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Faculty of Science

Factors involved in *Trypanosoma brucei* mitochondrial morphology

Ph.D. Thesis

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Annotation:

This PhD. thesis focuses on several molecules which are involved in mitochondrial morphology of *in the* parasitic protist *Trypanosoma brucei,* a eukaryotic system that enables the study of both operation modes of the complex in physiological settings. The results presented in this PhD thesis is divided to four topics: (i) Ultrastructural changes of the mitochondrion during the life cycle of *Trypanosoma brucei,* (ii) Intracytoplasmic-membrane development in alphaproteobacteria involves the homolog of the mitochondrial crista-developing protein Mic60, (iii) A novel group of dynamin-related proteins shared by eukaryotes and giant viruses is able to remodel mitochondria from within the matrix, (iv) The MICOS complex of trypanosomatids contains two cryptic mitofilin-domain proteins that may shape cristae.

Declaration

I hereby declare that I am the author of this dissertation and that I have used only those sources and literature detailed in the list of references.

České Budějovice, May 8th 2023

Shaghayegh Sheikh

This thesis originated from a partnership of **Faculty of Science**, **University of South Bohemia**, and **Institute of Parasitology AS CR**, supporting doctoral studies in the Molecular and Cell Biology and Genetics study programme.

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List of papers and author's contribution

The thesis is based on the following papers (listed chronologically):

I. Bílý T[1], **Sheikh S**[1], Mallet A, Bastin P, Pérez-Morga D, Lukeš J, Hashimi H. Ultrastructural changes of the mitochondrion during the life cycle of *Trypanosoma brucei*. Journal of Eukaryotic Microbiology. 2021 May;68(3):e12846. (IF: 3.88).
[1]: Bílý T and Sheikh S are sharing the first authorship.
Shaghayegh Sheikh made contributions to the project, including preparing figures, analysing data (e.g. computing coefficients of variation), and writing the manuscript.

II. Muñoz-Gómez SA, Cadena LR, Gardiner AT, Leger MM, **Sheikh S**, Connell LB, Bilý T, Kopejtka K, Beatty JT, Koblížek M, Roger AJ. Intracytoplasmic-membrane development in alphaproteobacteria involves the homolog of the mitochondrial crista-developing protein Mic60. Current Biology. 2023 Mar 27;33(6):1099-111. (IF: 10.9)
Shaghayegh Sheikh made contributions to both the early and final stages of the study. In the early stage, she prepared the cultivating media for the bacteria used in the study and conducted preliminary growth measurements. In the later stage, which was included in the published article, she cultivated the bacteria and performed electron microscopy. After analysing the electron microscopy images, she created a figure that included statistical analysis.

III. **Sheikh S**, Pánek T, Gahura O, Týč J, Záhonová K, Lukeš J, Eliáš M, Hashimi H. A novel group of dynamin-related proteins shared by eukaryotes and giant viruses is able to remodel mitochondria from within the matrix. Currently resubmitted to journal of Molecular Biology and Evolution. (IF: 8.8)
Shaghayegh Sheikh made significant contributions to the study, including performing cell cultivation and growth rate measurements, conducting digitonin fractionation, carbonate extraction, and proteinase K protection assays, as well as observing and analysing immunoflourescent and transmission electron microscopy images. In addition, she conducted 3D reconstruction of a cell,

measured membrane potential, analysed data using R Studio, and contributed to writing the manuscript.

IV. Unpublished results: The MICOS complex of trypanosomatids contains two cryptic mitofilin-domain proteins that may shape cristae.
Shaghayegh Sheikh made significant contributions to the study, including contributing to the experimental design, cultivating cells for growth measurement of T. brucei, and analysing electron microscopy images and performing Immunofluorescence assay. She measured the cristae and mitochondrial area, as well as cristae length using microscopy image browser software. She also conducted statistical analysis of all measurements.
In addition, she analysed data from immunolabled E. coli cells.

Co-author agreement

Doc. Hassan Hashimi, Ph.D., the supervisor of this Ph.D. thesis and co-author of papers **I-III**, fully acknowledges the stated contribution of Shaghayegh Sheikh to these manuscripts.

Doc. Hassan Hashimi, PhD.

8 May 2023
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1. Introduction

1.1 Mitochondria, a brief summary in early age of mitochondria research

The discovery of mitochondria is credited to Walther Flemming, who observed the organelle through light microscopy in the mid-1800s, shortly after the discovery of nuclei. The initial observation suggested that organelle appeared to be surrounded by a membrane (Ernster and Schatz, 1981). In 1898, Carl Benda named the organelle 'Mitochondria'. The name originated from the Greek word ''mitos'', which means thread, and ''kondrion'', meaning grain (Benda, 1898).

In 1930, the first steps towards the isolation of mitochondria from guinea-pig liver were taken by Bensley and Hoerr (Bensley and Hoerr, 1934). This later offered a better opportunity to study the biochemical aspects of this organelle. However, the detailed study of mitochondria was limited by the diffraction limit of light microscopy. Decades later, in 1952-1953, Palade and Sjostrand observed the structure of the mitochondrion by better-resolution electron microscopy (Palade, 1952, Sjöstrand, 1953). They proved that the mitochondrion is a double-membrane organelle, with an outer membrane and an inner membrane. Another observation was mitochondrial ridges or 'cristae mitochondriales', which are inner membrane protrusions inward from one side of the mitochondrion with free ends and not reaching to the other side of the mitochondrion, unlike septa (G. E. Palade, 1953, Palade, 1952). Furthermore, it was observed that cristae shapes differed from cell type to cell type. In salivary gland mitochondria, cristae are numerous and more regularly spaced; however, in endothelial cells, cristae are more irregularly spaced (Palade, 1952). The mitochondrial matrix, which is enveloped by the inner membrane of the mitochondrion, was suggested to be structureless and homogenous with different densities. These differences were suggested to depend on the pH of the sample during the fixing procedure (Palade, 1952).

In conclusion, the identification of mitochondria as a distinct organelle within cells was achieved through rigorous scientific investigation and meticulous observation. Subsequent studies revealed the intricate structure and diverse functions of this organelle, further enriching our understanding of its properties and role in cellular metabolism.

1.2 Mitochondria, general properties

Mitochondria are known to be necessary for generating ATP in aerobic eukaryotic cells. However, they are involved in many other processes such as iron-sulphur cluster (Fe-S) assembly, β-oxidation of fatty acids, and apoptosis (Figure 1). I will briefly discuss these three processes.

Fe-S clusters are small, inorganic cofactors that play a pivotal role in a variety of biological processes, including respiration, DNA repair, and gene expression (Beinert, 2000). In mitochondria, Fe-S clusters serve as electron carriers in the electron transport chain, and also play a role in the metabolism of iron and sulphur (Read et al., 2021). Until recently, it was widely believed that Fe-S cluster assembly was a fundamental function of mitochondria in most eukaryotes (Braymer and Lill, 2017). However, a recent study has challenged this view by suggesting that one carbon (1C) metabolism may actually be the essential function of mitochondria, even in species that have undergone significant mitochondrial reduction or loss (Zítek et al., 2022).

β-oxidation is a metabolic process that occurs in the mitochondria (Adeva-Andany et al., 2019). During β-oxidation, fatty acids are broken down to acetyl-CoA, which can then enter the citric acid cycle for additional energy production. The process of β-oxidation involves a series of enzymatic reactions that occur in the mitochondrial matrix, where fatty acids are transported from the cytosol into the mitochondria by a specific transport protein (Houten and Wanders, 2010).

Intrinsic apoptosis is a process of programmed cell death that occurs in response to various cellular stresses such as DNA damage and oxidative stress (Willis and Adams, 2005). Intrinsic apoptosis is initiated by the activation of pro-apoptotic proteins, such as Bcl-2-associated X protein and Bcl-2 homologous antagonist/killer. This activation causes the outer mitochondrial membrane to become permeable (Lindsten et al., 2000). This results in the release of cytochrome *c* from the mitochondria into the cytoplasm, where it forms the apoptosome complex. The apoptosome complex then activates caspase-3, which cleaves and activates other downstream caspases, leading to the degradation of cellular components and ultimately cell death (Lüthi and Martin, 2007, Dix et al., 2008).

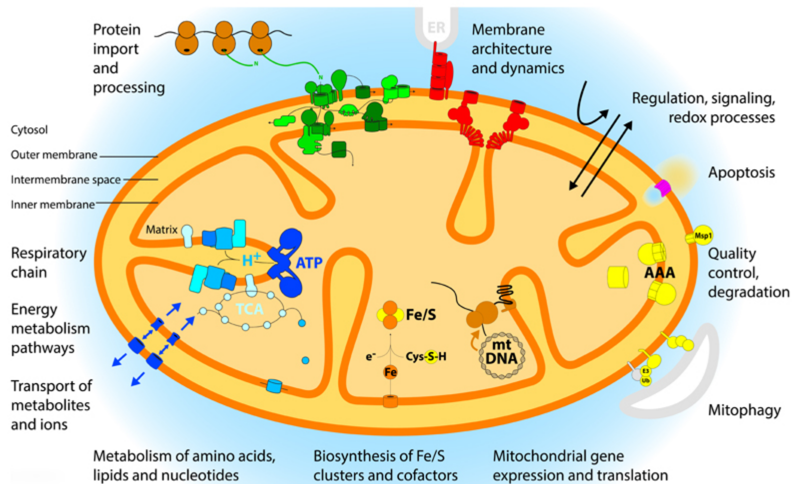

Figure 1. The graphic shows a wide range of functions that have been ascribed to mitochondrial proteins and protein complexes (Pfanner et al., 2019).

1.3 Mitochondria: origin and evolution

Eukaryotic cells show more complexity compared to prokaryotic cells due to the emergence of membranous compartments (Embley and Martin, 2006). In the process of emergence of eukaryotic cells named eukaryogenesis, the origin of mitochondria and acquisition of mitochondria is controversial (Emelyanov, 2003).

The endosymbiotic theory was first proposed by Konstantin Mereschkowsky (Mereschkowsky, 1920). He suggested that mitochondria and chloroplasts were once free-living bacteria that had been engulfed by early eukaryotic cells (Archibald, 2015, Mereschkowsky, 1920). However, this idea was largely ignored until the 1960s, when it was expanded and popularized by Lynn Margulis (Archibald, 2014, Sapp, 1994). She suggested that the similarities between mitochondria and bacteria were the result of a shared evolutionary history (Sagan, 1967). In 1998, Andersson and his colleagues sequenced the genome of Alphaproteobacterium *Rickettsia prowazakii* and compared the amino acid sequences encoded by mitochondrial and bacterial genomes (Andersson et al., 1998). They found that *R. prowazekii* had many similarities to mitochondria, including a circular genome structure, and similar gene content and organization. They

also found that many genes involved in mitochondrial energy metabolism and genetic processes were closely related to bacterial homologs, particularly to the ones from Alphaproteobacteria (Andersson et al., 1998).

Now we know that mitochondria evolved from an alphaproteobacterium that was engulfed by a relative of extant archea (Gray et al., 1999, Eme et al., 2017). However, it is difficult to pinpoint the precise alphaproteobacterial lineage, which is the most closely related to mitochondria (Muñoz-Gómez et al., 2019). The archeon host is thought to be closely related to Lokiarchaeota (Spang et al., 2015).

In addition, it is unclear whether the first mitochondria played a role in the initiation of eukaryogenesis (Hampl et al., 2019). According to mito-early theory, mitochondria existed in the last eukaryotic common ancestor (LECA), and the evolution of the eukaryotic cell was driven by the acquisition and diversification of mitochondrial functions (Lane and Martin, 2010) (Figure 2). The second hypothesis is mito-late, which argues that mitochondria were not present in the earliest eukaryotic cells, but instead was acquired later through endosymbiosis. Also the mito-late theory argues that the host cell of the mitochondrial endosymbiont had some cellular complexity such as the nucleus prior to the endosymbiosis (Ettema, 2016) (Figure 2). Another theory that is in more agreement with mito-late theory is mito-intermediate. Mito-intermediate theory argues that endomembrane compartments were acquired earlier and mostly contributed to eukaryogenesis (Pittis and Gabaldón, 2016). Also, it has been shown that LECA contained bacterial components from origins other than alphaproteobacteria. This implies that the host cell that acquired the mitochondrion was already a complex cell with a genome that contained pathways and processes from various bacterial origins. Therefore, it is likely that mitochondrial symbiosis occurred later in the evolution of eukaryotes, after the development of other eukaryotic features (Pittis and Gabaldón, 2016).

