Biogenesis of mitochondria in parasitic protist *Trypanosoma brucei*

Ph.D. Thesis

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I would like to thank my family, particularly my wife Veronika and my mother for their unwavering support. I would also like to thank all my colleagues for inspiring and pleasant working environment, especially lab assistant Míša and supervisor Jan Tachezy. Thank you all!

I declare that I substantially contributed to the results presented in this thesis.

Jan Mach
Prohlášení:

Prohlašuji, že jsem závěrečnou práci zpracoval samostatně a že jsem uvedl všechny použité informační zdroje a literaturu. Tato práce ani její podstatná část nebyla předložena k získání jiného nebo stejného akademického titulu.

V Praze, 26. 2. 2015

Jan Mach
Data presented in this thesis resulted from team collaboration at the Laboratory of Biochemical and Molecular Parasitology and during projects with our partners. I declare that the involvement of Mgr. Jan Mach in this work was substantial and that he contributed significantly to obtain the results.

Prof. RNDr. Jan Tachezy, PhD.
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Abstract

In last decade, investigations of mitochondria including their various reduced forms such as hydrogenosomes and mitosomes revealed unexpected diversity of this indispensable organelle. Interestingly, the single mitochondrion of parasitic protist *Trypanosoma brucei* is able to undergo remarkable functional and structural changes reflecting available carbon sources. Moreover, it was proposed that trypanosomes belong among the most ancient eukaryotes and as such, their mitochondria raised high attention of biologists. To contribute to the knowledge of mitochondrial biogenesis and function, we focused on studies of two key mitochondrial processes, the processing of preproteins that are imported to the mitochondria, and mechanism of pyruvate transport to these organelles. Moreover, we also investigated uptake of iron by *T. brucei*. This metal is essential for function of numerous proteins, particularly for iron-sulfur proteins in mitochondria.

Evolutionary history of trypanosomes and their mitochondrion is a question of debates. According to some reports, mitochondrion of trypanosomes represent an ancient form of this organelle, which is supported by identification of putative "archaic" translocase of the outer mitochondrial membrane (ATOM) and finding of only a single type of translocation pore in mitochondrial inner membrane. On the contrary, we identified and characterized mitochondrial processing peptidase within the mitochondrial matrix and two subunits of core proteins bound to the mitochondrial membrane with similar characters as those described in metazoans and fungi. Presence of highly evolved mitochondrial peptidases and comparable N-terminal mitochondrial presequences that target proteins to the trypanosomal mitochondrion do not support ancient character of this organelle. Further, we investigated an enigmatic mitochondrial pyruvate carrier (MPC) in inner mitochondrial membrane and characterized its function in procyclic and bloodstream forms of *T. brucei*. The character of *T. brucei* MPC appeared to be similar to previously discovered MPC in human, yeast and fruit fly. Additionally, we studied mechanism of iron uptake, which is poorly understood in procyclic form of *T. brucei*. We found that procyclic *T. brucei* is able to acquire iron from ferric complexes via a reductive mechanism, which can be beneficial for the parasite within the insect gut and which is comparable to mechanism used by yeast.

Taken together, our results do not support the ancient character of *T. brucei* mitochondrion. It seems more likely that it represents a highly evolved and versatile organelle, which is comparable in many characters with other eukaryotes.
1. Introduction

Compartmentalization is one of the major properties, which distinguish eukaryotic cell from prokaryotes. To distribute various pathways among distinct compartments has proved to be evolutionary successful step. It probably allowed eukaryotes to be more flexible and effective in metabolic processes, particularly in energy fixation (Martin et al, 2015). The beneficial effect is apparent from the appearance of wide diversification of eukaryotic lineages and evolving of multicellular organisms (Adl et al, 2012). Emerging of specific membrane-bound organelles such as nucleus, endoplasmic reticulum, peroxisomes, Golgi body, and others is in striking contrast when compared with simple cellular organization of prokaryotes. The endomembrane system originates possibly from the eukaryotic cell itself, whereas it has been generally accepted that mitochondria and chloroplast were acquired in the course of evolution by engulfing of bacterial endosymbionts.

One of the most studied eukaryotic organelle is the mitochondrion. The mitochondrion is believed to be a remnant of α-proteobacterial endosymbiont, which lived within a pre-eukaryotic or ancient eukaryotic cell (Pittis & Gabaldón, 2016; Lang et al, 1999). Its origin is apparent from the prokaryotic type of mitochondrial genome and double membrane separating its content from the rest of the cell. Importance and the ancient origin of mitochondrion is also reflected by its presence in all extant eukaryotes. Mitochondria were discovered more than 100 years ago but thorough investigation in past few decades revealed that it evolved in many different forms and functions. During the evolution, some mitochondria lost their DNA and many pathways, in extreme situation leaving just a single functional pathway within the whole organelle. In most organisms, mitochondria serve as ATP-producing organelle, utilizing tricarboxylic acid cycle, β-oxidation of fatty acids and oxidative phosphorylation. In addition, other essential pathways or their parts such as iron-sulfur cluster assembly, heme and steroids synthesis, amino acid metabolism and programmed cell death operate within mitochondria. Biogenesis of mitochondria is a highly complex process: 1) Since eukaryotic cell cannot create mitochondria de novo, it has to ensure their division and propagation during the reproduction and segregation to newly formed cells. 2) Most proteins and other compounds have to be transported to the mitochondria to ensure essential mitochondrial function. For this purpose, cells evolved an
elaborated protein import pathway and highly regulated transporters of other necessary compounds.

*Trypanosoma brucei* is a medically and veterinary important unicellular parasite of humans and other animals transmitted by tse-tse flies in Africa. Different environments inside these two types of hosts rise different demands on trypanosomal metabolism and response to the host immunity. A single mitochondrion that is present in the trypanosome cell is particularly interesting for its dramatic metabolic and morphological changes between bloodstream and insect forms. In this work, I review current knowledge on trypanosome biogenesis and metabolism and provide results of our investigations focused on selected functions of *T. brucei* mitochondrion that are compared with mitochondria of model eukaryotes *Saccharomyces cerevisiae* and *Homo sapiens.*
2. Trypanosomes

Trypanosomes are unicellular parasites of a wide range of vertebrates all over the world. They are members of the phylum Euglenozoa and order Kinetoplastida with their typical organelle kinetoplast, a dense concatenated network of circular mitochondrial DNA. Trypanosomes have complicated life cycle including the vertebrate host and the invertebrate vector, which is mostly blood-feeding insect. The most studied trypanosomes are parasites of humans: African *Trypanosoma brucei* that causes a sleeping sickness and *Trypanosoma cruzi* a causative agent of Chagas disease in Latin America.

2.1. Phylogenetic position

Solving the evolutionary relationship of trypanosomes is crucial for understanding of the intriguing biology of *T. brucei*. Evolutionary position of trypanosomes has been intensively studied but not resolved until recently (Lukeš et al., 2014; Simpson et al., 2002; Adl et al., 2012). Current studies base on phylogenetic analyses of multiple genes strongly suggested that the genus *Trypanosoma* is a monophyletic group within the family *Trypanosomatida*. The inner relationship within the *Trypanosomatida* group was reconstructed based on the SSU rRNA (Lukeš et al., 1997; Simpson et al., 2002) and more recently on glycosomal glyceraldehyde phosphate dehydrogenase (Hamilton et al., 2004). In addition to *Trypanosoma*, *Trypanosomatida* includes two dixenous monophyletic groups, genus *Phytomonas* and *Leishmania* and multiple monoxenous groups. The *Trypanosomatida* belong to the group *Kinetoplastea*. The position of *Kinetoplastea* on the phylogeny tree is controversial. Phylogenetic analysis based on SSU rRNA sequences produced long branches connecting kinetoplastids with diplonemids, and euglenids. The use of other genes like heat shock protein 70 and 90 (Simpson et al., 2004) and addition of more taxa to the analyses (Moreira et al., 2004) significantly strengthened the *Kinetoplastea* position.

Currently, the *Kinetoplastea* together with *Euglenida*, *Diplonemea* and *Symbiontida* form taxon *Euglenozoa*. *Euglenozoa* belongs to group with unique mitochondrial cristae *Discicristata* and based on the flagellar structure to the supergroup *Excavata* (Fig. 1).
2.2. Trypanosoma brucei

*Trypanosoma brucei* is one of the deadly but frequently neglected parasites causing serious and often fatal disease of humans and participates on economic losses by lowering fitness of cattle in sub-Saharan Africa (together with *T. vivax* and *T. congolense*). There are three subspecies *Trypanosoma brucei brucei*, *Trypanosoma brucei rhodesiense* and *Trypanosoma brucei gambiense*. While *T. brucei brucei* causes only livestock and wild animal disease called nagana, *T. b. gambiense* and *T. b. rhodesiense* are infectious to humans, causing human African trypanosomiasis (HAT) or sleeping sickness. *T. b. gambiense* is a causative agent of the chronic form of HAT in west and central Africa, whereas *T. b. rhodesiense* causes the acute form of HAT in southern and eastern Africa. There are reports of humans infected by other trypanosome species, but these cases are scarce (Truc *et al*, 2013). There are over 300 000 cases of HAT and more than 6 000 new cases reported annually (actual estimation is about 20 000 cases per year), the estimated population at risk is 65 million people (World Health Organization, 2013).
*T. brucei* is transmitted by blood-feeding insects Tse-tse flies (genus *Glossina*), which take the blood with “stumpy” forms of trypomastigotes (short-lived non-dividing transmission form without free part of flagella) from the infected host. Trypanosomes multiply in the gut as procyclic and mesocyclic forms and then they migrate to salivary glands where they transform to infectious metacyclic trypomastigotes. The metacyclic trypanosomes are inoculated to the mammalian host during the feeding of the fly. First, they multiply as a “long slender” trypomastigotes in the tissue around the place of inoculation, which can lead to skin inflammation, visible as “trypanosomal chancre”. Afterwards they invade lymphatic system, blood and finally they cross the blood-brain barrier and spread to the central nervous system. When trypanosomes in blood reach threshold density, they start to form so called “stumpy” forms, which are not dividing but highly infective for the tse-tse fly.

