TAC40/TAC60	DM (OM)	PC: yes BSF: yes	No	BA, BS, CF, EM, LM, LS, PC, TC	[8]
TAC60	60	- 2 TMD - N- and C-terminus IMS exposed - complex with TAC40/TAC42	DM (OM)	PC: yes BSF: yes	No	BA, BS, CF, EM, LM, LS, PC, TC	[8]
p166	166	- 1 TMD	DM (IM)? ULF?	PC: yes BSF: yes	No	BA, BS, CF, EM, LM, LS, PC, TC	[3, 9]
AEP-1		protein from alternatively edited mitochondrially encoded COX3 transcript	ULF (IM)	BSF: yes	ND	NA	[10]
TAC102	102	-internal mitochondrial targeting signal	ULF	PC: yes BSF: yes	No	BA, CF, EM, LM, LS, PC, TC	[11]
α–KDE2	41	- E2 subunit of α–ketoglutarate dehydrogenase -second function in TCA cycle	ULF Matrix	BSF: yes	ND	BA, BS, CF, EM, LM, LS, PC, TC	[12]

BA: *Blechomonas ayalai*
BS: *Bodo Saltans*
CF: *Crithidia fasciculata*
EM: *Endotrypanum monterogeii*
LM: *Leishmania major*
LS: *Leptomonas seymouri*
PC: *Paratrypanosoma confusum*
TC: *Trypanosoma cruzi* CL Brener Esmeraldo-like

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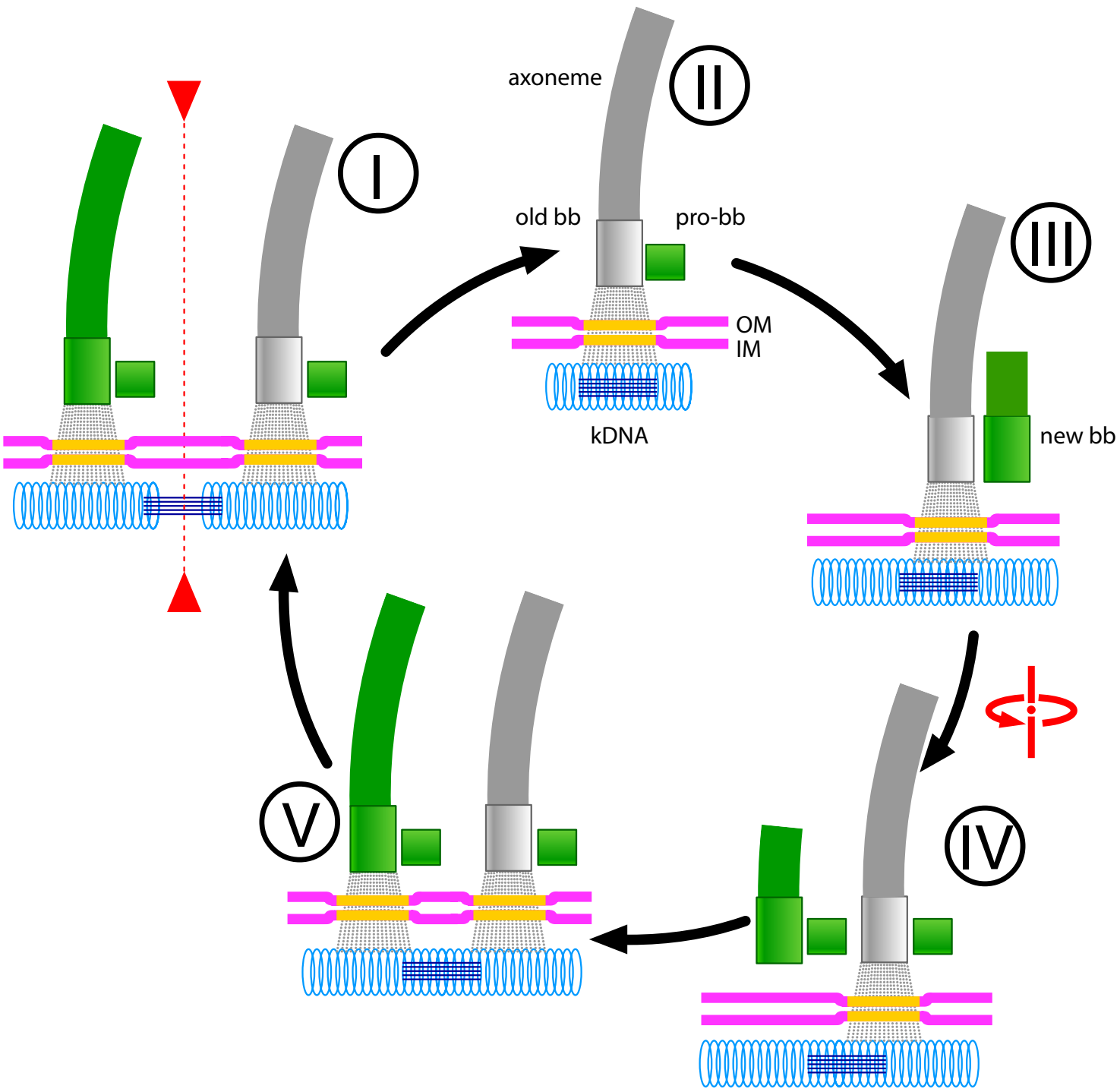
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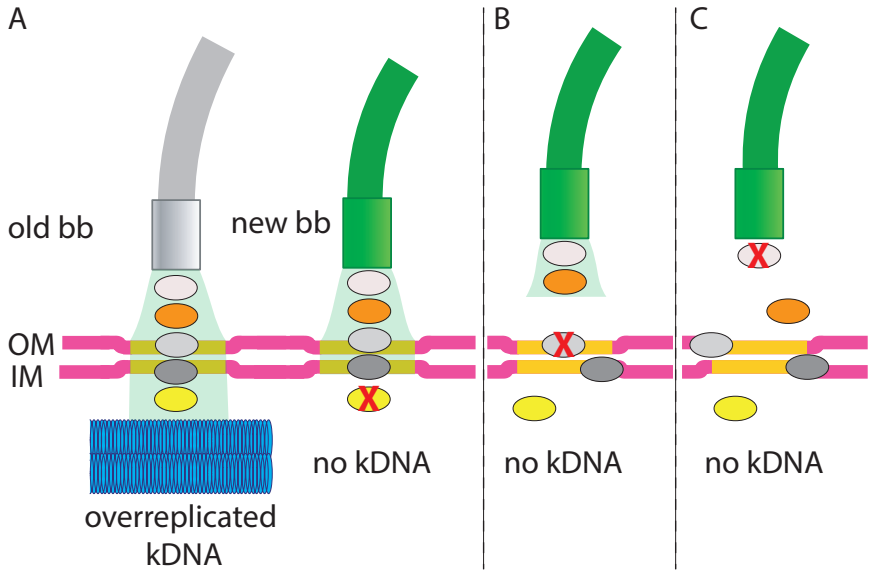
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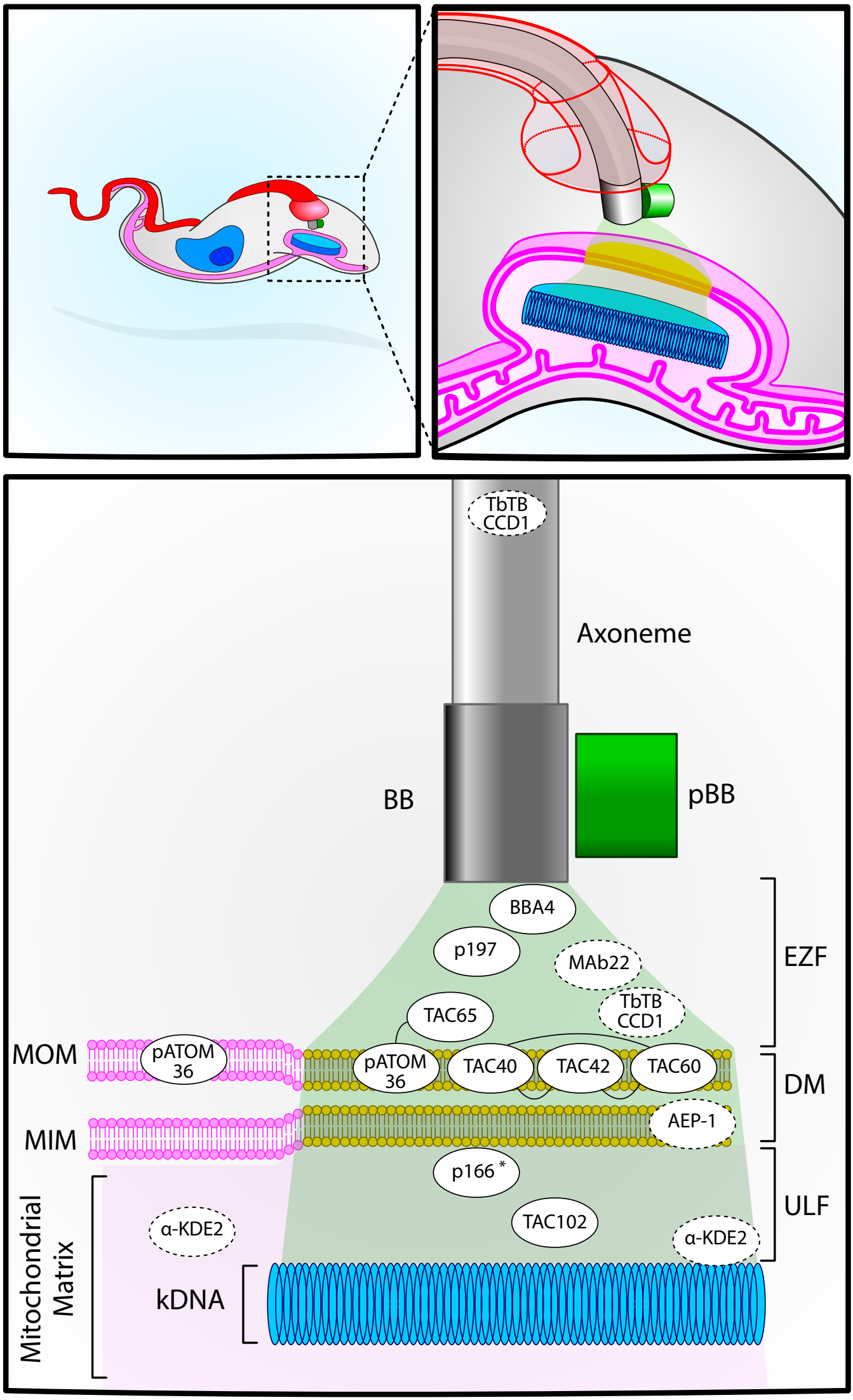
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