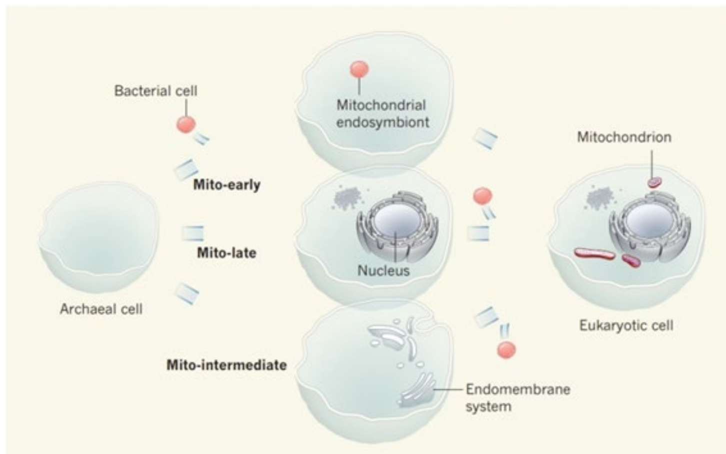

Figure 2. Different theories of mitochondria acquisition (Ettema, 2016).

The evolution of the mitochondrial ancestor from an independent endosymbiotic alphaproteobacterium involved several alterations, including, A) endosymbiont-encoded genes' transfer to the nucleus, B) The presence of β-barrel proteins in the outer membrane of mitochondria.

A) Endosymbiont-encoded genes' transfer to the nucleus. Although the majority of the protein-coding genes from alphaproteobacteria have been lost or migrated into the nuclear genome, a few remain, as do genes for tRNAs and ribosomal RNAs required for organellar translation of the remaining protein-coding genes (Ku et al., 2015). There are some exceptions to this rule. For example, some mitochondrial tRNAs genes are transferred to the nuclear genome in trypanosomatids (Zdeněk Verner et al., 2015). In this case, the nuclear-encoded tRNA are transported back into the mitochondria to carry out their function. Another example of mitochondrial encoded genes are subunits of the electron transport chain complexes (Gray, 2014). Mitochondrial genomes retain certain genes due to two main hypotheses. The first suggests that hydrophobic membrane proteins cannot be efficiently transferred through the hydrophilic channels of the translocator of the outer membrane (TOM) and translocator of the inner membrane (TIM) (Popot and de Vitry, 1990, von Heijne, 1986). The second hypothesis, known as 'colocation of gene and gene product for redox regulation of gene expression' (CoRR), proposes that gene expression can be modulated through redox signalling mechanisms

by having the gene and its corresponding protein product in close proximity within the cell (Allen, 2015). This mechanism provides tight control over gene expression and may prevent unwanted cross-talk with other signalling pathways.

B) The presence of β-barrel proteins in the outer membrane of mitochondria. One of the unique features of mitochondria is the presence of many β-barrel proteins in their outer membrane, which are also found in the outer membrane of alphaproteobacteria (Schulz, 2000). These proteins are of interest because they provide clues about the evolutionary origins of mitochondria. For example, one such protein is the β-barrel insertase Sam50, which is related to the prokaryotic protein BamA (Jiang et al., 2012). Another example is a pore-forming protein called TOM40 which has been modified from an existing β-barrel to facilitate protein import into mitochondria (Gabriel et al., 2003).

1.4 Mitochondria: Morphology and ultrastructure

Mitochondria are unique organelles with two membranes due to their endosymbiotic origin (Cavalier-Smith, 2006). The mitochondrial envelope comprises the outer membrane (OM) and inner membrane (IM), which form a double membrane that separates two sub-compartments, the intermembrane space (IMS) and the matrix. The IMS is the region between the OM and IM, while the matrix is enveloped by the IM. The IM is invaginated towards the matrix, forming structures called cristae (Figure 3). The regions of the IM that run parallel to the OM are referred to as the inner boundary membrane. The crista lumen or intracristal space refers to the inner space of the crista, and the connection of the IM and crista is called the crista junction (Bozelli Jr and Epand, 2020, Sjöstrand, 1953, George E Palade, 1953).

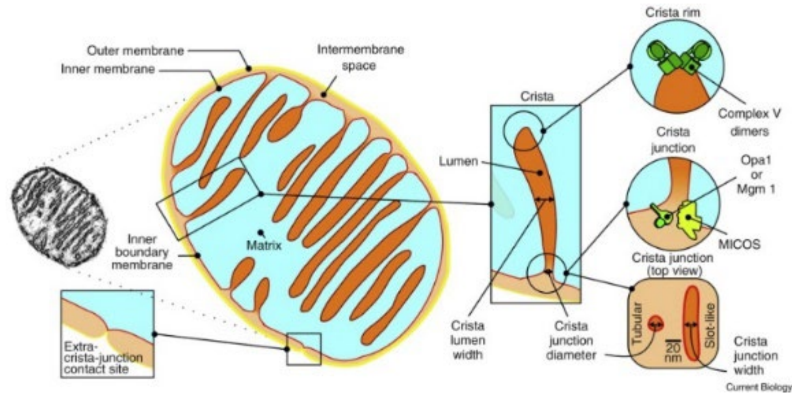

Figure 3. Schematic illustration of mitochondria (Pánek et al., 2020). From left to right, a reconstructed mitochondrion (Perkins et al., 1997) acts as the basis for the main scheme in the middle. The various compartments are marked. The darker orange colour in the crista lumen contrasts with the lighter shade in the IMS, indicating cytochrome *c* enrichment. Similarly, the darker red colour indicates a higher density of respiratory chain complexes I, III, IV, and V in crista membranes. A contact site between the IMs and OMs, specifically one occurring away from a crista junction, and crista from the schema are highlighted in boxes. The crista junctions and rims are further elaborated in circles, which depict the protein complexes which will be discussed in chapter 1.8.

1.5 Mitochondrial protein import

Mitochondrial protein import is a complex process that involves multiple pathways and protein complexes working together to ensure that newly synthesized proteins are properly targeted, translocated across mitochondrial membranes, and folded into their active conformations. Mitochondrial precursor proteins are synthesized in the cytosol with targeting signals that direct the proteins to mitochondria and into the correct mitochondrial compartment (Neupert, 1997).

There are at least five distinct (a-e) pathways that govern the import of proteins into mitochondria, which depend on different targeting signals (Horvath et al., 2015). The majority of these proteins require TOM complex as an entry site (Horvath et al., 2015). The pre-sequence pathway (a) is the most common pathway used by proteins (Vögtle et al., 2009). These preproteins contain a cleavable N-terminal targeting sequence that can create an amphipathic α-helix and is recognized by the TOM complex (Roise et al., 1986, Abe et al., 2000). Tom40, a β-barrel protein is responsible for translocating these preproteins across the OM (Hill et al., 1998, Bayrhuber et al., 2008). Once the preproteins reach the IMS, they are identified by the translocase of the inner membrane

(TIM) 23 complex and then transported to the matrix via the pre-sequence translocase-associated motor (PAM) with the assistance of ATP, or they are laterally released to the IM (Mokranjac and Neupert, 2010, Chacinska et al., 2005). In either case, the mitochondrial processing peptidase (MPP), located in the matrix, cleaves the N-terminal targeting sequence to produce the mature proteins (Hawlitschek et al., 1988, Taylor et al., 2001). However, in all other pathways, internal targeting signals remain intact and are not cleaved following protein import.

Proteins located within the IM that have multiple transmembrane domains, mostly carrier proteins, are brought into the mitochondria via the carrier pathway (b). In contrast to the pathway mentioned above, once these carrier proteins enter the mitochondria via the TOM complex, small TIM chaperones found in the IMS transport them to the TIM22 complex. Here, the proteins are inserted into the membrane in a way that is dependent on the membrane potential (Sirrenberg et al., 1996, Koehler et al., 1998, Rehling et al., 2004). On the other hand, numerous IMS proteins that have a specific cysteine motif are imported through the TOM complex and the mitochondrial intermembrane space import and assembly (MIA) pathway (c). Once the proteins are oxidized and form disulfide bonds with Mia40, they are released to the IMS (Chacinska et al., 2004, Herrmann and Riemer, 2012).

The OM has two main pathways for inserting proteins. The first pathway involves importing and inserting β-barrel proteins using the TOM complex, small TIM IMS chaperones, and the SAM complex (d) (Wiedemann et al., 2003, Höhr et al., 2015). Other OM proteins, such as receptors that have one or more transmembrane helices, are inserted using the mitochondrial import (MIM) complex (e), which involves Mim1, Mim2, and the TOM complex (Becker et al., 2008, Hulett et al., 2008, Dimmer et al., 2012).

1.6 Respiratory chain complexes: an overview

The electron transport chain (ETC) is a series of protein complexes situated in the IM membrane of eukaryotic cells and the cytoplasmic membrane of prokaryotic cells (Cecchini, 2003). The ETC complexes are crucial in the mechanism of oxidative phosphorylation, which produces ATP, the primary energy source for the cell. These complexes, comprising I to IV, are incorporated into the cristae and are encoded by genes in both the mitochondria and the nucleus. Moreover, complex V is situated on the cristae rims (Dudkina et al., 2010).

Complex I is an L-shaped structure consisting of several subunits, including flavin mononucleotide (FMN) and several Fe-S clusters (Lennarz and Lane, 2013). It catalyses the transfer of electrons from NADH to coenzyme Q, pumping protons from the mitochondrial matrix to the IMS and generating an electrochemical gradient used by ATP synthase to produce ATP (Hatefi, 1985) (Figure 4).

Complex II also known as succinate dehydrogenase plays a critical role in both the citric acid cycle and the ETC of aerobic respiration (Cecchini, 2003). In human, Complex II is composed of four protein subunits encoded by nuclear genes (Sun et al., 2005). Complex II contains two hydrophilic subunits, SDHA and SDHB, located on the matrix side of the IM. These subunits hold the binding site for succinate, three Fe-S clusters, and a flavoprotein bound to a FAD cofactor (Bezawork-Geleta et al., 2017). In addition, the other two hydrophobic subunits, SDHC and SDHD, serve as anchors for the complex to the IM. In the citric acid cycle, Complex II catalyzes the oxidation of succinate to fumarate while simultaneously reducing FAD to FADH2. The FADH2 produced by Complex II then donates its electrons to coenzyme Q (Cecchini, 2003) (Figure 4).

Complex III or cytochrome *c* reductase receives electrons from coenzyme Q and transfers them to cytochrome *c*, a soluble protein that acts as an electron carrier between Complex III and Complex IV. The transfer of electrons in Complex III is coupled with the transport of protons across the IM and contributes directly to the generation of the membrane potential (Nicholls, 2013). Complex III consists of several subunits, including cytochrome b, cytochrome c1, and the Fe-S protein Rieske (Saraste, 1999) (Figure 4).

Complex IV or cytochrome *c* oxidase (COX), receives electrons from cytochrome *c* and uses them to reduce molecular oxygen (O_2) to water (H_2O). This reaction is coupled with the transport of protons across the IM, generating a proton gradient that powers the synthesis of ATP through oxidative phosphorylation (Cogliati et al., 2018, Capaldi, 1990) (Figure 4).

Complex V or F_0F_1-ATP synthase is composed of two main domains, the F_1 and F_0, which are connected by a central stalk (Pedersen and Amzel, 1993, Boyer, 1997). The F_1 is located on the matrix side of the mitochondrial inner membrane and contains the catalytic subunits responsible for ATP synthesis, while the F_0 is embedded in the membrane and contains a proton channel that drives the rotation of a rotor-like structure. As protons flow through the F_0 channel, they cause the rotor to rotate, leading

to a conformational change in the catalytic subunits of the F_1 portion, which ultimately leads to the synthesis of ATP from ADP and inorganic phosphate (forward mode) (Figure 4). In the mitochondria, ATP synthase forms a dimer which will be discussed in details in chapter 1.8.