### 2.3. *T. brucei* cell biology

*T. brucei* cell appearance is remarkably polymorphic during its life cycle. Here, I will describe general characteristics of the cell together with specific features of main trypanosomal forms, insect gut-dwelling procyclic form (PCF) and long slender mammalian bloodstream form (BSF).

In general, the cell has a single long flagellum emerging from its posterior part and oriented toward the anterior part. It consists of typical flagellar axoneme (9+2 arrangement of microtubules) and paraflagellar rod, an unusual structure found only in kinetoplastids and a few related organisms. The function and structure of the paraflagellar rod has not been fully determined yet, although it was suggested that it can act as a biomechanical spring to absorb and transmit energy (Hughes et al., 2012) and may act as a flagellar Ca\(^{2+}\) and cAMP regulator (Langousis & Hill, 2014; Oberholzer et al., 2007). The flagellum is connected along the cell body and only tip of anterior part is free. It is attached to the body by junctional complexes that bind the flagellum and plasma membrane creating undulating membrane. Flagellum originates in specialized plasma membrane invagination called the flagellar pocket. The flagellar pocket is exclusive place for endocytosis (pinocytosis) and exocytosis, which makes it the only entrance to the cell and most important interacting place with the host. The flagellum is not only used for motility of the cell but also has an adhesion function, which is important for the attachment to the tse-tse fly salivary glands.
Even though, the flagellar pocket is the most important host-parasite interaction site, the rest of the cell is exposed to the hostile environment in the host body as well. *T. brucei* forms specialized protein coat that shields cell surface against the immune response of the host acting as a physical barrier that covers any vital trypanosomal epitopes. Within the mammalian host, the cell surface is covered by a coat formed by Variable Surface Glycoproteins (VSG), while in the insect, VSG is replaced by procyclins. Many genes in the genome encode VSGs, however, only a single gene is actually expressed on the surface. Immune suppression of the parasite population expressing major VSG leads to selection of another population expressing different VSG coat. That makes impossible for the host to raise effective immune response against whole population of the parasite and is effective only against the parasites with the “old” VSGs. This periodical fight of the immune system against the waves of the parasite populations with different VSG types causes exhaustion of immunity.

*T. brucei* mitochondrion is a single large net-like organelle spreads all over the cell body in PCF and highly reduced in BSF. During the life cycle, mitochondrion undergoes not only changes in the shape and volume but also in the metabolic activities. It contains a large amount of DNA in form of concatenated network of 5000 to 10 000 minicircles (0,5-10 kb each) and 40 to 50 maxicircles (20-40 kb each) forming the dense structure, the kinetoplast. Maxicircles encode two ribosomal RNAs, one guide RNA (gRNA) and a set of core subunits of mitochondrial respiratory complexes (Alfonzo *et al*, 1997; Benne, 1985). Minicircles encode guide RNAs (gRNA), small 50-60 nucleotides long gRNAs that are used for editing of the mitochondrial mRNA encoded in maxicircles (Corell *et al*, 1993). Originally it was thought that the mitochondrial genome contains only pseudogenes, but later on, Benne *et al* (1986) found that mRNA must be first edited by the addition or deletion of uridine residues based on the binding of gRNA. Interestingly, no tRNAs are encoded in the mitochondrial genome and all of them originate from nuclear DNA. Consequently, all tRNA operating in mitochondria are imported from the cytosol.

As there is just a single mitochondrion per cell, it has to be ensured that during the cell division, mitochondrion is also divided and separated to both the newly formed cells. Therefore, the mitochondrion division is tightly coordinated with the cell division via association with the basal body of the flagellum (Ogbadoyi *et al*, 2003).

The glycosome is a peculiar organelle originally found in trypanosomes and later in other organisms. This organelle is a specific form of peroxisome (Opperdoes, 1984). It is
enveloped by a single membrane with its biogenesis is dependent on peroxins machinery and contains a dense protein matrix. The name glycosomes was given to the organelle because of the presence of first seven glycolytic enzymes (Fig 2.). The size of glycosomes and the amount of glycolytic enzymes depend on the life cycle or more precisely on the environment. Glycosomes in PCF are smaller containing 40-50% of the glycolytic enzymes out of all the protein content, while in BSF they are larger and contain over 90% of glycolytic enzymes (Gualdrón-López et al., 2012). The glycosome also contains enzymes for other processes such as the pentose-phosphate pathway, β-oxidation of fatty acids, purine salvage and biosynthetic pathways for pyrimidines, ether-lipids and squalenes. However, it lacks the typical enzyme for peroxisomes, the peroxidase.

### 2.4. *T. brucei* as a molecular biology model

*T. brucei* is the most widely used representative of kinetoplastids to study specific pathways of trypanosomes but also various eukaryotic pathways in general. Nowadays, both stages are easily maintained in vitro in the liquid semi-defined medium (Baltz et al., 1985; Brun & Schönenberger, 1979). In addition, BSF can be maintained in vivo in mice. Both nuclear and mitochondrial genomes were sequenced for different strains (Aslett et al., 2010). Many methods, which depend on the ability to deliver foreign DNA to the cell and its recombination to the genome have proved to be useful in studies of different cell aspects from protein function to the cell morphology and biology. DNA can be inserted to the *T. brucei* by electroporation in the form of the circular plasmid, which remains in the cell for a short time or in the form of a linear fragment, which can be incorporated by homologue recombination to the specific site in the genome. The cell transformation allows for example expression of homologous as well as heterologous proteins to test their function, cellular localization etc. Importantly, it allows to develop methods of reverse genetics including gene knock-out methods (Sommer et al., 1996) as well as gene silencing using RNA interference (Ngô et al., 1998). These genetic manipulations are useful not only for study of the *T. brucei* but also to study different proteins and pathways of organisms, for which methods of reverse genetics are not amenable.
3. Mitochondria

Mitochondrion is the indispensable organelle of all eukaryotes. It was originally acquired by the endosymbiosis of α-proteobacteria by common eukaryotic ancestor approximately 1.5 - 2 billion years ago (Doležal et al., 2006; Margulis, 1970). Most mitochondria contain their own genome but some eukaryotes possess reduced forms of mitochondria without mitochondrial DNA (van der Giezen & Tovar, 2005). Mitochondrion is known as a compartment that is responsible for the energy metabolism [tricarboxylic acid cycle (TCA) and oxidative phosphorylation], biosynthesis of various important compounds such as biotin, heme, and iron-sulfur (Fe-S) clusters. All these pathways are dependent on cytosolic molecules and proteins, which has to be actively imported to the mitochondrion while other metabolites have to be exported outside. Only few proteins are encoded and translated directly in the mitochondria. These proteins are usually core components of the oxidative phosphorylation pathway. Hydrogenosomes and mitosomes are the most reduced mitochondria (Embley & Martin, 2006). These organelles lost most of typical mitochondrial pathways including the TCA cycle and oxidative phosphorylation. The only identified pathway in most of the mitosomes is Fe-S cluster assembly, which suggests that the formation of Fe-S clusters might be the only essential mitochondrial function (Tovar et al., 2003).

3.1. Mitochondrial membrane

Mitochondrion is surrounded by a double membrane, therefore it consists of four subcompartments: outer membrane (OM), intermembrane space (IMS), inner membrane (IM) and matrix. Besides other transporters, OM contains many copies of pore-forming protein called VDAC, which is at physiological conditions permeable for most of the uncharged molecules up to 5 kDa weight (Mannella, 1992). On the contrary, IM is impenetrable for most of the molecules and ions, which has to be actively transported into and out of the mitochondrion by different transporters. Proteins have to be imported to the mitochondrial subcompartments by the translocase of outer membrane (TOM) and the translocase of inner membrane (TIM). Importantly, IM serves as an electrochemical barrier used as a driving force for energy fixation in form of ATP via oxidative phosphorylation pathway. To maintain physiological function of the mitochondrion, correct amount of
metabolites, ions and other small solutes have to be imported or exported from the mitochondrion. Therefore IM contains many transporters of different solutes, the most important transporters of small solutes are carriers of ATP/ADP, Pi, coenzyme A, malate, pyruvate and others, which belong to the large family of mitochondrial carrier proteins (Scheffler, 2007). The pyruvate transporter was for many years enigmatic. Whereas the pyruvate transport was biochemically characterized in the 1970s (Halestrap, 1975; Papa et al., 1971), molecular identity of mitochondrial pyruvate carrier was identified only recently in (Bricker et al., 2012; Herzig et al., 2012; Li et al., 2014). Most significant ions transported cross IM are H⁺, Na⁺, K⁺, Ca²⁺, Mg²⁺ and trace metal Fe²⁺, Cu²⁺, Zn²⁺, and others (Scheffler, 2007).

3.2. Mitochondrial energy metabolism

Mitochondrion is a powerhouse of the cell, producing utilizable energy source for the key cellular processes. The tricarboxylic acid cycle (TCA, also known as the Krebs cycle), oxidative phosphorylation (OXPHOS) and β-oxidation of fatty acids are the core pathways of the energy metabolism in the mitochondria.