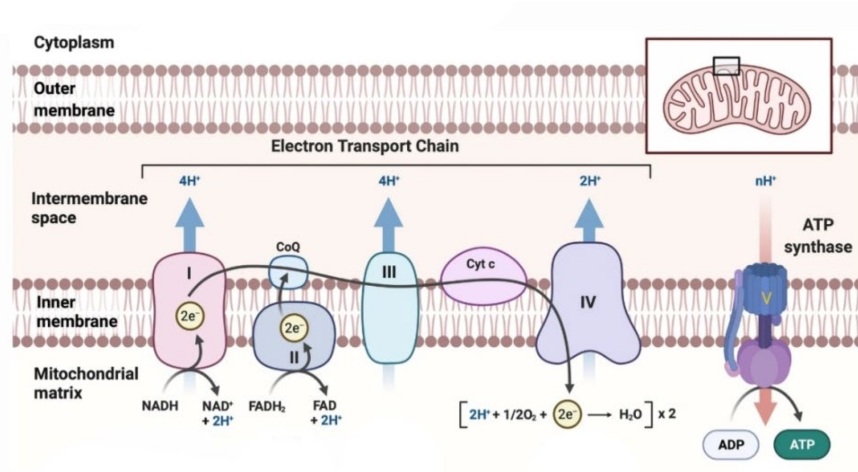

Figure 4. A schematic illustration of inner membrane of mitochondria comprising oxidative phosphorylation complexes (Wu et al., 2022).

1.6.1 Respiratory chain complexes in *Trypanosoma brucei*

To better understand the characteristics of the respiratory chain complexes in *Trypanosoma brucei*, it is necessary to provide a brief introduction to the model organism studied in this doctoral thesis - *T. brucei*. The life cycle of *T. brucei* is characterized by a complex interplay between two hosts, a tsetse fly and a mammal (Vickerman, 1985). This parasitic organism resides in the bloodstream of an infected mammal as the bloodstream form (BSF). Following ingestion by the tsetse fly, the parasite undergoes a series of complex differentiation events. The stage which is known as procyclic form (PCF) resides the midgut of the insect vector (Matthews, 2005). For further details, please refer to the chapter on the *T. brucei* life cycle 1.10.1.

The biological significance of complex I is still unclear in *Trypanosoma brucei* as it does not contribute to the electron transfer from NADH or proton translocation in either PCF

or BSF (Surve et al., 2012). However, Complex I is necessary for the development of the parasite through its life cycle stages (Doleželová et al., 2020). It has been proposed that Complex I may involve in the production of reactive oxygen species and the modulation of the parasite's response to oxidative stress (Duarte and Tomás, 2014). Similar to Complex I, Complex II does not play a vital role in BSF but it is essential for the intermediate stages of development in insect (Alkhaldi et al., 2016).

Besides the canonical ETC complexes, *T. brucei* possesses two other protein complexes that are involved in the electron entry and exit from the ETC, namely, type II alternative dehydrogenase (NDH2) and Trypanosoma Alternative Oxidase (TAO). NDH2 is functionally similar to complex I, except that it does not pump protons into the IMS (Fang and Beattie, 2002). Our knowledge regarding NDH2 is narrow and this protein needs more investigation. TAO has a function similar to complex IV, as it catalyses the reduction of oxygen to water, yet it does not pump protons to the IMS. The viability of the BSF of *T. brucei* is strongly reliant on the presence of TAO, as TAO facilitates the production of ATP within the parasite (Chaudhuri et al., 2006). Complexes III and IV activities are completely absent in the long slender and short stumpy bloodstream forms of the parasite. Individual Complex III and IV subunit expression is triggered early in the differentiation to PCF, possibly even when the stumpy forms first encounter the environment of the fly's midgut following a blood meal (Naguleswaran et al., 2021). I will elaborate on life cycle stages of *T. brucei* in chapter 1.10.1.

In PCF the membrane potential is generated by complexes III and IV, and the F_0F_1-ATP synthase serves the purpose of synthesising ATP. However, in the absence of active complexes III and IV in the BSF, the F_0F_1-ATP synthase reverses its function and extrudes protons into the mitochondrial IMS, thereby generating membrane potential at the expense of ATP hydrolysis (Schnaufer et al., 2005, Brown et al., 2006, Nolan and Voorheis, 1992).

1.7 Cristae diversity

Mitochondrial cristae are structures found in the mitochondria of aerobic eukaryotes, and they exhibit diverse morphologies (Joubert and Puff, 2021). Cristae junction morphotypes can be categorized into two major classes: tubular and slot-like. While the tubular shape is more commonly observed among eukaryotes, the slot-like shape is only observed in potato plants and three different species of ascomycete fungi (Pánek et al.,

2020) (Figure 5). However, the difference in the width of the slot-like and diameter of tubular cristae junctions is not significant (Pánek et al., 2020).

The morphology of cristae can be further sub-categorized into two major classes: the tubulo-vesicular class and the flat class. The tubulo-vesicular class includes tubular, vesicular, and irregular tubule-vesicular morphologies, while the flat class encompasses both discoidal and lamellar cristae (Figure 5). Recent research, based on available datasets and published transmission electron microscopy micrographs, shows that the tubulo-vesicular class is the most abundant, with tubular-shaped cristae being the most prevalent among the 226 species examined (Pánek et al., 2020) (Figure 5).

Interestingly, different sub-categories of cristae are observed in opisthokonts (e. g, yeast and humans), in contrast with SAR (Stramenopiles-Alveolates-Rhizaria) which encompasses multi and unicellular species that exhibit exclusively tubulo-vesicular morphotype. In Amoebozoa, which contains diverse amoebae such as the slime mold *Dictyostelium discoideum*, tubulo-vesicular cristae are reported exclusively. In Euglenozoa from the Discoba clade, discoidal cristae are reported in *T. brucei*, and lamellar cristae are found in the majority of diplonemids (Pánek et al., 2020, Tashyreva et al., 2018). *T. brucei*, the model organism I studied belongs to this group, for more information please go to chapter 1.10.

Currently, the physiology role of cristae is not completely understood. Two potential explanations have been proposed to explain why cristae form. The first hypothesis suggests that the intracristal compartment acts as a reservoir for protons, which are used for ATP synthesis. This hypothesis proposes that the increased surface area of the IM, caused by cristae folding, creates more ATP synthase complexes, which use the proton gradient generated by the ETC to produce ATP. Protons accumulate in the intracristal space, providing a local concentration of protons for ATP synthesis (Mannella et al., 1994, Wolf et al., 2019). The second theory suggests that the ETC and ATP synthase are enriched in these membranes to position the oxidative phosphorylation complexes closely together. This allows the producers and consumers of the proton motive force to be kinetically coupled through a process known as lateral membrane proton diffusion (Mannella, 2020, Toth et al., 2020).

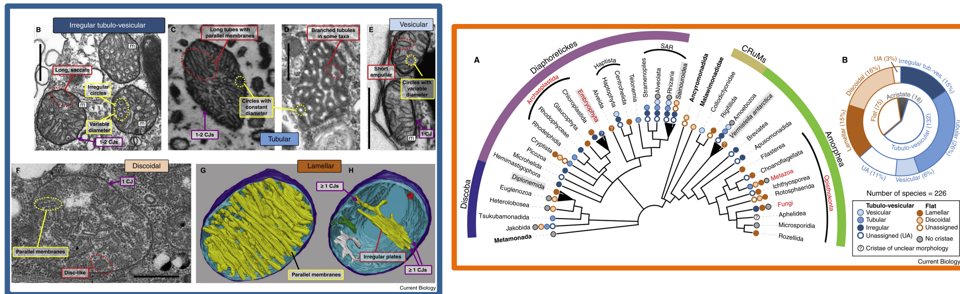

Figure 5. The dark blue box shows the variety of cristae morphotypes (Pánek et al., 2020)**.** B-F are images from transmission electron microscopy, exhibiting a range of cristae morphotypes. Yellow boxes highlight typical characteristics of cristae in cross-section; red boxes highlight typical characteristics of cristae in longitudinal section. B: irregular tubulo-vesicular cristae of a male gamete of the brown alga *Ectocarpus siliculosus* (Maier, 1997). C: tubular cristae of the ciliate *Spirostomum ambiguum* (Finley et al., 1964). D: tubular cristae of the amoebozoan *Flamella arnhemensis* (Kudryavtsev et al., 2009). E: Vesicular cristae of the alveolate *Parvilucifera sinerae (Garcés and Hoppenrath, 2010).* F: Discoidal cristae of *Stephanopogon pattersoni* (Lee et al., 2014). G: The entire model showing all cristae in yellow, inner boundary membrane in light blue, and the OM in dark blue. H: OM, inner boundary membrane and four representative cristae in different colours. G and H are the three dimensional model of chick cerebellum which is generated from the tomograms (Frey and Mannella, 2000). **The orang box exhibits the distribution of crista morphotypes in eukaryotic tree of life** (Pánek et al., 2020). A: distribution of morphotypes on a phylogenetic tree of eukaryotes. B: total abundance of crista morphotypes in eukaryotes.

1.7.1 The mitochondrial contact site and cristae organization system (MICOS), an overview

The MICOS complex is located at the cristae junctions and plays a vital role in the cristae junction development and maintenance of contact sites between the OM and IM. The latter is mediated by MICOS interacting with OM proteins such as TOM40 and Sam50 (Ott et al., 2012, Körner et al., 2012, Zerbes et al., 2016). In some organisms, MICOS is involved in protein import and maintaining the contact site for lipid trafficking between OM and IM (M. Michaud et al., 2016, Aaltonen et al., 2016, Wollweber et al., 2017, Kaurov et al., 2018).

Initial studies upon discovery of this complex by different scientists led to different nomenclature (K. von der Malsburg et al., 2011, S. Hoppins et al., 2011, Max Harner et al., 2011, Alkhaja et al., 2012). However, the nomenclature has been harmonized for the MICOS complex (Pfanner et al., 2014). MICOS complex is composed of six subunits in fungi (M. Harner et al., 2011), and seven subunits in mammals (Kozjak-Pavlovic, 2017,

Darshi et al., 2011) (Figure 6). Recently, the MICOS complex has been characterized outside of opisthokonts, in the Discoba clade member *T. brucei* (Kaurov et al., 2018), which will be discussed in chapter 1.7.1.4. The MICOS complex in yeast and human are categorized into two main subcomplexes that are centred on Mic10 and Mic60. These subunits are highly conserved and found in every eukaryotic lineage studied in this respect (Sergio A Muñoz-Gómez et al., 2015, M. A. Huynen et al., 2016). Mic60 and Mic10 are core subunits of the MICOS complex, as their absence causes the complex to dissociate, resulting in a near-complete loss of crista junctions and aberrant changes in mitochondrial morphology (Karina von der Malsburg et al., 2011, Max Harner et al., 2011, Suzanne Hoppins et al., 2011, G. B. John et al., 2005, Alkhaja et al., 2012, Stephan et al., 2020). On the other hand, if other MICOS subunits are absent, the impact is milder, and the mitochondria exhibit intermediate characteristics.

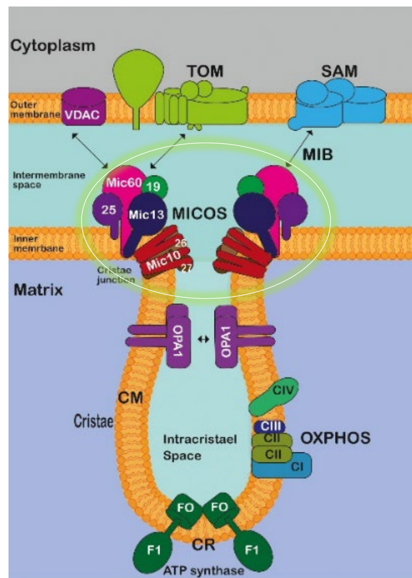

Figure 6. MICOS in mammalian cell (Mukherjee et al., 2021).

1.7.1.1 Mic60 is an ancient conserved protein

Mic60 is the largest subunit of MICOS (Karina von der Malsburg et al., 2011, Mukherjee et al., 2021). Prior to the discovery of the MICOS complex, Mic60 was observed to exhibit significant enrichment at the crista junctions, and was called mitofilin/Fcj1 (Regina Rabl et al., 2009, G. B. John et al., 2005). Structurally; Mic60 is targeted to the mitochondria by the N-terminal targeting sequence and is anchored in the IM by its transmembrane domain. Mic60 possesses a central coiled-coil domain, suggesting the protein-protein interaction platform (Gieffers et al., 1997) (Figure 7). *In vitro* and *in vivo* analysis show that the intermembrane (mitofilin) domain of Mic60 has lipid-binding properties and can induce high membrane curvatures even when the transmembrane domain is absent (Hessenberger et al., 2017, Tarasenko et al., 2017). A recent study shows that thermostable-yeast Mic60 forms a bow tie-shaped tetrameric assembly with help of the central coiled-coil domain. Mic19 promotes tetramerization into an active MICOS subcomplex. Therefore, this heterotetrameric assembly tailors a convex-shaped dimeric membrane-binding module to bind to the highly curved cristae junction membrane (Figure 7) (Bock-Bierbaum et al., 2022).

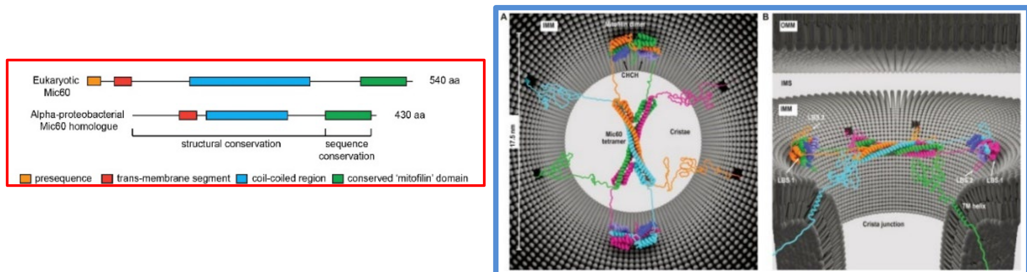

Figure 7. Red box shows the schematic domain architecture of Mic60, which is conserved in both eukaryotes and prokaryotes (S. A. Muñoz-Gómez et al., 2015). **Blue box exhibits model of Mic60-Mic19 function at cristae junctions (Bock-Bierbaum et al., 2022).** A: Top view. B: side view showing the proposed architecture of the Mic60-Mic19 complex at cristae junctions. Each monomer is labelled with a different colour.

In mammalian cells, Mic19 interacts directly with Sam50 and Mic60 to form Sam50–Mic19–Mic60 axis, which together builds a supercomplex called mitochondrial intermembrane space bridging complex (MIB) (M. A. Huynen et al., 2016, Tang et al., 2020). It has been shown that Mic60 and Mic19 interact with Sam50 to maintain cristae

structure (Ding et al., 2015). In line with that, depletion of Mic60 or Mic19 causes destabilization of Sam50 which is closely involved in cristae organization (Sastri et al., 2017).

The knockdown or knockout of *mic60* in mammalian cells results in the loss of cristae junctions and their attachment to the OM and altering the overall mitochondrial IM shape to stacks of elongated cristae (Stephan et al., 2020, Li et al., 2016). The loss of cristae junctions and the organization of cristae membrane into membrane stacks inside the matrix are likewise caused by the deletion of the *mic60* gene in yeast (G. B. John et al., 2005).

Homologues of Mic60 have been found in alphaproteobacteria and share a conserved domain architecture (transmembrane domain, coiled-coil domain and mitofilin domain), suggesting a conserved function (Sergio A Muñoz-Gómez et al., 2015). In line with this, some alphaproteobacteria (*e.g.* purple bacteria) form intracytoplasmic membranes (ICM) which are bioenergetic sub-compartments. Recently, the role of Mic60 in ICM has been confirmed experimentally by disruption or overexpression of alphaMic60 in *Rhodobacter sphaeroides* and *Rhodopseudomonas palustris* (Muñoz-Gómez et al., 2023). Furthermore, it has been demonstrated that Mic60 in alphaproteobacteria has a physical interaction with BamA, the homolog of Sam50. This interaction possibly has a role in maintaining the contact site at intracytoplasmic junctions, between bacterial outer envelope and cytoplasmic membrane (Figure 8) (Muñoz-Gómez et al., 2023).

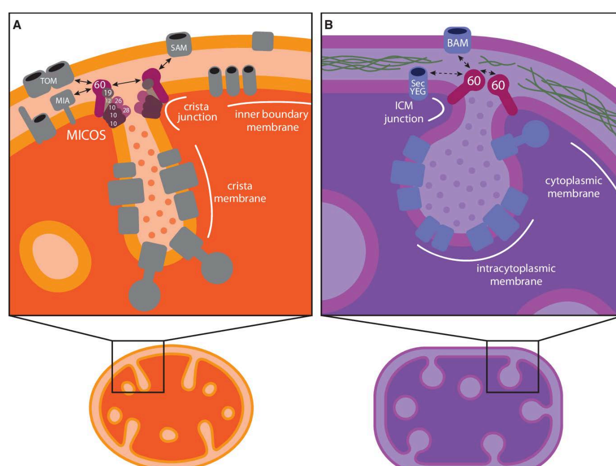

Figure 8. Alphaproteobacteria and mitochondria both likely use MICOS for the same purpose (Muñoz-Gomez et al., 2017). A: To maintain and stabilize cristae, MICOS develops contact sites and cristae junctions in the mitochondrion of *Saccharomyces cerevisiae*. B: AlphaMic60 is considered to play a role in the formation of ICM junctions and contact sites in alphaproteobacteria in order to stabilize bioenergetic ICMs.

1.7.1.2 Mic10 and other MICOS subunits

MIC10/MINOS1 is another core subunit of the MICOS complex (Mukherjee et al., 2021). Mic10 is composed of two alpha-helices transmembrane domains anchored in the IM of mitochondria and are connected by a short positively charged loop (Bohnert et al., 2015). Both termini protrude to the IMS (Figure 9). A highly conserved GxGxGxG motif of Mic10 regulates the Mic10 oligomerization which is necessary for membrane bending and promoting the formation of the crista junctions (Barbot et al., 2015, Bohnert et al., 2015, Kaurov et al., 2018).

In HeLa cells, Mic10 knockout resulted in a reduced number of cristae junctions and cristae shape alteration from lamellar to tubular (Stephan et al., 2020). Hence, Mic10 is most likely responsible for cristae development. The *in vitro* analysis of isolated Mic10 from yeast demonstrated that Mic10 has remodelled the spherical lipid bi-layer of liposomes into a tubular shape (Barbot et al., 2015). The interesting point is the diameters of Mic10-generated tubules were highly similar to that of cristae junctions, having a 10 and 30 nm in width.

Recently, studies have shown that Mic10 has functions in two populations. 1) as a core subunit of the MICOS complex which has been discussed earlier, and 2) as the partner of the ATP synthase (Rampelt et al., 2022, Cadena et al., 2021). In yeast, Mic10 is in

direct association with the ATP synthase. A study investigated the possible role of Mic10 in mitochondrial physiology by creating a fusion protein of Atp21 (subunit *e* of ATP synthase which has a role in dimerization and oligomerization) and Mic10 (Rampelt et al., 2022). It was found that Mic10 was required for optimal mitochondrial respiration and growth under conditions of high energy demand (Rampelt et al., 2022).

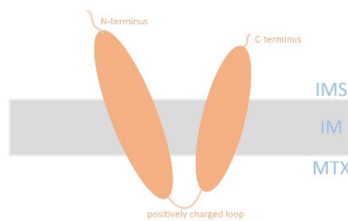

Figure 9. Schematic illustration of Mic10 topology in mitochondrial membrane.

1.7.1.3 MICOS, the subcomplex architecture and properties

As mentioned earlier, the MICOS complex in yeast and human is divided into two subcomplexes around Mic10 and Mic60 core subunits. Yeast Mic60-subcomplex contains Mic60 and Mic19 which contribute to the formation of contact sites by interaction with Sam50 and TOM (Körner et al., 2012, Zerbes et al., 2012). The Mic10 subcomplex on the other hand consists of Mic10, Mic12 (QIL in metazoa), Mic26 and Mic27 and appears to be mainly mediating cristae formation (Schorr and van der Laan, 2018). Mic19 has a paralog in vertebrates, known as Mic25. The loss of Mic19 or Mic25 results in disassembly and reduction of the Mic60 sub-complex components and irregular cristae architecture (Darshi et al., 2011, An et al., 2012, Tang et al., 2020, Ding et al., 2015). Mic12/13 (also known as QIL1) connects Mic60 and Mic10 subcomplexes (Guarani et al., 2015).

In the Mic10 subcomplex, Mic26 and Mic27 are paralogs derived from independent gene duplications in yeast and vertebrates, respectively (Martijn A Huynen et al., 2016, S. A. Muñoz-Gómez et al., 2015). Mic26 and Mic27 belong to the Apolipoprotein O family and they play a role in stabilizing Mic10's oligomeric state as well as the dynamics of the Mic10 sub-complex assembly (Koob et al., 2015, Weber et al., 2013). The yeast Mic27 was discovered to have a stabilizing impact on Mic10 oligomers. However, the oligomerization of Mic10 takes place independently of Mic27 (Bohnert et al., 2015,

Zerbes et al., 2016). Moreover, Mic26 and Mic27 in yeast appear to have opposing roles, as Mic26 destabilizes Mic10 oligomers (Rampelt et al., 2018). The Mic10 subcomplex necessitates cardiolipin to maintain its stability (Rampelt et al., 2018). Cardiolipin binds to human Mic27, and deleting Mic26 and Mic27 leads to reduced levels of cardiolipin in mitochondria, as demonstrated by studies on both yeast and humans (Weber et al., 2013, Anand et al., 2020).

The MICOS complex was discovered over a decade ago and since then there were many studies performed to understand the mechanism of this complex. While it is known that MICOS serves as a protein interaction hub, the molecular details of these interactions, including their stability, binding motifs, and kinetics, are not fully understood. To investigate these biophysical parameters, *in vitro* reconstitution assays are often necessary but challenging to perform, particularly when multiple integral membrane proteins are involved. Nevertheless, to comprehend the hierarchies of interaction cascades and cooperative effects of multiple proteins and complexes, these difficult and laborious approaches are essential.

1.7.1.4 MICOS in *T. brucei*

Extensive investigations of the MICOS complex have been conducted in opisthokonts such as *Saccharomyces cerevisiae*, *Caenorhabditis elegans*, and *Homo sapiens* (Ji Young Mun et al., 2010, Head et al., 2011). However, this complex has also been studied in non-opisthokonts, including *Arabidopsis thaliana* and the unicellular parasite *T. brucei* (Kaurov et al., 2018, M. Michaud et al., 2016). The MICOS complex was thoroughly investigated in *T. brucei* (Kaurov et al., 2018). However, the study on *A. thaliana* was focused on Mic60 in which it was shown that Mic60 mediates contact site and involves in lipid trafficking (Morgane Michaud et al., 2016).