3.2.1. Tricarboxylic acid cycle

Glucose is a principle source of energy for most eukaryotic cells. In the cytosol, glucose enters glycolysis, which metabolizes this hexose to pyruvate. The overall yield of glycolysis is two NADH and two ATP molecules per one glucose. Pyruvate can be further metabolized in the cytosol to lactate by lactate dehydrogenase or ethanol by alcohol dehydrogenase. Another possible end-product of glycolysis is glycerol produced by glycerol kinase from glycerol-3-phosphate. Most of the pyruvate is usually transported to the mitochondrion and further metabolized by pyruvate dehydrogenase to acetyl coenzyme A (Ac-CoA), which enters the TCA cycle. In the TCA cycle Ac-CoA is converted to different intermediates, some of them also serve as crucial substrates for other mitochondrial processes. A single TCA cycle produces one molecule of adenine dinucleotide (FADH₂) and three molecules of reduced nicotinamide adenine dinucleotides (NADH), which are afterwards used for ATP production in OXPHOS.
Initial part of *T. brucei* glycolysis takes place within glycosomes. The glycosomal metabolism converts glucose into 3-phosphoglycerate (3-PGA) and glycerol (Fig. 2) (Opperdoes & Borst, 1977). The overall fixation of energy in form of ATP and NADH within glycosome is in balance, all produced ATP and NADH is consumed (Michels *et al*., 2006). Advantage of glycolysis compartmentalization is explained by the necessity to increase the efficiency of glycolysis by putting glycolytic enzymes together with two enzymes of glycerol metabolism in one organelle, creating specific conditions. Ability to replace whole pathways within glycosome during the change between host glucose rich and vector glucose poor environments, supports metabolic flexibility of trypanosomes (Opperdoes & Borst, 1977; Hammond *et al*., 1985; Gualdrón-López *et al*., 2012).

Glycerol and 3-phosphoglycerate (3-PGA) are transported from glycosome to cytosol. 3-PGA is metabolized to phosphoenolpyruvate, which is either transformed to pyruvate generating one ATP or transported back to glycosome. Phosphoenolpyruvate is converted in the glycosome to succinate and excreted as an end-product. The net ATP yield of glycolysis is only one molecule of ATP per glucose. Pyruvate is the main excreted end-product of BSF, while in the PCF is mostly transported to mitochondrion. Pyruvate in BSF, and L-proline and L-threonine in PCF are the main substrates entering the mitochondrial energetic metabolism. Acetate and succinate are mitochondrial end-products of pyruvate catabolism. L-threonine is converted to acetate and L-glycine, while succinate is end-product of L-proline metabolism.

In *T. brucei*, mitochondrial TCA cycle does not work as a cycle, despite the fact that all the TCA cycle enzymes are present (Fig. 3) (van Hellemond *et al*., 2005; Tielens & van Hellemond, 2009; Verner *et al*., 2015). Moreover, in the BSF mitochondrion, TCA cycle enzymes are either missing or undetectable (Tielens & van Hellemond, 2009). Unlike in usual mitochondrion, Ac-CoA does not enter the TCA cycle but is transformed to acetate either by Ac-CoA thioesterase (ACH) or by acetate:succinate CoA-transferase (ASCT) (Millerioux *et al*., 2012). Activity of ASCT in the presence of succinate leads to the production of succinyl-CoA, which is reconverted to succinate by succinyl-CoA synthetase with concomitant production of ATP (van Hellemond *et al*., 1998). Ac-CoA produced from pyruvate is imported from the cytosol and from L-threonine, which *T. brucei* imports from the medium as a carbon source (Bringaud *et al*., 2006). L-threonine is converted to Ac-CoA and L-glycine. Moreover, PCF can also use L-proline and L-glutamine, as they are main carbon sources of the PCF *T. brucei* in the hemolymph of
the tse-tse flies. Both the amino acids are transported to the mitochondria, converted to glutamate and then to 2-oxoglutarate, which enters TCA cycle. Alongside conversion of proline to glutamate, FADH₂ and NADH are produced. Another NADH is produced by glutamate dehydrogenase (Bringaud et al., 2006). It should be mentioned that pyruvate is necessary for the transamination of glutamate, which leads to the production of 2-oxoglutarate and alanine. 2-oxoglutarate is transformed to succinyl-CoA and further to succinate, which is exported out of the cell. Succinate is also produced by mitochondrial fumarase and fumarate reductase from cytosol imported malate.

Figure 2. Schematic representation of glycolysis of bloodstream form T. brucei (Michels et al., 2006). Abbreviations: 1,3BPGA, 1,3-bisphosphoglycerate; DHAP, dihydroxyacetone phosphate; F-6-P, fructose-6-phosphate; FBP, fructose-1,6-bisphosphate; G-3-P, glyceraldehyde-3-phosphate; G-6-P, glucose-6-phosphate; Gly-3-P, glycerol-3-phosphate;
PEP, phosphoenolpyruvate; 3-PGA, 3-phosphoglycerate; Pi, inorganic phosphate; UQ, ubiquinone pool. Enzymes: 1, hexokinase; 2, glucose-6-phosphate isomerase; 3, phosphofructokinase; 4, aldolase; 5, triosephosphate isomerase; 6, glycerol-3-phosphate dehydrogenase; 7, glycerol kinase; 8, glyceraldehyde-3-phosphatedehydrogenase; 9, glycosomal phosphoglycerate kinase; 10, phosphoglycerate mutase; 11, enolase; 12, pyruvate kinase; 13, FAD-dependent glycerol-3-phosphatedehydrogenase; 14, alternative oxidase.

3.2.2. Fatty acid biosynthesis

*T. brucei* as other eukaryotic cells has to synthesize their own lipids from available sources. Eukaryotes produces fatty acids by two distinct pathways: Fatty Acid Synthesis type I (FAS I) (Smith, 1994) consisting of one or two large polypeptides with multiple catalytic domains or type II (FAS II) that is formed by multiple components (enzymes, carriers) that are encoded by separate genes (White *et al*., 2005). FAS I is found in the cytosol of mammalian and fungal cells, while FAS II is present in mitochondria and plastids.

For a long time it was believed that trypanosomes are unable to synthesize fatty acids *de novo* (Dixon *et al*., 1971). More recently, FAS II system was found in the *T. brucei* mitochondrion and it was assumed that FAS II is responsible for all *de novo* synthesized lipids (Paul *et al*., 2001; Stephens *et al*., 2007). Instead, Lee *et al*. (2006) found that FAS II system produces only small part of the lipids, while most of lipids are synthesized by unique set of microsomal elongases that are present in the endoplasmatic reticulum (ER) (Lee *et al*., 2006).

Ac-CoA, an essential component for the lipid biosynthesis, is produced from pyruvate or threonine in the mitochondria of *T. brucei*, while the lipid biosynthesis takes place mainly in the ER. Recently, it was found that unlike other eukaryotes, which use citrate/malate shuttle to export acetyl equivalents to the cytosol, *Trypanosoma* is the only one that export acetate (Rivière *et al*., 2009). Acetate crosses mitochondrial membranes probably by passive diffusion and afterwards it is converted to Ac-CoA by Acetyl-CoA synthetase.
3.2.3. Oxidative phosphorylation

Using electron-chain oxidative phosphorylation, cells create proton gradient on the mitochondrial IM and utilize it to gain required energy for ATP production by ATP synthase so called complex V. Unlike other ATP producing pathways, it is highly efficient; it can produce up to 36 ATP molecules from one glucose compared to two ATPs from glycolysis. OXPHOS pathway consists of four complexes labeled I-IV that co-operate on proton translocation from mitochondrial matrix to IMS. Complex I and II oxidizes NADH and FADH, respectively, translocating protons and providing electrons for transport. Electrons pass via ubiquinone to cytochrome $c$ using complex III. Cytochrome $c$ delivers electrons to the complex IV, which catalyzes the reduction of oxygen, a terminal electron acceptor, to water. The final step of OXPHOS is the production of ATP by ATP synthase (complex V), a large complex that works like a molecular motor powered by the proton-motive force. All complexes of OXPHOS, except for ATP synthase, contain iron in the form of heme or Fe–S clusters (Lill & Kispal, 2000; Kim et al., 2012).

*T. brucei* OXPHOS differs greatly from the most studied pathway of yeast and human (Fig. 3). Most pronounce difference is the presence of two unusual enzymes, which can supply electrons to the OXPHOS pathway and an alternative terminal oxidase. FAD-dependent glycerol-3-phosphate dehydrogenase (G3PDH) (Guerra et al., 2006) and an alternative NADH dehydrogenase (NDH2) (Fang & Beattie, 2002; Verner et al., 2012) are both able to deliver electrons to ubiquinone, while complex I is present but probably it does not contribute to electron transport (Surve et al., 2012). NDH2 is a single enzyme alternative to complex I facing the IMS. The function of G3PDH is coupled with glycosomal NAD-dependent glycerol-3-phosphate dehydrogenase to form the glycerol-3-phosphate:dihyroxyacetone phosphate shuttle (G3P:DHAP). This shuttle helps to regenerate NAD$^+$ reduced during glycolysis in the glycosomes. Trypanosoma alternative oxidase (TAO) is capable to transfer electrons from ubiquinol to oxygen, which is reduced to water. G3P:DHAP and TAO are produced mainly in the BSF, when glycolysis is the main source of ATP and there is need for NAD$^+$ regeneration. (Chaudhuri et al., 1998; Škodová et al., 2013).

Based on the recent proteomic study by Acestor et al (2011), all four complexes of OXPHOS as well as complex V were shown to be present in the PCF *T. brucei*
mitochondrion. On the contrary, complex III and complex IV together with cytochrome c are absent in the BSF (Tielens & Van Hellemond, 1998).

Although most of the DNA sequences and proteins of complex I subunits has been found (Opperdoes & Michels, 2008; Pagliarini et al, 2008; Kannan & Burger, 2008; Panigrahi et al, 2008), the activity does not seem significant (Verner et al, 2011). Ablation of several core subunits by RNAi in PCF and BSF does not significantly affect cell metabolism and growth (Verner et al, 2011; Surve et al, 2012). Based on these observations we can deduce that T. brucei does not use complex I or in very limited extent and instead it uses NDH2 and G3PDH as electron donors. Complex II, the integral enzyme of TCA cycle, is present and active in T. brucei IM, but it is essential only for PCF grown without glucose using amino acids as a primary carbon source (Coustou et al, 2008). Based on the comparative proteomics, complex II is more abundant in the PCF (Butter et al, 2012; Verner et al, 2015). As mentioned above complexes III and IV are absent in BSF T. brucei, but they are essential for PCF (Horváth et al, 2005).

Last part of the OXPHOS pathway is ATP synthase (complex V), which is essential in both T. brucei stages (Schnaufer et al, 2005; Zíková et al, 2009). In PCF, it has similar function as in the typical mitochondrion and produces ATP from ADP and inorganic phosphate energizing the reaction from electrochemical gradient of protons on IM. Most peculiarly, in the BSF it operates in the opposite way. Since the ATP from OXPHOS is no longer necessary, OXPHOS complexes that pump protons from mitochondrial matrix to IMS are not present. Consequently, protons are not pumped from the mitochondrial matrix to the IMS, but the need for proton gradient on IM is still required for protein translocation to mitochondria and transport of different substrates. This contradiction is solved by the ATP synthase, which works in the reverse direction, pumping H⁺ from the matrix to IMS, while ATP is hydrolyzed, producing ADP and Pi (Schnaufer et al, 2005).
Figure 3. Mitochondrial energy metabolism based on Verner et al, (2015). Schematic representation of mitochondrial energy metabolism in T. brucei. Black lines depict pathways found in T. brucei; white lines depict pathways found in mammalian cells; purple lines depict reactions present only in the BSF of T. brucei; pink lines depict transit of electrons in the respiratory chain. Pathways with a green background represent those present in both PCF and BSF trypanosomes. Pathways with blue background are present in BSF only; orange represents pathways upregulated in BSF; yellow background and dotted lines indicate putative proteins whose activity has not been experimentally verified. Enzymes: (1) citrate synthase; (2) and (3) aconitase; (4) isocitrate dehydrogenase; (5) a-ketoglutarate dehydrogenase (2-oxoglutarate); (6) succinyl-CoA synthetase; (7) succinate dehydrogenase/complex II; (8) fumarase; (9) malic enzyme; (10) malate dehydrogenase; (11) fumarase; (12) fumarate reductase; (13) L-proline dehydrogenase; (14) pyrroline-5-carboxylase; (15) L-glutamine deaminase; (16) glutamate dehydrogenase; (17) asparagine synthetase; (18) aspartate aminotransferase; (19) L-threonine dehydrogenase;
(20) Ac-CoA:glycine C-acetyltransferase; (21) succinyl CoA synthetase; (22) acetate:succinate CoA transferase; (23) pyruvate dehydrogenase; (24) alternative NADH:ubiquinone oxidoreductase (rotenone-insensitive); (25) glycerol-3-phosphate dehydrogenase; (26) succinate dehydrogenase. Abbreviations: Ac-CoA, acetyl Coenzyme A; AOX, alternative oxidase; C, cytochrome c; cl, cII, cIII, cIV, and cV, respiratory chain complexes; DHAP, dihydroxyacetone phosphate; G3P, glycerol 3-phosphate; MCP, mitochondrial carrier protein; Pi, inorganic phosphate; OM, mitochondrial outer membrane; S-AdoMet, S-adenosylmethionine; Ub, ubiquinone.

3.3. Protein translocation

During endosymbiont-to-mitochondrion transition, most of the bacterial genes were either transferred to the host cell nucleus or lost. Consequently, large numbers of mitochondrial proteins are translated on free ribosomes in the cytosol and they need to be delivered into the mitochondria. Therefore, mitochondrial functions are largely dependent on the protein import from cytosol. Thus, eukaryotes had to develop precise and highly specific pathway to recognize and efficiently transport proteins to the correct location in the mitochondrion. Proteins are translocated mostly post-translationally, however recently it has been reported that some of the mitochondrial proteins can be imported also co-translationally from ribosomes attached or very close to OM (Williams et al, 2014; Schulz et al, 2015). More than 99% of the *T. brucei* mitochondrial proteins are nuclear encoded (Panigrahi et al, 2009) and have to be imported to the mitochondrion.

3.3.1. Mitochondrial targeting sequences

The first step of the protein import to the mitochondrion is the translocation across the OM. Mitochondrial precursor proteins are recognized by specific receptors, which followed by their import to the correct mitochondria sub-compartment. Recognition and sorting of proteins to the correct destination is based on the mitochondrial targeting sequences (MTS).

There are three types of MTS (fig.4): 1) The most common MTS is the N-terminal peptide extension of the preprotein (N-MTS), which is removed upon import to the mitochondria (Vögtle et al, 2009). The length and amino acid composition of the N-MTSs
are highly variable, which usually form positively charged amphipathic α-helix, recognized by mitochondrial membrane translocases. It often contains partially conserved cleavage site where MTS is proteolytically cleaved from the preprotein by mitochondrial processing peptidases. This extension is present in more than 60% of mitochondrial precursor proteins (Vögtle et al, 2009). 2) Non-cleavable MTSs are located mostly internally and remains within the mature protein. The amino acid sequences and the location of non-cleavable MTSs are not conserved. We can distinguish six different kinds of signals for translocation (Chacinska et al, 2009). 3) Non-cleavable internal signal for intermembrane space, which contains cysteine inside the signal sequence and around it. Cysteine residues form a transient disulfide bridge with IMS translocase Mia40 that arrest the substrate within IMS (Milenkovic et al, 2009).
Figure 4. Targeting and Sorting Signals of Mitochondrial Precursor Proteins (Chacinska et al., 2009). MPP, mitochondrial processing peptidase; IMP, inner membrane peptidase complex; MIA, mitochondrial intermembrane space assembly; Mim1, putative insertase of the mitochondrial outer membrane; PAM, presequence translocase-associated motor; SAM, sorting and assembly machinery; Tim9-Tim10, chaperone complex in the intermembrane space; TIM22, carrier translocase of the inner membrane; TIM23, presequence translocase of the inner membrane. TOM, translocase of the outer membrane; the putative signal directing β-barrel precursors through the TOM complex is shown in light blue. The mitochondrial intermembrane space signal for the MIA pathway is shown in red.

N-MTSs are typically present in precursor proteins that are targeted to mitochondrial matrix. Canonical N-MTSs are approximately 15-50 amino acids (AAs) long that form amphipathic α-helices, although presequences shorter than 10 amino acids and longer than 90 were also observed (Mossmann et al., 2012). Amino acid sequences of N-MTSs are usually rich in hydrophobic and basic residues, such as arginine (R) that provide the net positive charge, otherwise they are not conserved (Gakh et al., 2002) except the cleavage site motif, which is recognized by mitochondrial processing peptidase. The
cleavage site typically contains arginine, which is at position -2 (R-2) from the cleavage site (C-terminus of MTS) (Huang et al, 2009; von Heijne, 1986; Mossmann et al, 2012), aromatic and bulky hydrophobic AAs at the +1 position, and hydrophilic AAs at the +2 and +3 positions (Shimokata et al, 1997; Song et al, 1998; Mossmann et al, 2012).

A number of mitochondrial precursor proteins do not contain cleavable N-MTS and the mitochondrial signal is embedded within the mature protein. These proteins include mitochondrial outer membrane proteins, most of the intermembrane space proteins, most of inner membrane proteins and a few matrix proteins. Primary amino acid sequence of these targeting signals and its position within the protein is highly variable (Kutik et al, 2008; Beilharz et al, 2003; Brandner et al, 2005; Chacinska et al, 2009; Milenkovic et al, 2007). For summary of different MTSs, please see Figure 4.

Mitochondrial preproteins of T. brucei possess N-MTSs with similar properties as other studied eukaryotic MTSs. Initial analysis of a few mitochondrial preproteins indicated that N-MTSs are considerably shorter in T. brucei (between 8 to 20 amino acids) (Hauser et al, 1996; Bertrand & Hajduk, 2000; Häusler et al, 1997; Priest & Hajduk, 1995), which was interpreted as a sign of a primitive organelle (Schneider et al, 2008). However, later it was shown that some mitochondrial preproteins also possess longer N-MTS and interestingly, T. brucei is also able to deliver proteins with canonical N-MTS from other organisms to the mitochondrion (Long et al, 2008b; Hamilton et al, 2014; Krnáčová et al, 2012).

Import of T. brucei mitochondrial precursor proteins with non-cleavable MTS to mitochondria is still poorly understood. However, we can estimate from the first results, that these MTS are similar to those found in other eukaryotes (Basu et al, 2013; Eckers et al, 2012).