Except for Mic10, other MICOS homologs were not found in *T. brucei*. Mic10 in *T. brucei* is represented by two paralogues, TbMic10-1 and TbMic10-2. The former showed higher degree of similarity to conventional yeast Mic10 in terms of glycine residue amounts within one of the predicted transmembrane domains (Kaurov et al., 2018). In addition, Mic10-1 mediates the crosstalk between MICOS and ATP synthase (Cadena et al., 2021). This function of Mic10-1 is similar to its homologue in yeast (Cadena et al., 2021). Trypanosomal MICOS is divided into two subcomplexes: the membrane integral subcomplex containing Mic10-1/2-Mic16-Mic60 and the peripheral soluble

intermembrane space complex Mic32-Mic34-Mic17-Mic40-Mic20 (Figure 10) (Eichenberger et al., 2019). Surprisingly, the homolog of Mic60 found in eukaryotes and alphaproteobacteria is missing in *T. brucei*. However, a putative Mic60 which is composed of a conserved coil coiled domain, transmembrane domain, and mitochondrial targeting sequence (MTS) but lacking a mitofilin domain had been reported (Kaurov et al., 2018, Sergio A Muñoz-Gómez et al., 2015, Hashimi, 2019). It is possible that one of the soluble subunits possessing the mitofilin domain may serve as a Mic60 replacement. I will discuss this possibility in chapter of unpublished results, chapter 2.2.

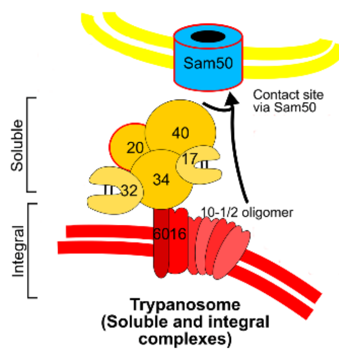

Figure 10. MICOS complex in *T. brucei* (Hashimi, 2019)

1.8 F_0F_1-ATP synthase dimerization

All mitochondrial ATP synthases occurs as dimers unlike in bacteria and chloroplasts (Dudkina et al., 2005, Arnold et al., 1998, Kühlbrandt, 2019). Dimerization of complex V induces positive curvature at cristae rims and is ultimately involved in shaping the different types of cristae (Kühlbrandt, 2019, Pánek et al., 2020). Thus, not only it plays a role in ATP synthesis but also in shaping cristae. Thus, the widespread presence of dimers implies that the latter role is already stablished in LECA (Davies et al., 2012, Dudkina et al., 2006, Sinha and Wideman, 2022, Gahura et al., 2022).

Solving the structure of ATP synthase dimers by cryogenic electron microscopy methods was accomplished in different organisms such as mammals (Gu et al., 2019, Pinke et al., 2020, Spikes et al., 2020), *S. cerevisiae* (Guo et al., 2017), *Polytomella* (Murphy et al.,

2019) *Euglena* (Mühleip et al., 2019), *Tetrahymena* (Flygaard et al., 2020) and *Toxoplasma* (Mühleip et al., 2021). It is evident that the dimers take on various shapes, which could have different effects on the morphology of cristae. The majority of structures are V-shaped dimers, meaning the angle originating from the dimer interface at the F_0 moiety pries apart at each F_1 head (Type I, II, IV). However, there are exceptions like Type III dimers which exhibit as U-shaped, with each monomer erected in parallel to each other (Figure 11) (Pánek et al., 2020, Kühlbrandt, 2019).

Type I: Opisthokonts are known for their Type I dimers, which have an average angle of ~86° (Guo et al., 2017, Hahn et al., 2016). The dimers typically form loosely packed rows at the tightly curved edges of lamellar cristae (Davies et al., 2011) (Figure 11).

Type II: This type is characterized by chlorophyll-less green alga *Polytomella sp*. and its close relative *Chlamydomonas reinhardtii*. A proteomics analysis has uncovered that Polytomella harbours additional subunits associated with ATP synthase, which potentially comprise the constituent components of both the peripheral stalk and the dimerization interface (Vázquez-Acevedo et al., 2016). The angle between central stalks is about 56°, The dimer rows are uneven, which may be associated with irregular tubulo-vesicular cristae (Figure 11) (van Lis et al., 2005, Vázquez-Acevedo et al., 2016, Kühlbrandt, 2019, Pánek et al., 2020).

Type III: Type III dimers are predominantly found in ciliates such as *Paramecium* sp. and *Tetrahymena* sp. (Flygaard et al., 2020, Mühleip et al., 2016). Recently, *Toxoplasma* sp. dimer has also been included in this category (Gahura et al., 2021, Sinha and Wideman, 2022, Salunke et al., 2018). The type III dimers have laterally displaced peripheral stalks and a wide area of protein densities connecting the two monomers on both sides of the membrane at the dimer interface. As a result, there is no bending of the membrane at the dimer interface, which leads to the U-shape of type III dimers instead of the V-shape. The assembly of type III dimers into rows generates membrane curvature, resulting in the formation of helical tubular cristae (Flygaard et al., 2020, Mühleip et al., 2016). Interestingly, although the overall structure of the apicomplexan dimers is similar to that of ciliates, they do not form rows but instead form pentagonal pyramids with icosahedral symmetry at the rims of their bulbous cristae (Mühleip et al., 2021) (Figure 11) .

Type IV: Mitochondrial ATP synthase dimers of *E. gracilis* and *T. brucei* are characterized as type IV. The euglenid and kinetoplastid structures have smaller dimer angles of 45°

and 60°, respectively, with novel subunits accumulating around the periphery of both the F_1 and F_0 sectors (Figure 11). Type IV dimers consist of tightly packed ladder-like assemblies composed of three to six dimers, which generate a discoidal cristae morphology. The rows of these dimers interlock with each other by the interdigitation of the two F_1 domains from one dimer with the monomers of the adjacent dimer. This results in the closest neighbouring monomer belonging to different dimers, both across and along the row (Mühleip et al., 2017).

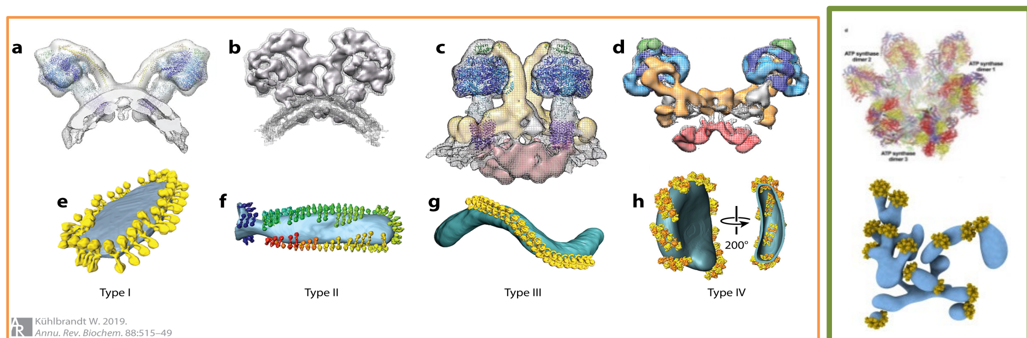

Figure 11. Orange box shows Cryo-ET of mitochondrial ATP synthase dimers (Kühlbrandt, 2019). a–d: the subtomogram averages, e-h: 3D volumes of cristae vesicles: *Saccharomyces cerevisiae* (a, e), *Polytomella sp*. (b, f), *Paramecium tetraurelia* (c, g), and *Euglena gracilis* (d, h). different subunits are drawn in different colours. Dimer rows are yellow in panels e and g. Panel f's short dimer ribbons are rainbow coloured. At the ridges of disk-like cristae, alternating interdigitated dimers (yellow and orange) form short ribbons in panel h. The membrane is a light blue colour. **Green box shows pentagonal pyramids of ATP synthase in *T.gondii*** (Mühleip et al., 2021). The upper image is the atomic model of the hexamer with different coloured subunits in *T. gondii*. The lower image shows the segmentation of mitochondrial membranes in blue with the dimers in yellow.

1.9 Mgm1/Opa1

Proteins that are found in opisthokonts and they likely have a fundamental role in cristae architecture are Mitochondrial Genome Maintenance (Mgm1) in yeast and its mammalian presumed-homolog Optic Atrophy 1 (Opa1). Mgm1 and Opa1 belong to the dynamin-related GTPase family, which are involved in membrane fusion and fission (Wong et al., 2003, Cipolat et al., 2004, Meeusen et al., 2006, Westermann, 2010).

Structurally, Opa1 consists of the guanosine triphosphate (GTPase) domain and at the N-terminus, it possesses a MTS and transmembrane domain (Figure 12). The protein is

targeted to mitochondria by its MTS and is cleaved by a MPP to give rise to the long isoforms of Opa1 (L-Opa1). L-Opa1 is cleaved by two metalloproteases cleaving different sites to form S-Opa1: the ATP-dependent protease yeast mitochondrial DNA escape 1-like (YME1L) and the zinc metalloprotease overlapping with *m*-AAA protease (OMA1) (Käser et al., 2003, Griparic et al., 2007). The long isoform is anchored to the IM and the short isoform is peripherally attached to the IM or diffuses to the IMS as it lacks a transmembrane domain (Herlan et al., 2004, Song et al., 2007, Ishihara et al., 2006, Ehses et al., 2009, Head et al., 2009, Anand et al., 2014).

Opa1 have been shown to play a role in maintaining the integrity of the mitochondrial cristae (Frezza et al., 2006, Sara Cogliati et al., 2013). Opa1 also regulates the formation of respiratory chain supercomplexes, which are necessary for proper electron transport and the prevention of electron leakage (Sara Cogliati et al., 2013, Varanita et al., 2015). Research has demonstrated that the oligomerization of Opa1 at the cristae junctions plays a key role in controlling the width of both the cristae junctions and the cristae lumen. This is critical for the induction of pro-apoptotic-dependent cytochrome *c* release upon Opa1 cleavage (Anand et al., 2014, Kasahara and Scorrano, 2014).

Opa1 interacts with protein complexes such as ATP synthase and MICOS. It has been shown that the Opa1 levels correlated with ATP synthase oligomerization. In fact, upon deletion of Opa1, ATP synthase dimers and monomers were less abundant and ATP synthase activity reduced, suggesting that the cristae formation can affect on row formation of ATP synthase (Quintana-Cabrera et al., 2018). The role of Opa1 in cristae formation and in the maintenance of the mitochondrial architecture supported by the observation of septa formation in knockdown models of Opa1 and the association of Opa1 with Mic60 (Stephan et al., 2020). In another study they showed that Opa1 is epistatic to Mic60, meaning that Opa1 acts upstream of Mic60 in the regulation of cristae morphology (Glytsou et al., 2016).

Studies have shown that Mgm1 is processed by a rhomboid-type protease, Pcp1, which cleaves the protein at a single site (Herlan et al., 2003). This cleavage results in two distinct isoforms, namely the long N-terminally anchored isoform to the IM (L-Mgm1) and a shorter soluble form (S-Mgm1) (Herlan et al., 2003, Esser et al., 2002). The S-Mgm1 stalk forms tetramers and helical assemblies inside the membrane tubules *in vitro*, which resemble the shape and dimension of cristae junctions (Faelber et al., 2019). The stable membrane association of S-Mgm1 with L-Mgm1, which has a transmembrane domain, may promote membrane curvature (Yan et al., 2020).

Studies have shown that S-Opa1 and S-Mgm1 have similar roles. In the case of S-Opa1, it has been shown to form tubules *in vitro* when added to liposomes containing cardiolipin, which is a unique phospholipid found in the IM. This suggests that S-Opa1 may play a role in shaping the IM by inducing membrane curvature and tubulation (Ban et al., 2010). Similarly, S-Mgm1 actively binds to cardiolipin and phosphatidylserine, which are negatively charged non-bilayer-forming lipids enriched in the IM (Rujiviphat et al., 2015).

The relationship between Mgm1 and Opa1 maybe not be as clear as supposed to be for the members of the dynamin-related family. They are cleaved by different mitochondrial proteases at different sites (Herlan et al., 2003) . In line with this, a study shows that when Opa1 exogenously is expressed in yeast, it is cleaved by *m*- AAA and not by rhomboid protease (Duvezin-Caubet et al., 2007). It is suggesting that Opa1 and Mgm1 have independently evolved from holozoan and fungal dynamin, respectively and that do not share a common ancestor (S. A. Muñoz-Gómez et al., 2015). This topic is explained in more depth in chapter 2.1.3 (Sheikh et al., 2023).