3.3.2. Mitochondrial protein import complexes

Mitochondrial protein precursors are translocated from the cytosol to mitochondria by mitochondrial translocation machinery. This machinery consists of multiple proteins and protein complexes, localized in both outer and inner membranes as well as IMS. Translocation machinery not only import proteins to mitochondria, but it also sorts them to different mitochondrial sub-compartments.
The first step of newly cytosol-synthesized mitochondrial preproteins is their recognition and translocation through the translocase of the outer mitochondrial membrane (TOM). TOM complex consists of three receptor proteins (Tom70, Tom22 and Tom20), the channel-forming protein Tom40 and three other proteins (Tom5, Tom6 and Tom7), responsible for assembly and stability of the complex. α-helical amphipathic N-MTS is recognized by the cytosol-facing receptor Tom20 and Tom22, based on the hydrophobicity (Brix et al., 1997), whereas Tom70 recognizes integral protein of IM without N-MTS. Next, the preprotein is transferred through the pore formed by Tom40 with contribution of Tom5 (Dietmeier et al., 1997).

After passing through OM it is retained by intermembrane space domain of Tom22 for further sorting. Matrix proteins are transferred to the translocase of the inner mitochondrial membrane complex TIM23. TIM23 consists of pore-forming proteins Tim23 and Tim17, presequence receptor Tim50, Tim21 and recently identified Mgr2 (Ieva et al., 2014; Gebert et al., 2012; Chacinska et al., 2005). Preproteins bind to the Tim50 and then, are pulled through Tim23 formed channel (Geissler et al., 2002). Transport of the preprotein through IM requires ATP-driven presequence translocase-associated motor complex (PAM) and membrane potential. Core protein of PAM complex is the ATP-dependent mitochondrial heat shock protein 70 (mtHSP70), which is regulated by Mge1, Pam16, Pam17 and Pam18. Tim44 anchors whole PAM complex to TIM23 complex from the matrix side of the membrane (Becker et al., 2012; Neupert & Herrmann, 2007). Some of the IM proteins are integrated to the membrane directly by TIM23 complex, while others are transported to the matrix and subsequently integrated to IM by oxidase assembly complex (OXA). OXA is a complex formed by dimers or oligomers of Oxa1. OXA complex also insert several hydrophobic subunits of respiratory chain complexes encoded in mitochondrial genome (Bohnert et al., 2015; Krüger et al., 2012; Dolezal et al., 2006).

Integral proteins of IM without cleavable MTS are recognized in the cytosol by Tom70 receptor and transported to IMS by Tom40. Hydrophobic preproteins are transferred through IMS by small Tim chaperons (Tim9 and Tim10) and by means of membrane potential integrated to the IM by TIM22 complex. It consists of pore-forming protein Tim22, Tim18 and Tim54 and to this structure Tim9, Tim10, Tim12, Tim8-Tim13 complex and Sdh3 are bound from the IMS side (Neupert & Herrmann, 2007; Chacinska et al., 2009; Rehling et al., 2003; Gebert et al., 2011; Hoppins & Nargang, 2004).
Many proteins in IMS such as Tim9 and Tim10 are sorted based on specific sorting signal, which contains conserved cysteines. This signal is recognized by mitochondrial intermembrane space assembly (MIA) machinery (Milenkovic et al., 2009; Stojanovski et al., 2008). Core of MIA machinery consists of two proteins, Mia40 and Erv1. Mia40 binds proteins with signal sequence via disulfide bridge formation trapping the translocated protein in the IMS (Chacinska et al., 2004). Afterwards, Mia40 catalyzes the forming of intramolecular disulfide bridges in the translocated protein, which causes release of the protein and reduction of Mia40. Sulfhydryl oxidase Erv1 catalyzes reoxidation of Mia40 transferring electrons to cytochrome c (Terziyska et al., 2007; Neal et al., 2015).

Two classes of proteins are inserted to OM: β-barrel proteins containing multiple β-strands, which are of bacterial origin, and α-helical proteins, which are probably eukaryotic. Both classes of proteins are translocated by TOM complex, but distinct machineries arrange the integration to OM.

β-barrel proteins are recognized and transported across OM by TOM complex followed by delivery of the proteins by small Tim chaperons to sorting and assembly machinery (SAM) complex, which is responsible for integration of the proteins. SAM complex and its function is conserved from bacteria to humans. It consists of channel-forming Sam50 (member of Omp85 protein family) and two proteins facing cytosol, Sam35 and Sam37. Recent data suggest that SAM complex forms supercomplex with TOM complex by the means of Tom22, which would avoid the necessity of small Tim chaperons in the pathway (Qiu et al., 2013; Bohnert et al., 2015). It was further shown that protein Mdm10, which is associated with SAM complex, is required for the biogenesis of Tom40. Interestingly, Mdm10 is also subunit of the endoplasmic reticulum-mitochondria encounter structure (ERMES) (van der Laan et al., 2012).

Mitochondrial OM proteins containing α-helical transmembrane domains such as Tom20 and Tom70 are bound in the cytosol by TOM complex but they do not pass via Tom40 channel. Instead, they are incorporated to OM via the mitochondrial import machinery (MIM) complex, which consists of Mim1 and Mim2 proteins (Dimmer et al., 2012).

Translocases of *T. brucei* appears to be highly divergent from most of studied opisthokonts (Fig. 5). Despite much effort, typical TOM complex was not identified in *T. brucei* genome or proteome. However, homologous beta barrel protein in OM was recently described that appeared to be essential for the mitochondrial protein import.
(Pusnik et al., 2011, 2012). It was named archaic translocase of the outer mitochondrial membrane (ATOM40) as the phylogenetic analysis suggested that ATOM40 is related to bacterial β-barrel Omp85 family, which has been shown to be required for protein translocation in the outer membranes of bacteria (Pusnik et al., 2011). Contrary to this concept, detail analysis by Žárský et al. (2012) suggested that ATOM40 is a divergent eukaryotic Tom40, thus questioning the concept of the ancient trypanosomal mitochondria. Later, another five subunits of ATOM complex were described: ATOM69, ATOM46, ATOM14, ATOM12 and ATOM11 (Mani et al., 2015). Except ATOM14, which shows limited similarity to Tom22, all subunits of ATOM complex seems to be unique for Kinetoplastids.

Schneider et al. (2008) identified a homolog of Sam50 in T. brucei genome. Although no Sam35 and Sam37 were found, proteins sharing sequence similarity with metaxins can be recognized in the genome. Metaxins are proteins known to be associated with SAM complex participating in preprotein import into mammalian and plant mitochondria (Armstrong et al., 1997, 1999; Lister et al., 2004).

Despite an effort to identify components of TIM complexes, only a single gene for pore-forming protein Tim17 was found in T. brucei (Singha et al., 2008). Interestingly, neither Tim22 nor Tim23 were identified, indicating that a single multifunctional pore possibly fulfills the function of Tim22 and Tim23 complexes. Lately, Tim50 homolog of TIM23 complex was found (Duncan et al., 2013) and three novel proteins of T. brucei TIM complex, TbTim47, TbTim54 and TbTim62. These proteins form complex with TbTim17 of molecular weight about 1100 kDa, which is much larger than in other eukaryotes (Singha et al., 2012; Gentle et al., 2007). Singha et al., (2015) discovered that TbTim62 is a structural backbone of the large TIM complex. It was also shown, that TIM complex in T. brucei is responsible for tRNA import to the mitochondria (Tschopp et al., 2011).

Integral proteins of IM are inserted to IM by TIM22 complex with contribution of small Tims. Although TIM22 is possibly not present in T. brucei, small Tim chaperons (homologs of Tim9, Tim10, Tim8 or Tim13) were found in the genome (Gentle et al., 2007). The PAM complex is likely present in T. brucei as most of the core subunits (Tim44, Pam18, Mge1 and mtHSP70) were identified in the genome (Schneider et al., 2008). Interestingly, Erv1 homolog of MIA machinery is present, while Mia40 seems to be absent (Allen et al., 2008). It was shown that TbErv1 is essential for mitochondrial biogenesis but does not function as canonical MIA complex (Basu et al., 2013). Moreover
Eckers et al (2012) were unable to replace Saccharomyces cerevisiae Erv1 with close relative to T. brucei, Leishmania tarentolae Erv1 probably due to altered cysteine patterns and protein architectures, even though the sulfhydryl:cytochrome c oxidoreductase activity was shown in both yeast and Leishmania Erv1 homologs. Currently it is unclear what is the substrate of TbErv1 and its exact function.

OXA machinery integrates mostly mitochondrial encoded proteins to IM. Both Oxa1 and Oxa2 genes homologs were identified in T. brucei genome. This is especially intriguing as it was suggested that Oxa2 recently derived from Oxa1 by gene duplication (Preuss et al, 2005) whereas kinetoplastids are believed to diverge very early in eukaryotic evolution (Mani et al, 2015; Cavalier-Smith, 2010).

Figure 5. Schematic contemporary perspective to T. brucei mitochondrial protein translocation and processing (according to Verner et al (2015)). Proteins from cytosol are translocated via complex of archaic translocase of the outer mitochondrial membrane (ATOM) to the intermembrane space (IMS). Sorting and assembly machinery (Sam50 and metaxin) inserts proteins to outer mitochondrial membrane (OM). Proteins targeted towards mitochondrial matrix or IM are further transported by translocase of the inner
mitochondrial membrane (TIM) complex and mitochondrial heat shock protein 70 (mtHSP70), presequence translocase-associated motor (Pam18) and mitochondrial GrpE protein (Mge1). Transmembrane proteins are integrated to IM also by oxidase assembly complex (Oxa). Mitochondrial targeting sequence is cleaved by mitochondrial processing peptidase (MPP) and further by intermediate cleaving peptidase (Icp55) or mitochondrial intermediate peptidase (MIP). Substrate for inner membrane peptidase (Imp2) is unknown.

### 3.3.3. Mitochondrial processing peptidases

Most of the proteins imported to the mitochondrial matrix have N-MTS, which has to be proteolytically removed upon translocation for the maturation and proper folding. Processing of N-MTS in the mitochondrial matrix is executed by mitochondrial processing peptidase (MPP). MPP is a zinc-dependent metallopeptidase that consists of α and β subunits (Schneider et al., 1990; Géli, 1993). The β subunit possesses conserved metal binding motif (HXXEHXₐE) within its catalytic site and is responsible for the cleavage of N-MTS (Kitada et al., 1995; Striebel et al., 1996), while the α subunit possesses conserved glycine rich loop domain that serves for recognition of substrates (Nagao et al., 2000; Dvořáková-Holá et al., 2010; Kučera et al., 2013). Core I (Cp1) and core II (Cp2) components of the mitochondrial respiratory complex III are homologs of β-MPP and α-MPP, respectively (Braun and Schmitz 1995). These proteins were recognized to cleave off the presequence of Rieske protein precursor (Deng et al., 2001). Sequence homology of Cp1 and Cp2 with β-MPP and α-MPP is apparent, however the zinc-binding motif and glycine rich loop are incomplete or missing in the metazoan core proteins. Interestingly, in plants no matrix forms of MPP were found, and N-MTSSs of mitochondrial matrix proteins are processed by Cp1/2 proteins that possess complete zinc-binding and glycine rich loop motifs (Eriksson et al., 1996; Gencic et al., 1991).

After cleavage by MPP, some proteins have to be further processed for correct function. Mitochondrial intermediate peptidase (MIP also known as Oct1) cleaves another eight amino acids after MPP cleavage site (F/L/I)XX(T/S)XXXX↓X (Gavel & von Heijne, 1990; Gakh et al., 2002; Chew et al., 1996). It is a soluble monomeric metalloprotease in the mitochondrial matrix with the zinc-binding motif and two conserved cysteines responsible for the protein stability (Gakh et al., 2002; Chew et al., 1996). Recent identification of substrates in yeast shed more light on the cleavage site motif and
confirmed predominantly arginine at-10 position, phenylalanine and leucine at position -8, and the abundance of serine and threonine, which are distributed across positions -5, -6 and -7 from the cleavage site (Mossmann et al., 2012).

The intermediate cleaving peptidase of 55 kDa (Icp55) is another mitochondrial matrix peptidase, which processes some proteins after MPP. It removes only one amino acid next to MPP cleavage site. The cleavage site of Icp55 R-X-(F/L/Y)↓(S/A) was found in the plant and yeast mitochondria (Huang et al., 2009; Timmer et al., 2007) before identification of responsible peptidase (Vögtle et al., 2009; Naamati et al., 2009). Icp55 is attached to IM facing matrix and interestingly, it was found in nucleus (Naamati et al., 2009). Proposed reason for the dual Icp55 localization is dual localization of IscS (Nbp1), the Icp55 substrate. Interestingly, in nucleus Icp55 removes three amino acids from IscS, which is somehow processed by MPP in mitochondria (Naamati et al., 2009). The cleavage of specific AA residues by MIP or Icp55 is important for stabilization of mature proteins (Vögtle et al., 2011; Sriram et al., 2011).

The inner membrane peptidase (IMP) is an integral protein complex within the IM, facing IMS. In yeast, it is composed of two catalytic subunits Imp1 and Imp2, and non-catalytic protein Som1 (Nunnari et al., 1993; Schneider et al., 1994; Jan et al., 2000). IMP processes both nuclear and mitochondrial encoded precursors and some of them need to be first cleaved by MPP (Gasser et al., 1982; Esser et al., 2004; Hahne et al., 1994). Unusual feature of IMP is that each catalytic subunit cleaves distinct set of substrates. Only one subunit of IMP complex, homolog of yeast Imp2, was found in T. brucei genome (Schneider et al., 2008).

T. brucei has similar range of mitochondrial proteins as other eukaryotes and albeit its translocase machinery is divergent, the MTSs are comparable. Thus, we would expect also similar processing peptidases, which process mitochondrial precursors. Indeed, homologs of all main processing peptidases were found in T. brucei genome. Both MPP subunits as well as Cp1 and Cp2 were identified (Desy et al., 2012; Mach et al., 2013). T. brucei genome also contains a homolog of MIP, at least three homologs of Icp55 (Verner et al., 2015), and one subunit of IMP, homolog of Imp2.
4. Iron

Iron is an essential nutrient for all living organisms. It is so widely used because of flexibility of its redox potentials and capacity to undergo electron transfer and acid-base reactions (Crichton, 2001). Iron is of utmost importance for all organisms. Iron requirements are especially high for parasites, which have to propagate much faster than their host (Šuťák et al, 2008). However, there are two sides of iron. On one side, it is necessary component of multiple proteins and pathways that are essential for cell survival, including nucleic acid synthesis/DNA replication, lipid synthesis, protein translation, energy metabolism, oxygen sensing/transport, and oxidative defense (Nairz et al, 2014; Šuťák et al, 2008). On the other side, free non-bound iron is highly toxic, catalyzing production of reactive oxygen species (ROS) by Fenton’s reaction, which can damage lipids, DNA, proteins, and other cellular components (for review see Dixon & Stockwell, 2014). Therefore, iron level in all cells has to be under strict control.

In metazoans, most of the iron is stored within the cells and only small amount is extracellular and bound in specialized proteins to prevent parasites to gain access to the iron. Therefore, to establish infection within the host, the parasites developed various mechanisms for iron acquisition (Weinberg, 2000; Šuťák et al, 2008; Doherty, 2007).

4.1. Iron import

There is several different pathways used by organisms to acquire iron from the environment. The character of the pathway depends on the form of the available iron. Parasites such as T. brucei, which lives in different hosts, have to be very malleable and adapt their iron import mechanisms.

Bloodstream form of T. brucei acquires iron by receptor-mediated endocytosis of host (mammalian) transferrin within the flagellar pocket. The receptor is the hetero-dimeric protein complex consisting of ESAG6 and ESAG7. ESAG6 possesses glycosylphosphatidylinositol-anchor, which embeds receptor in the membrane (Steverding, 2000; Chaudhri et al, 1994). The receptor is internalized upon binding of transferrin by receptor-mediated endocytosis, and iron is released in the lysosome after acidification of the endosome (Taylor & Kelly, 2010). Translocation of iron to the cytosol requires endosomal ferric reductase and divalent metal transporters (Taylor et al, 2013; Guerinot,
2000; Basu et al, 2016). Iron is taken up also in a form of heme, more precisely as a compound of haptoglobin related protein and hemoglobin from the blood. BSF high affinity haptoglobin-hemoglobin receptor (HpHbR) is localized in the plasma membrane within the flagellar pocket (Higgins et al, 2013; Vanhollebeke et al, 2008). It was recently shown that an ability of this receptors to bind human lytic factor is responsible for human innate immunity to *T. brucei brucei* (Vanhollebeke et al, 2008; Higgins et al, 2013). Apart from transferrin, *T. brucei* is able to acquire iron from iron-binding glycoprotein lactoferrin (Tanaka et al, 2004).

It could be estimated that iron requirements are much higher in the PCF then in BSF taking to account that BSF mitochondrion loses most of pathways that are dependent on Fe-S cluster and heme dependent proteins. PCF dwells within the insect midgut, where blood digestion occurs. Transferrin and hemoglobin is also degraded and thus PCF *T. brucei* cannot utilize the same transporters as BSF. Recently, it was shown that *Leishmania amazonensis* Lhr1 receptor can import free heme from the environment (Huynh et al, 2012). The same mechanism is probably used by trypanosomes, as the homolog of Lhr1 was found in *T. brucei* genome (Basu et al, 2016). Although, large amounts of ferrous iron are released, it is not known how *T. brucei* PCF takes up free iron from insect environment.

Within the cell, iron is needed mostly in mitochondria for heme synthesis and mitochondrial Fe-S cluster assembly. It was shown in *S. cerevisiae* that mitochondrial IM transporter, heterodimeric complex of Mrs3 and Mrs4 import ferrous iron into the mitochondria in a membrane potential-dependent manner (Mühlenhoff et al, 2003; Foury & Roganti, 2002). Carrier *Tb*MCP17 with high sequence similarity to Mrs3 was found in *T. brucei* genome and localized to mitochondria (Colasante et al, 2009). Recently, Mittra et al, (2016) characterized MIT1, homolog of yeast Mrs3 and human mitoferrin-1 in *Leishmania amazonensis* and *T. brucei* and showed that it is essential for PCF but not for BSF.

### 4.2. Iron-sulfur clusters

Fe-S clusters are simple inorganic complexes consisting of iron and inorganic sulfur that are coordinated mostly by cysteine amino acid residues in the Fe-S proteins. Fe-S proteins were described in variety of proteins of almost all living organisms. We can
find them in many reactions including oxidative phosphorylation pathway, TCA cycle, amino acids biosynthesis, DNA metabolism, tRNA modification and essential proteins including ferredoxins, hydrogenases, nitrogenases and many others. Based on their distribution across all life forms and cellular pathways, we can imply its ancient origin (Lill, 2009). Fe-S clusters are assembled by several different systems (Blanc et al, 2015). Most of the studied eukaryotes assemble Fe-S clusters in mitochondria by mitochondrial iron-sulfur cluster (ISC) pathway and in the cytosol by cytosolic iron-sulfur cluster assembly (CIA) pathway. Interestingly, ISC pathway is necessary for Fe-S cluster production in the cytosol. Even in organisms with highly reduced form of mitochondria, the ISC pathway is usually maintained, indicating that Fe-S cluster assembly pathway can be the reason for retain of mitochondria (Lill, 2009; Goldberg et al, 2008).