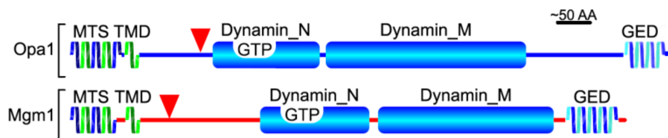

Figure 12. Domain architectures of the mitochondrion-targeted dynamin related proteins, Mgm1 and Opa1. Red arrowheads show site of S1 proteolytic cleavage for each protein which leads to shorter and soluble isoform for Opa1 and Mgm1. Mitochondrial targeting sequence (MTS), transmembrane domain (TMD), GTPase effector domain (GED).

1.9.1 Dynamin related proteins

Dynamin is a family of large, multidomain GTPases that are involved in a wide range of cellular processes, including endocytosis, vesicle trafficking, and mitochondrial fission (Praefcke and McMahon, 2004). The dynamin family includes classical dynamin, which is the best-characterized member of the family, as well as several dynamin-related proteins (DRPs) (Ford and Chappie, 2019).

Dynamin-1, also known as classical dynamin, is a large protein that consists of several domains, including a GTPase domain, a middle domain, a pleckstrin homology (PH) domain, a GTPase effector domain (GED), and a proline-rich domain (PRD) (Muhlberg et al., 1997). Dynamin-1 has a critical role in clathrin-mediated endocytosis, which is the

process of internalizing extracellular molecules and membrane proteins through the creation of clathrin-coated vesicles (Takei and Haucke, 2001).

DRPs are involved in a wide range of cellular processes, including mitochondrial fusion and fission (Adebayo et al., 2021). Dynamin-related protein 1 (Drp1) is a DRP that plays a critical role in mitochondrial fission in mammalian cells. Drp1 has several distinct domains, including an N-terminal GTPase domain, a middle domain resembling dynamin, a variable domain, and a C-terminal GTPase effector domain (GED) (Otera et al., 2013). The GTPase domain links to signalling elements called bundle signalling elements (BSEs) and to a stalk domain that facilitates Drp1's attachment to membranes, oligomerization, and conformational changes (Fröhlich et al., 2013, Mears et al., 2011). It has been shown that in opisthokonts, an enzyme called the endoplasmic reticulum (ER) phospholipid hydrolase helps to squeeze mitochondria at their contact points with the ER (Nguyen and Voeltz, 2022). This squeezing allows for the Drp1 to be recruited to the site of fission, where it assembles into a spiral structure around the mitochondria and constricts it, leading to membrane fission (Nguyen and Voeltz, 2022). The yeast homolog of Drp1 is known as Dnm1 and also plays a similar role in mitochondrial fission (Ingerman et al., 2005).

Mitofusins (MFN1/2) and fuzzy onions 1 (Fzo1) in mammals and yeast, respectively, are involved in mitochondrial fusion. The MFNs structure is proposed to be U-shaped due to the presence of a cytosolic N-terminal GTPase domain, a coiled-coil heptad-repeat (HR1) domain, and two transmembrane domains that are anchored close to each other in the OM and cross the OM (Daste et al., 2018). Additionally, a coiled-coil heptad-repeat (HR2) domain is located at the C-terminal (Koshiba et al., 2004, Daniele et al., 2014).

A study demonstrated that Ugo1 protein acts as a molecular bridge connecting Fzo1 and Mgm1, which allows the two GTPases to come into close contact and promote their interaction in order to facilitate mitochondrial fusion (Sesaki and Jensen, 2004). MFN1 and MFN2 exhibit significant similarities in mammals, but they also display noticeable differences. MFN1 is more evenly distributed throughout the OM, whereas MFN2 is localized mainly at the contact sites between mitochondria and ER. In addition, MFN1 and MFN2 have different GTPase activities, with MFN1 having a higher GTPase activity than MFN2. This difference in GTPase activity may contribute to differences in their roles in mitochondrial fusion (Ishihara et al., 2004, Eura et al., 2003). MFN1 and MFN2 interact with different proteins, which may contribute to their functional differences.

For example, MFN2 interacts with the protein Opa1, which is involved in the regulation of mitochondrial cristae morphology. In mammals, Slc25a46 has been suggested as the homologue of Ugo1 (Abrams et al., 2015). Slc25a46 interacts with Opa1, Mfn2, and Mic60, which may control IM architecture (Janer et al., 2016).

1.9.1.1 Giant viruses use DRPs homologous genes to manipulate the host's organelle

Andre Lwoff introduced the criteria for distinguishing between viruses and living cells (Lwoff and Tournier, 1966). These criteria state that viruses have a DNA or RNA genome, cannot divide by themselves, do not encode a protein translation apparatus, and cannot synthesize the ATP they consume for replication (Lwoff and Tournier, 1966). The use of sterilizing filters to isolate viruses was based on the belief that microorganisms that could be seen by light microscopy or retained by sterilizing filters were not viruses. The discovery of giant viruses, following a pneumonia outbreak in Bradford, England, marked a significant shift. After years of research, scientists identified a giant virus, which they named Mimivirus, as the causative agent (Xiao et al., 2005). The Mimivirus, which was discovered living inside the amoeba *Acanthamoeba polyphaga*, is categorized into the nucleocytoplasmic large DNA viruses (NCLVD) group (Scola et al., 2003, Raoult et al., 2004). Several eukaryotic lineages including opisthokonts have been documented to be infected giant virus (Schulz et al., 2020, Mönttinen et al., 2021, Sun et al., 2020).

Giant viruses are a fascinating group of viruses that have been found to possess genes that are similar to those found in their host cells (Cunha et al., 2020). The presence of genes from different domains of life suggests that lateral gene transfer (LGT) has played a significant role in their evolution (Boyer et al., 2010). Giant viruses may use these genes to manipulate the host for reproduction (Charrier et al., 2008, Cock et al., 2010). In the following paragraph I will mention some of these genes.

The DRP gene, which is found in *Cafeteria roenbergensis* virus and *Clandestinovirus*, highlights the viruses' ability to manipulate host cell membranes and mitochondria for their replication (Rolland et al., 2021, Ford et al., 2011, Fischer et al., 2014). Also, Coccolithovirus and Mimivirus have been found to encode genes for SNAR proteins. These proteins are involved in secretion and endosomal trafficking, suggesting that giant viruses use them to interfere with vesicle trafficking for viral production (Abergel and Claverie, 2020, dos Santos Oliveira et al., 2021, Rodrigues et al., 2021, Colson et al.,

2017, Sobhy, 2017, Khalifeh et al., 2022). Therefore, it is suggested that DRPs may themselves contribute to successful pathogen entry and propagation in the host.

1.10 Trypanosoma, an attractive model organism

The Trypanosomatidae are a diverse family of flagellate protozoan parasites that belong to the Discoba supergroup (Burki et al., 2020). The Discoba supergroup is believed to radiate from the LECA around 1.9 billion years ago, and is only distantly related to opisthokonts (Eme et al., 2014). Trypanosoma belongs to the kinetoplastida group and has a uniquely structured mitochondrion genome known as kinetoplast (kDNA), which makes it interesting to study mitochondrial function and evolution (Lukeš et al., 2002). Additionally, *T. brucei* is easy to cultivate and genetically manipulate, which allows researchers to study various aspects such as gene expression, metabolic changes, and cristae biogenesis (Hashimi, 2019). Furthermore, *T. brucei* is available as pleomorphic cell line that can differentiate from bloodstream long slender form to PCF *in vitro* (see chapter 1.10.1), providing researchers with a unique opportunity to study the different life cycle stages of the parasite (Katelyn Fenn and Keith R Matthews, 2007). Finally, by comparing *T. brucei* with well-studied organisms such as *S. cerevisiae* and *M. musculus*, researchers can gain insights into the conserved properties of mitochondria and their evolution as well as other aspects of cell biology (Hashimi, 2019).

1.10.1 *T. brucei* life cycle

T. brucei is a parasitic organism that undergoes various life cycle stages, which occur in both mammalian hosts and fly vectors, specifically *Glossina spp.* (Vickerman, 1985). Throughout these stages, significant changes occur, including alterations in morphology, ultrastructure, gene expression profile, cellular metabolism, surface protein coats, and mitochondrial physiology (Smith et al., 2017).

The life cycle of *T. brucei* begins when a tsetse fly bites an infected mammal, such as a cow, human, or wild animal (Cox, 2004). The mammal carries BSFs, including long slender and short stumpy forms, with the majority of the latter concentrated in the blood, dermis, and subcutis (Capewell et al., 2016). The BSFs can switch the expression of a variable surface glycoprotein to evade the host immune system (Pays et al., 2004). Once the long slender forms reach high density, they differentiate into growth-arrested short stumpy forms (Rojas and Matthews, 2019). The short stumpy forms or long slender forms are taken by the tsetse fly when it bites the host (Shapiro et al., 1984, Bass and Wang, 1991). In the fly, as the parasite moves from the midgut, where it is

called PCF, to the salivary glands, it undergoes several stages of development, which are accompanied by changes in morphology and surface proteome (Szöőr et al., 2020). During its journey from the midgut to the salivary glands of the tsetse fly, *T. brucei* undergoes differentiation into various life cycle stages (K. Fenn and K. R. Matthews, 2007). Once transmitted to a new host via the tsetse fly's blood meal uptake, the parasite continues to differentiate and ultimately divides into long slender BSFs (Katelyn Fenn and Keith R Matthews, 2007) (Figure 13).

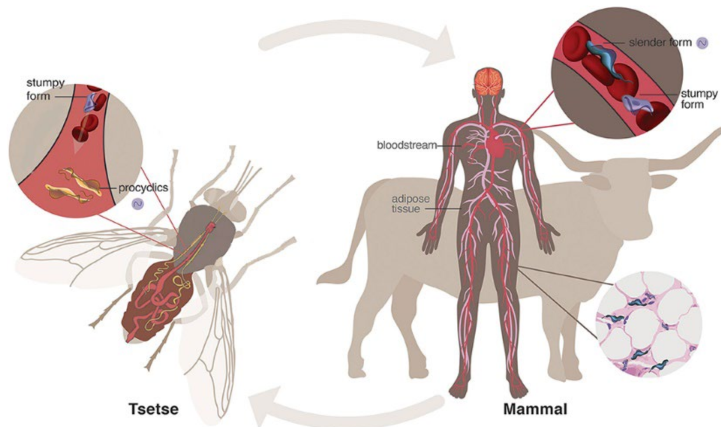

Figure 13. Life cycle of *T. brucei* *(Rijo-Ferreira and Takahashi, 2020)*.

1.10.2 *T. brucei* mitochondrion

T. brucei's ability to rapidly adapt to different environments is closely tied to changes in its metabolism, many of which occur in the mitochondrion (Smith et al., 2017). In the bloodstream, the primary source of energy for *T. brucei* is glucose, which is abundant in the bloodstream of the mammalian host. Glucose is taken up by a glucose transporter and metabolized to pyruvate through the glycolytic pathway to generate ATP (Haanstra et al., 2016). The glycosome compartmentalizes most of the enzymes involved in glycolysis needed for ATP generation (Michels et al., 2006). Conventional oxidative phosphorylation does not take place in the BSF and complex III and IV of electron transport chains are absent (A. Zíková et al., 2017).

The PCF is found in the tsetse fly midgut, which contains abundant proline. Thus, this amino acid is the main energy source as this amino acid in this life cycle stage. Proline is

used as the main source of carbon for the tricarboxylic acid cycle and the production of ATP (Haindrich et al., 2021). The metabolism of proline in *T. brucei* involves two enzymes, FAD-dependent proline dehydrogenase and pyrroline-5-carboxylate dehydrogenase. The supply of electrons to ubiquinone can occur via two pathways: either directly by proline dehydrogenase, or indirectly through the use of reduced NADH molecules generated by pyrroline-5-carboxylate dehydrogenase. In the second scenario, the electrons from NADH are then oxidized by either complex I or NDH2 (Paes et al., 2013, Marchese et al., 2020).