In mitochondria, elemental sulfur is provided by the conversion of cysteine to alanine, which is catalyzed by cysteine desulfsurase complex IscS-Isd11 through a persulfide intermediate (IscS is Nfs1 in yeast) (Zheng et al, 1994; Adam et al, 2006; Wiedemann et al, 2006). Sulfur is further reduced to S²⁻ probably by ferredoxin reductase and ferredoxin as an electron donor (Lange et al, 2000; Li et al, 2001). Iron is imported to mitochondria from the cytosol as Fe²⁺ and somehow interacts with frataxin. However, it is still under discussion what is the exact role of frataxin in FeS assembly (Stehling et al, 2014; Adinolfi et al, 2009; Stemmler et al, 2010; Gerber et al, 2003). Iron and sulfur are assembled to [2Fe-2S] cluster on the scaffold made by IscU protein (Garland et al, 1999). Chaperons Mge1, Jac1 and Hsp70 deliver [2Fe-2S] cluster (Kampinga & Craig, 2010; Vickery & Cupp-Vickery, 2007; Dutkiewicz et al, 2004; Uzarska et al, 2013) to monothiol glutaredoxin (Grx5) (Shakamuri et al, 2012). Then it is transferred to target protein or converted by IscA1, IscA2 and Iba57 into [4Fe–4S] cluster (Mühlenhoff et al, 2011; Gelling et al, 2008), which is inserted to different proteins by specific ISC targeting factors (Nfu, Ind1, Aim1) (Stehling et al, 2014; Cameron et al, 2011; Navarro-Sastre et al, 2011; Bych et al, 2008).

Unknown sulfur-containing compound is exported from mitochondria to the cytosol, where it is necessary for CIA pathway. This compound is transported by ABC transporter Atm1 of the IM (Kispal et al, 1999) with involvement of sulphhydryl oxidase Erv1 and glutathione (Lange et al, 2001; Sipos et al, 2002).

All ISC machinery components are present in the genome of *T. brucei* (Manta et al, 2013; Verner et al, 2015). Homologs of IscS and IscU were shown to be essential for the
function of FeS proteins that are involved in mitochondrial energy metabolism of PCF. Their downregulation caused metabolic changes that resemble function of downregulated mitochondria in BSF (Šmíd et al., 2006). TbIsd11 and TbIscS was shown to participate not only in Fe-S cluster assembly but also in tRNA thiolation, connecting two seemingly unrelated pathways (Paris et al., 2010). Even though, ferredoxin reductase homolog and two homologs of human ferredoxins TbFdxA and TbFdxB were found in T. brucei (Basu et al., 2016; Verner et al., 2015) only TbFdxA localized to mitochondria where it is essential for Fe-S cluster assembly (Changmai et al., 2013). Function of Tbfrataxin was studied together with its necessity for T. brucei growth. It is essential and its ablation highly reduces both cytosolic and mitochondrial activities of Fe-S enzymes (Long et al., 2008a, 2008b, 2008c). TbIsa1, and TbIsa2 homologs of IscA1, and IscA2, respectively are essential for PCF but neither of them for BSF (Long et al., 2011). Homologs of both components of sulfur-containing compound export pathway are essential for CIA pathway and thus for both stages of T. brucei (Horáková et al., 2015; Basu et al., 2013).

Figure 6. Schematic representation of mitochondrial iron-sulfur (ISC) machinery in mitochondria of T. brucei (adapted from Lukeš & Basu (2015)). Ferrous iron (red dot) is delivered from the cytosolic iron pool to the ISC machinery by MCP17. The role of frataxin in utilization of iron in the mitochondria is essential but still unknown (broken
Sulphur is acquired from cysteine by cysteine desulfurase complex (IscS-Isd11) and reduced by ferredoxin reductase (FdxR) and ferredoxin (FdxA). Iron and sulphur are delivered to the iron-sulfur cluster assembly enzyme (IscU) scaffold and with assistance of chaperons heat-shock protein 70 (Hsp70), J-type accessory chaperone (Jac1), mitochondrial GrpE protein (Mge1) and glutaredoxin (Tb1-C-Grx1) incorporated to the target apoproteins. Examples of mitochondrial Fe-S proteins are succinate dehydrogenase (SDH) and mitochondrial aconitase and fumarase. Sulphur bound to unknown carrier (X-S) is transported from the mitochondria by mitochondrial ABC transporter (Atm1) and sulfhydryl oxidase Erv1 to the cytosolic iron-sulfur cluster assembly (CIA) machinery.
4.3. Heme metabolism

Heme belongs to the tetapyrrol family of compounds, so called “cofactors of life” together with chlorophyll or cobalamin (vitamin B12). It occurs in almost all organisms. Heme contains iron, which is coordinated in the porphyrin ring. It is widely utilized in various biological functions such as electron transfer, transport of diatomic gases and a number of chemical catalysis. Heme is well known as a cofactor of hemoglobin that transport oxygen and carbon dioxide in the blood; however, heme is also necessary for the oxidative phosphorylation pathway. Aside the most common heme $b$, which is mostly found in hemoglobin and myoglobin, there are another two major types: heme $a$ and heme $c$ that are involved in many cell processes including oxidative phosphorylation.

Most organisms synthesize their own heme. Heme synthesis appears to be an ancient pathway and eukaryotes apparently utilizes pathways from endosymbiotic predecessors of mitochondria and/or chloroplast, therefore it is usually associated with these cell compartments (Kořený et al., 2013). The heme synthesis pathway is fairly conserved among all organisms except the synthesis of the heme precursor 5-aminolevulinic acid (ALA). While $\alpha$-proteobacteria and most non-photosynthetic eukaryotes uses ALA-synthetase for condensation of glycine and succinyl-CoA to synthesize ALA, other organisms use three consecutive steps to create ALA. The remaining seven steps of the pathway leading to protoheme are conserved in all organisms that are able to synthesize heme.

However, some organisms lost their ability to synthesize heme. Most of the organisms without heme synthesis pathway are able to obtain heme from exogenous sources by feeding on other organisms (Caenorhabditis elegans) or taking heme from mammalian blood by parasitism (tick Boophilus microplus, Trypanosoma brucei) (Kořený et al., 2010). Other group of organisms without capability to produce heme are anaerobic parasitic protists with highly reduced mitochondria such as Giardia, Cryptosporidium, Blastocystis and Trichomonas (Kořený et al., 2013, 2010). Utmost examples are Entamoeba histolytica, which probably lost all hemoproteins (Pyrih et al., 2014). Most interestingly, it was found that plant pathogenic kinetoplastid Phytomonas serpens can live entirely without hemoproteins, but utilizes heme when it is available (Kořený et al., 2012).

Trypanosomes belong among organisms which lost its heme synthesis pathway and need to acquire heme from the host (Kořený et al., 2010; Salzman et al., 1982; Lombardo et
Moreover, trypanosomes lack heme oxygenase and ferrochelatase that are required for the heme catabolism and release of iron. Therefore, it is believed that acquired heme is not further processed but directly incorporated into heme-proteins (Kořený et al., 2010). *T. brucei* incorporates heme into variety of proteins of sterols and polyunsaturated fatty acids biosynthesis, respiration, oxidative stress response, and detoxification (Tripodi et al., 2011). Interestingly, Vanhollebeke et al., (2008) discovered that BSF *T. brucei* are able to survive *in vitro* and *in vivo* without HpHbR, which indicates similar dispensability of heme as for *P. serpens*. On the contrary, PCF *T. brucei* has to be maintained *in vitro* with heme-derived compounds like hemin. In spite of the high toxicity of the heme, it is not known how heme is transported within the cell (Tripodi et al., 2011; Severance & Hamza, 2009).
5. Aims

To contribute to the greater understanding of evolution and function of *T. brucei* mitochondria, I focused on following aims:

1) To characterize processing of mitochondrial targeting sequences with focus on:
   a. Characterization of N-terminal mitochondrial targeting sequences.
   b. Characterization of mitochondrial processing peptidase
   c. Phylogenetic analysis of mitochondrial core proteins and mitochondrial processing peptidase.
2) To characterize putative pyruvate transporter in different *T. brucei* life stages.
3) To characterize mechanisms of iron uptake in insect stage of *T. brucei.*
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7. List of publications and author contribution


Cell cultivation, cloning of TbMPP to plasmids for localization in *T. brucei* and overexpression in *E. coli*, preparation of recombinant proteins and antibodies in rats, isolation of native TbMPP, immunofluorescent and western blot localization, isolation of mitochondria, enzymatic activities measurement, MPP cleavage assay and in silico analysis.


Cell cultivation, uptake and evaluation of radioactively labeled iron, isolation of mitochondria, measurement of enzymatic activities and blue native electrophoresis (together with Róbert Šuťák).