The morphology of the mitochondria in *T. brucei* undergoes a significant change during the two life cycle stages of the parasite discussed in chapter 2.1.1. In the PCF, the mitochondria are expansive, reticulated, and decorated with discoidal cristae. The cristae occupy between 2-13% of the mitochondrial volume (Bílý et al., 2021). This morphology is associated with the presence of complex III and IV of the ETC (Zdeněk Verner et al., 2015). However, in the BSF, the mitochondria are reduced in size and have a tubular shape along the side of the cell opposite to the attached flagellum. They have only a few extensions and the cristae morphology is stub-like (Hughes et al., 2017, Jakob et al., 2016, Bílý et al., 2021). This morphology is associated with the lack of complex III and IV of the ETC, which are typically enriched within cristae. The cristae occupy only 1-2% of the mitochondrial volume in the bloodstream form (Figure14) (Bílý et al., 2021, A. Zíková et al., 2017, Vickerman, 1965, S. Cogliati et al., 2013). Despite the differences in overall mitochondria and cristae morphology, kDNA shows identical between life cycle stages (Bílý et al., 2021).

Cristae morphology has been extensively studied in long slender and PCFs, but little is known about their morphology during the transition from short stumpy to PCF (Bílý et al., 2021). Given that the short stumpy form can survive for several days in a mammal, it would be interesting to study cristae formation during this life cycle stage (Turner et al., 1995, MacGregor et al., 2011).

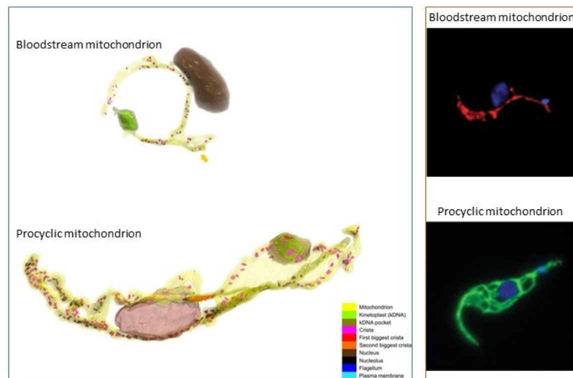

Figure 14. On left box is a three dimension of mitochondria in BSF and PCF of *T .brucei* (Bílý et al., 2021)**. Right box exhibits immunofluorescence analysis of a mitochondrial with Hsp70,** blue is showing the DAPI -stained Kinetoplast and nucleus (Alena Zíková et al., 2017).

2. Results

2.1 Published results

2.1.1 Ultrastructural changes of the mitochondrion during the life cycle of *Trypanosoma brucei*

2.1.2 Intracytoplasmic-membrane development in alphaproteobacteria involves the homolog of the mitochondrial crista-developing protein Mic60

2.1.3 A novel group of dynamin-related proteins shared by eukaryotes and giant viruses is able to remodel mitochondria from within the matrix

2.2 Unpublished results

2.2.1 The MICOS complex of trypanosomatids contains two cryptic mitofilin-domain proteins that may shape cristae

3. Conclusion remarks

The main conclusions of this Ph.D. thesis can be summarised as follows:

Results § 2.1.1: Ultrastructural changes of the mitochondrion during the life cycle of *Trypanosoma brucei*

1. PCF generally has a higher proportion of the inner mitochondrial membrane surface area compared to BSF, despite using the whole mitochondrial surface area as a proxy due to an inability to fully delineate the inner and outer mitochondrial membranes.
2. The mean surface area of PCF cristae is six times higher than that of BSF.
3. BSF cells have a lower total mitochondrial volume ranging from 0.9 to 1.5 μm^3 compared to PCF cells which occupy 2.5-3 μm^3.
4. According to the analysis, the volume of crista in PCF varies between 3% to 13% of the total mitochondrial volume. This suggests that PCF trypanosomes possess a broad range of crista volumes, which indicates the possibility of capturing individual crista in different states of respiration.
5. The size of BSF cristae is smaller than that of PCF, occupying about 10 times less volume which may correlate with PCF mitochondria are rich in electron transport chain complexes III and IV.
6. In BSF, it appears that the cristae number is maintained as the number of cristae is doubled as it was undergoing of mitosis.

Results § 2.1.2: Intracytoplasmic-membrane development in alphaproteobacteria involves the homolog of the mitochondrial crista-developing protein Mic60

1. Orf52, a protein downstream of Mic60 and co-transcribed with it in *R. sphaeroides* and *Magnetospirillum gryphiswaldense*, is an integral membrane protein with two transmembrane segments, contains tetratricopeptide repeat motifs.
2. Mic60 and Orf52 are found in a broad and dense phylogenetic distribution in alphaproteobacteria, and their presence overlaps with species reported to develop extensive ICMs, suggesting that they are ancestrally present and required by extant species that either have or lack the capacity to develop ICMs.
3. Alphaproteobacterial Mic60 has similar secondary and tertiary structures to its eukaryotic homolog, including a conserved amphipathic helix that aids in membrane

binding and bending but differs in having a smaller central coiled-coil segment and a transmembrane domain farther from the N-terminus.

4. Knocking out *mic60* and *orf52* genes in two different types of purple alphaproteobacteria resulted in slower photoheterotrophic growth rates and suggested that these genes affect the development of photosynthetic ICMs, with the impact varying between species.
5. The area occupied by lamellar ICMs increased in *R. palustris* knockout and overexpression strains compared to the wild-type, with statistically significant increases in the deleted-Orf52 and overexpressed-Orf52 strains.
6. Deletion or overexpression of Mic60 in *R. sphaeroides* resulted in a significant increase in the number of tubular ICMs observed per cell compare to deletion or overexpression of Orf52. In addition, Mic60-overexpressed cells exhibited a higher incidence of branching ICMs.
7. Alphaproteobacterial Mic60 and Orf52 physically interact with BamA, homologue of Sam50.
8. It is suggested that the structural and functional similarities between Mic60 in alphaproteobacteria and its mitochondrial homolog support the idea that ICMs and cristae are evolutionarily related. This implies that cristae likely evolved from ICMs in the last common ancestor of mitochondria and the alphaproteobacteria, before the endosymbiotic event.

<u>Results § 2.1.3:</u> A novel group of dynamin-related proteins shared by eukaryotes and giant viruses is able to remodel mitochondria from within the matrix

1. The study reports the existence of DRPs encoded by viruses within the Nucleocytoviricota phylum, including within the Mimiviridae family.
2. The study identified a novel group of mitochondrial dynamin-related proteins, named MidX, which is present in eukaryotic and viral genomes and likely transferred from eukaryotes to viruses.
3. The study confirmed the mitochondrial localization of the MidX protein from Hyperionvirus in the matrix-facing leaflet of the IM in *T. brucei*, indicating its potential role in mitochondrial dynamics.
4. MidX heterologous expression in *T. brucei* caused a decrease in mitochondrial membrane potential and altered mitochondrial morphology by inducing cytoplasmic protrusions, mitochondrial volume expansion, and multi-layered

membranes. Therefore, MidX has the capacity to massively remodel mitochondrial membranes.

5. Using AlphaFold2, four *de novo* models of MidX proteins were found to have a closed conformation with a distinct absence of a short helix and a VD organization that differed from other DRPs, highlighting the uniqueness of MidX.
6. Phylogenetic analyses using a comprehensive dataset of eukaryotic and viral DRPs never recovered a close relationship between Opa1 and Mgm1. Hence, Opa1 and Mgm1 do not share a common ancestor. Instead, Mgm1 seems to have a closer phylogenetic relationship to MidX than to Opa1.
7. The 3D structures of the Mgm1 and Opa1 revealed a high similarity between Mgm1 and Opa1, suggesting the convergent evolution.

<u>Results § 2.2.1:</u> The MICOS complex of trypanosomatids contains two cryptic mitofilin-domain proteins that may shape cristae

1. Heterologous expression of Mic34 and Mic40 proteins as a fusion with MBP in E. *coli* results in the emergence of vesicles in the periplasm, suggesting the capability of Mic34 and Mic40 in membrane remodelling.
2. Inducible RNA interference silencing of Mic34 or Mic40 in PCF *T. brucei* resulted in growth inhibition, reduced branching of mitochondria and a tubular mitochondrial shape after three days, which became severe by day five, suggesting the involvement of Mic34 and Mic40 in mitochondrial morphology. At this point it is hard to discern if the effect due to impaired IMS protein import or due to membrane remodelling.
3. The study investigated the effect of Mic34 RNAi and Mic40 RNAi by TEM which showed higher number of cristae in Mic34 RNAi but significant decrease in cristae area and cristae length. On the other hand, Mic40 RNAi showed a noticeable decrease in cristae number but the cristae area showed less decrease compare to Mic34 RNAi and cristae length showed no change compare to wild-type. Therefore, the reduced area is not in direct correlation with reduced number of cristae.

4. Summary of results and Future perspective

This doctoral thesis focuses on several molecules involved in the mitochondrial morphology of *T. brucei*, which is an ideal subject for my PhD study for several reasons. Firstly, during its life cycle, this parasite naturally undergoes significant changes in mitochondrial morphology, and a 3D model of mitochondria and cristae was critical for examining the inquiry discussed in Results § 2.1.1. Additionally, the study delved into the role of prokaryotic Mic60 in two groups of purple bacteria ICMs, the findings of which are presented in Results § 2.1.2. Furthermore, since Mic60 is conserved in both prokaryotes and eukaryotes, a section of the PhD thesis explored the diverged mitofilin-like proteins, Mic34 and Mic40, in *T. brucei*, with the inquiry's outcomes discussed in Results § 2.2.1. Lastly, *T. brucei* has only one mitochondrion and lacks DRP proteins such as Mgm1 and Opa1, presenting an opportunity to study the potential involvement of MidX, a DRP molecule found in NCLDVs, in mitochondrial morphology.

Part of the PhD thesis is involved in creating a 3D reconstruction of the mitochondria and cristae morphology in two main stages of *T. brucei*'s life cycle by focused ion beam scanning electron microscopy (FIB-SEM) technique. The FIB-SEM technique used in this study for 3D reconstruction of mitochondria and cristae to provide high-resolution of whole or nearly whole mitochondria. Although FIB-SEM technique has some limitations, such as the lack of statistical significance due to a small sample size, it was able to give us qualitative insights. The results in Results § 2.1.1 shows that the volume of mitochondria in BSF cells is smaller compared to PCF cells, which is in agreement with previous observation (Brown et al., 1973). Furthermore, the PCF mitochondrion has a larger surface area compared to the BSF organelle, which is consistent with its more reticulated structure compared to the generally cylindrical shape of the BSF mitochondrion (Vickerman, 1985). Unexpectedly, when BSF cells were cultured *in vitro*, we observed that the mitochondria had a high number of stub-like cristae, which occupied up to 2% of the total mitochondrial volume and up to 15% of the organelle's total surface area. Another unexpected observation was a wide distribution of cristae volumes in PCF cells and nucleus-proximal mitochondrial regions, which can be explained by previous observations that cristae can expand and contract based on their bioenergetic state (Hackenbrock, 1966, Mannella, 2020). This phenomenon has been observed in isolated mitochondria and human hepatocytes (Dlasková et al., 2019). Each crista within a human mitochondrion exhibits different membrane potential due to different energetic fluxes (Wolf et al., 2019). The heterogeneity of cristae volumes in

PCF may be due to capturing individual cristae in different respiration states (Horvath et al., 2005).

The unexpected results of our study highlight the necessity for a more comprehensive comprehension of the morphological transformations that arise during the transition between various life cycle stages, with a particular focus on the short stumpy stage. Short stumpy stage is an intermediate stage that emerges from the long slender differentiation and is predominantly found in blood, dermis, and subcutis of an infected mammal (Capewell et al., 2016). The parasite may start some of the changes in short stumpy stage in assembly of some of the complexes which may accompany with maturation of cristae. Our understanding of cristae shape of the short stumpy form is limited to IFA images showing overall mitochondrial morphology (Tyler et al., 1997). The study demonstrated that the mitochondria in the short stumpy form are neither tubular and narrow like those in the long slender form, nor do they have the elaborated branching structures of PCF mitochondria (Tyler et al., 1997). Therefore, creating a 3D reconstruction of both the mitochondria and cristae from short stumpy stage to PCF stage would be beneficial in tracing the entire transition from stub-like cristae to mature discoidal cristae.

However, if we examine the cristae from a bioenergetic standpoint, their function is mirrored. BSF utilizes glycolysis to produce ATP and does not rely heavily on oxidative phosphorylation complexes (Alena Zíková et al., 2017). However, PCF relies heavily on oxidative phosphorylation (Zdeněk Verner et al., 2015). The accommodation of these complexes at the cristae makes it evident why mature cristae are present in PCF and immature cristae in long slender BSF.

The MICOS complex is another molecule which is responsible for maintaining the cristae shape by inducing the negative curvature at cristae junctions so that can also influence the overall shape of cristae. The MICOS complex has been extensively studied for over a decade in various organisms. It has garnered attention from both evolutionary and functional perspectives. From an evolutionary standpoint, the complex consists of different subunits, with one subunit, Mic60, being conserved across the eukaryotic tree of life and alphaproteobacteria (S. A. Muñoz-Gómez et al., 2015). Mic60 has been extensively studied in opisthokonts and serves two functions: forming the cristae junction and maintaining contact sites between the OM and IM. Results § 2.1.2. is presented data that the secondary and tertiary structure of Mic60 is conserved between alphaproteobacteria and eukaryotes (Muñoz-Gómez et al., 2023).

The disruption of alphaproteobacterial Mic60 in two studied purple non-sulphur alphaproteobacteria showed that it is involved in ICM formation and thus phototropic growth. In addition, Mic60 has been found to physically interact with BamA, a homolog of Sam50. The congruity between alphaproteobacterial Mic60 and its mitochondrial counterpart, with respect to both structural and functional features, provides compelling evidence that cristae may have arisen from ICMs that were present in the last common ancestor of mitochondria and its sister group, the alphaproteobacteria, and thus have a pre-endosymbiotic origin. Should this hypothesis prove correct, it would suggest that the bioenergetic ICMs could have preadapted the first mitochondrial ancestor to evolve into an efficient bioenergetic or respiratory organelle.

Although our study has elucidated the role of Mic60 in two groups of alphaproteobacteria, yet a crucial query regarding the molecular mechanism responsible for the maintenance of Mic60's contact site with BamA remains unresolved. Recently, a study on yeast demonstrated that Mic60 can form an elongated, bow-tie shaped tetramer facilitated by Mic19 (Bock-Bierbaum et al., 2022). However, the absence of a Mic19 homolog in bacteria makes it challenging to investigate the mechanism underlying how Mic60 can cause constriction at cristae junctions.

From a functional perspective, MICOS displays divergence, especially in *T. brucei*. Unlike in yeast and mammals, the complex's entire architecture is sorted into peripheral and integral subunits rather than subcomplexes cantered around Mic10 and Mic60 (Eichenberger et al., 2019, Mukherjee et al., 2021). Peripheral subunits have been found to play a critical role, as their absence can drastically alter cristae morphology and potentially impair protein import (Eichenberger et al., 2019, Kaurov et al., 2018). In *T. brucei*, no homologues of Mic60 have been found (Kaurov et al., 2018). However, as discussed in Results § 2.2.1, two proteins, Mic34 and Mic40, were identified to have mitofilin domain, a signature domain of canonical Mic60, which suggests that they may have similar functions. Our preliminary findings indicate that the morphology of Mic34 RNAi and Mic40 RNAi appears similar in IFA images which change from elaborated branched mitochondria to tubular shape mitochondria. In line with this, previous results indicated that when Erv1 is knocked down, the mitochondria undergo a shift from a branched to a tubular shape (Haindrich et al., 2017). Therefore, based on a previous report it suggests that both Mic34 RNAi and Mic40 RNAi mitochondrial morphology alteration may result in impaired IMS protein import.

Upon conducting a more in-depth examination using TEM, we have observed discernible variations between Mic34 RNAi and Mic40 RNAi, which may indicate distinct roles of Mic34 and Mic40. Specifically, in our TEM analysis, we have noted that while Mic40 RNAi exhibited a reduction in the number of cristae in the mitochondrion, the total area and length of cristae did not display a significant alteration. In contrast, Mic34 RNAi was associated with an increased number of cristae, but a considerable decrease in both the area and length of cristae. These findings suggest that Mic34 and Mic40 may have slightly different roles in shaping crista. Based on the distinctive cristae remodelling patterns of Mic34 RNAi and Mic40 RNAi, one could hypothesize that one of the proteins plays a more significant role in protein import, while the other is more involved in membrane remodelling. One possible way to understand better and observe the real differences of mitochondria and cristae shapes in Mic34 RNAi and Mic40 RNAi is a 3D reconstruction by FIB-SEM which can give us a clear shape of organelle. Furthermore, it is possible to investigate the disparity in membrane remodelling between Mic34 and Mic40 through *in vitro* analysis using large unilamellar vesicles consisting of phospholipids found in the IM. This would enable the examination of the distinct interactions of Mic34 and Mic40 with the membrane phospholipids and potential remodelling. Previous research has demonstrated that yeast Mic60 has the capacity to deform liposomes and generate tubular structures (Tarasenko et al., 2017, Hessenberger et al., 2017). However, I suggest that performing this experimental approach for Mic34 and Mic40 may pose some challenges. While the previous study did not provide data on the number of vesicles upon expressing yeast MBP-Mic60 in *E. coli*, we suggest a side-by-side systematic comparison of *E. coli* expressing either yeast MBP-Mic60, trypanosomal MBP-Mic34 or MBP-Mic40 and quantifying the emergence of vesicles (Tarasenko et al., 2017).

While we base our hypothesis on the presence of the mitofilin domain and its function in membrane remodelling, it is important to note that there is another crucial site in the structure of Mic60 that plays a significant role in the remodelling of mitochondrial membranes. Recent studies have shown that canonical Mic60 can deform membranes, which may introduce curvature at crista junctions (Hessenberger et al., 2017, Tarasenko et al., 2017). This membrane-deforming capability is dependent on a lipid-binding site (LBS) located between the central coiled-coils and the mitofilin domain. The LBS consists of two α-helices (LBS1 and LBS2), where the first is amphipathic and presumably integrates into the IM (Hessenberger et al., 2017). LBS1 is particularly vital for Mic60's function, as its removal or mutation results in the loss of membrane binding and

deformation, and also leads to phenotypes that are similar to those obtained when the entire *mic60* gene is deleted in yeast (Hessenberger et al., 2017). Therefore, to further understand the abilities of Mic34 and Mic40 in membrane remodelling, investigating the presence of LBS in their structure would be another approach.

In this PhD thesis, we touched a topic about behaviour of mitochondria, which are highly dynamic organelles (Kondadi et al., 2019). Our investigation focused on a viral DRP protein that shares similarities with proteins that are involved in mitochondrial dynamics. The results of this study are presented in Results § 2.1.3. Two proteins from DRP family, Mgm1 and Opa1, participate in mitochondrial fusion in yeast and mammals, respectively (Herlan et al., 2003, Anand et al., 2014). However, our study discussed in Results § 2.1.3 challenges the widely held belief that Opa1 and Mgm1 are homologs, since we discovered that they are not sister lineages by molecular phylogenetics. The 3D structure of Mgm1 and Opa1 reveals a high similarity, which suggest the convergent evolution of these two proteins. In addition, we identified a gene called MidX, which is a member of the DRP family and appears to share a common ancestor with Mgm1. This gene is present in NCLDVs and six eukaryotic lineages. To investigate the impact of MidX on mitochondria, we heterologously expressed Hyperion viral MidX in *T. brucei*. Our results indicate that MidX significantly affects mitochondria morphology by operating from within the matrix, in contrast to the way Opa1 and Mgm1 alter the IM structure in the IMS. However, the exact mechanism by which MidX remodels mitochondria remains unclear. One potential avenue for future studies could be to reconstitute MidX into large unilamellar vesicles composed of cardiolipin, phosphatidylserine, and phosphatidic acid, which can stimulate the GTPase activity (Rujiviphat et al., 2009). A study demonstrated that that Mgm1 interaction with IM phospholipids and presence of S-Mgm1 resulted in aggregation of large unilamellar vesicles (Rujiviphat et al., 2009). Therefore, one could investigate MidX membrane remodelling properties and compare them to Mgm1, which, despite having a common ancestor, has different membrane localization and predicted 3D structure.

In the end, although *T. brucei* may be more known as it is causative agent of African Sleeping Sickness, it is a valuable model organism for investigating various aspects of cell biology (Büscher et al., 2017, Hashimi, 2019). It is one of the most genetically accessible protozoan organisms, and advanced reverse genetics methods have been successfully used to quickly characterize proteins (Lukeš et al., 2010). *T. brucei* is easy to grow *in vitro* without the need for animal models, and it is safe to handle as it cannot infect humans (Zhou et al., 2014). This unicellular protist has several organelles found

in all eukaryotes or only in trypanosomes, making it an excellent system for studying cell division, organelle biogenesis, and protein trafficking (Matthews, 2005). *T. brucei* has unique features such as an intricate mitochondrial DNA network, a highly complex RNA editing machinery of mitochondrial transcripts, polycistronic RNA molecules that are processed to mRNAs by trans-splicing (Lukeš et al., 2010).

Rather than being intimidated or discouraged by the vast array of organisms in the tree of life, we should approach it with curiosity and wonder. Investigating different biological systems can broaden our understanding and offer a thrilling experience, even if our focus is purely on acquiring new knowledge. It is understandable that some may harbour concerns that studying "lower" eukaryotes may not provide insights applicable to typical (Opisthokont) model organisms. However, it is important to keep an open mind and recognize that any system that can effectively address our scientific questions is worth exploring, even if it involves venturing beyond the boundaries of higher eukaryotes. By embracing the diversity of life, we open up new avenues of inquiry and opportunities for discovery, ultimately advancing our understanding of the natural world.

5. List of abbreviations:

In alphabetical order:

ATP: Adenosine triphosphate
BSE: Bundle signalling elements
BSF: *Trypanosoma brucei* bloodstream form
CoRR: Colocation of gene and gene product for redox regulation of gene expression
COX: Cytochrome c oxidase
DRP: Dynamin-related proteins
Drp1: Dynamin-related protein 1
ER: Endoplasmic reticulum
ETC: Electron transport chain
Fe-S: Iron-Sulphur cluster
FIB-SEM: focused ion beam scanning electron microscopy
FMN: Flavin mononucleotide
Fzo1: Fuzzy onions 1
GED: GTPase effector domain
GED: GTPase effector domain
GTP: Guanosine triphosphate
HR: Heptad repeat
IBM: Inner boundary membrane
ICM: intracytoplasmic membrane
IM: Mitochondrial inner membrane
IMS: Intermembrane space
LECA: Last eukaryotic common ancestor
LGT: Lateral gene transfer
L-Mgm1: Long isoforms of Mgm1
L-Opa1: Long isoforms of Opa1
MFN: Mitofusins
Mgm1: Mitochondrial Genome Maintenance 1
MIA: Mitochondrial intermembrane space import and assembly

MIB: Mitochondrial intermembrane space bridging complex
MICOS: Mitochondrial contact site and cristae organizing system
MPP: Mitochondrial processing peptidase
MTS: Mitochondrial targeting sequence
NCLVD: Nucleocytoplasmic large DNA viruses
NDH2: Alternative dehydrogenase
OM: Mitochondrial outer membrane
Opa1: Optic Atrophy 1
PAM: Pre-sequence translocase-associated motor
PCF: *Trypanosoma brucei* procyclic form
PH: Pleckstrin homology domain
PRD: Proline-rich domain
S-Mgm1: Short isoforms of Mgm1
TAO: Trypanosoma Alternative Oxidase
TIM: Translocator of the inner membrane
TOM: Translocator of the outer membrane

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7. Curriculum vitae

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EDUCATION

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CONFERENCES

. Oral presentation at 50th Jírovec's Protozoological Days, Czech Republic 2021. A dynamin-like protein from Mimivirus may shed light on the evolutionary history of mitochondria remodelling in opisthokonts.

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