Cell cultivation (together with Jitka Štáfková), in silico analysis, localization by fluorescent microscopy (together with Jitka Štáfková), cell fractionation, measurement of enzymatic activities, uptake of radioactively labeled pyruvate and its evaluation (together with Zdeněk Verner), HPLC analysis.
7.1. Publication 1: Mach et al., 2013

An Advanced System of the Mitochondrial Processing Peptidase and Core Protein Family in *Trypanosoma brucei* and Multiple Origins of the Core I Subunit in Eukaryotes

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**Abstract**

Mitochondrial processing peptidase (MPP) consists of α and β subunits that catalyze the cleavage of N-terminal mitochondrial-targeting sequences (N-MTSs) and deliver preproteins to the mitochondria. In plants, both MPP subunits are associated with the respiratory complex bc₁, which has been proposed to represent an ancestral form. Subsequent duplication of MPP subunits resulted in separate sets of genes encoding soluble MPP in the matrix and core proteins (cp1 and cp2) of the membrane-embedded bc₁ complex. As only α-MPP was duplicated in *Neurospora*, it single β-MPP functions in both MPP and bc₁ complexes. Herein, we investigated the MPP/core protein family and N-MTSs in the kinetoplastid *Trypanosoma brucei*, which is often considered one of the most ancient eukaryotes. Analysis of N-MTSs predicted in 336 mitochondrial proteins showed that trypanosomal N-MTSs were comparable with N-MTSs from other organisms. N-MTS cleavage is mediated by a standard heterodimeric MPP, which is present in the matrix of procyclic and bloodstream trypanosomes, and its expression is essential for the parasite. Distinct Genes encode cp1 and cp2, and in the bloodstream forms the expression of cp1 is downregulated along with the bc₁ complex. Phylogenetic analysis revealed that all eukaryotic lineages include members with a *Neurospora*-type MPP/core protein family, whereas cp1 evolved independently in metazoans, some fungi and kinetoplastids. Evolution of cp1 allowed the independent regulation of respiration and protein import, which is essential for the procyclic and bloodstream forms of *T. brucei*. These results indicate that *T. brucei* possesses a highly derived MPP/core protein family that likely evolved in response to its complex life cycle and does not appear to have an ancient character proposed earlier for this eukaryote.

**Key words:** mitochondrial processing peptidase, bc₁ complex, mitochondrial targeting sequence, trypanosome, evolution.

**Introduction**

Parasitic protists of the *Trypanosoma brucei* family are causative agents of African sleeping sickness in humans and nagana of domestic animals. Trypanosomes undergo a complex life cycle, with the procyclic form (PF) inhabiting the gut of a tsetse fly and the bloodstream form (BF) being pathogenic in vertebrate hosts. The cyclic changes between the BF and PF are accompanied by dramatic changes in the parasite’s metabolism, particularly by the activation of the citric acid cycle and respiratory chain in the mitochondrion of the PF and their downregulation, followed by a switch to glycolysis in the BF (Clayton and Michels 1996; Besteiro et al. 2005). *Trypanosoma brucei* belongs to the class Kinetoplastea, which commonly have a large network of mitochondrial DNA termed the kinetoplast. Together with related euglenids and diplomonads, kinetoplastid flagellates form the taxon Euglenozoa within the eukaryotic supergroup Excavata (Adl et al. 2012). Several lineages of excavates were previously considered to be among the most ancient eukaryotes based on the ostensible absence of mitochondria (Cavalier-Smith 1987). However, the “amitochondriate” hypothesis eroded when hydrogenosomes of *Trichomonas* and mitosomes of *Giardia* were recognized as anaerobic reduced forms of mitochondria (Embley and Martin 2006). More recently,
kinetoplastids, such as *T. brucei*, were proposed to be the most ancient eukaryotes based on some unique features of their mitochondria, particularly mitochondrial protein import (Cavalier-Smith 2010).

During eukaryogenesis, the majority of genes encoded in the genome of the proteobacterial ancestor of mitochondria were lost or transferred to the nucleus. Consequently, more than 95% of proteins in the contemporary mitochondria are encoded in the nucleus, synthesized in the cytosol and imported into the mitochondrial membranes or matrix (Lill and Neupert 1996; Neupert and Herrmann 2007). Correct protein targeting and translocation is assured mainly by targeting sequences at the amino termini (N-MTS) of imported mitochondrial preproteins (Chacinska et al. 2009). The N-MTS is initially recognized by a receptor in the outer membrane translocase (TOM) complex. The preprotein is then translocated across the β-barrel-shaped Tom40 channel and interacts with the inner membrane translocase machinery (TIM), which mediates its translocation through the inner membrane. Upon translocation, the N-MTSs are cleaved by the mitochondrial processing peptidase (MPP). *Trypanosoma brucei* possesses a considerably divergent Tom40 that had not been recognized in initial bioinformatic searches of its genome, and a structurally similar voltage-dependent anion channel (VDAC) has been found as the only mitochondrial porin (Pusnik et al. 2009). More recent studies have revealed the presence of another import channel called the archaic translocase of the outer mitochondrial membrane (ATOM) (Pusnik et al. 2011). ATOM has been suggested to represent an ortholog of the bacterial Omp85 protein family, which is distinct from Tom40 and may represent an evolutionarily ancestral protein transport system. However, later this view has been challenged by Zarsky et al. (2012) who found that ATOM is most likely a divergent ortholog of a mitochondrial TOM40.

A typical mitochondrial system of inner membrane translocases includes the TIM23 complex, which is dedicated to the import of matrix proteins, and the TIM22 complex, which mediates the insertion of proteins into the inner membrane (Bauer et al. 2000). However, another unique feature of the *T. brucei* import machinery is that only a single member of the Tim17/22/23 protein family has been found in its genome, suggesting that a single TIM complex facilitates the functions of both TIM23 and TIM22 in the single mitochondrion of this unicellular parasite (Schneider et al. 2008). Finally, analysis of the N-MTSs of five mitochondrial preproteins revealed that they are considerably shorter in *T. brucei* (usually 8–20 amino acid residues [AA]) (Priest and Hajduk 1995; Hauser et al. 1996; Häusler et al. 1997; Bertrand and Hajduk 2000) compared with the average length of N-MTSs in ophistokonts and plants (20–80 AA in yeast/mammals; 20–70 AA in plants) (Burri and Keeling 2007; Huang et al. 2009). Although long N-MTSs were rarely documented in *T. brucei* (Long et al. 2008), the generally short nature of its N-MTSs was interpreted as a primitive feature of the import system (Schneider et al. 2008; Pusnik et al. 2009).

Primary sequences of AA residues in N-MTSs are usually rich in hydrophobic and basic residues, such as arginine (R), which allow for the formation of amphipathic α-helices and are generally not conserved (Gakh et al. 2002). The proximal arginine is either at position −2 (R-2) or −3 (R-3) from the cleavage site, whereas the distal arginines or other basic residues contribute to the overall positive net charge of N-MTSs (Moriwaki et al. 1999; Taylor et al. 2001; Huang et al. 2009). The site of N-MTSs cleavage contains a loosely conserved motif, with aromatic and bulky hydrophobic AA at the +1 position and hydrophilic AA at the +2 and +3 positions (Shimokata et al. 1997; Song et al. 1998). A recent proteomic analysis of the amino termini of the mature mitochondrial proteins in yeast revealed that N-MTSs with the R-2 motif are processed by MPP, whereas those with R-3 motif undergo two-step processing by MPP and lcp5S, with the latter enzyme removing just a single AA, such as tyrosine, leucine, or phenylalanine (Vogtle et al. 2009).

MPP is a zinc-dependent metallopeptidase that consists of two homologous subunits, α and β, which are both required for its processing activity (Schneider et al. 1990; Géli 1993). The α-MPP subunit possesses a highly conserved glycine-rich loop required for the recognition of N-MTSs (Nagao et al. 2000; Dvorakova-Hola et al. 2010), whereas the metal-binding motif HXXEX,xE of β-MPP is the catalytic site responsible for cleavage of the peptide bond (Kitada et al. 1995; Striebel et al. 1996). In various eukaryotes, such as metazoans and Saccharomyces cerevisiae, MPP is present in the mitochondrial matrix as a general presequence processing enzyme. In addition, these organisms contain core I (cp1) and core II (cp2) proteins, which are homologous to β-MPP and α-MPP, respectively (Braun and Schmitz 1995). The cp1/cp2 proteins are components of the mitochondrial ubiquinol–cytochrome c oxidoreductase (bc1) complex of the respiratory chain (Deng et al. 2001; Zara et al. 2004). The core proteins process a precursor of the Rieske protein, and the cleaved presequence is retained as subunit IX of the bc1 complex (Deng et al. 2001).

Unlike α-MPP, cp2 does not contain a glycine-rich loop, and compared with β-MPP, the Zn-binding motif of mammalian cp1 is incomplete (Gencic et al. 1991). None of the Zn-binding ligands are present in cp1/cp2 of *S. cerevisiae* (Braun and Schmitz 1995). Interestingly, in plants, the core proteins are identical to the α- and β-MPP subunits, and the majority of the MPP activity is associated with the bc1 complex (Eriksson et al. 1996). Thus, in addition to its core function in the respiratory chain, the bc1 complex of plants is involved in general N-MTS processing. To distinguish plant MPP subunits from cp1/cp2, we designate herein the former as cb1-α-MPP and cb1-β-MPP. It has been proposed that MPP evolved from a single subunit of a bacterial protease, followed by gene duplication and specialization that gave rise to a heterodimeric enzyme in mitochondria (Kitada et al. 2007), although more recent studies
suggested that the heterodimeric protease was formed already in bacteria (Maruyama et al. 2011). Subsequently, another gene duplication resulted in the appearance of the membrane-associated core proteins and soluble MPP subunits. The association between MPP and the bc1 complex in plants suggests that a heterodimeric MPP was part of this complex before the second gene duplication occurred, with the complete set of cp1/cp2 and soluble heterodimeric MPP representing a more recent phylogenetic event (Braun and Schmitz 1995).

The proposed ancient character of the T. brucei import system prompted us to re-evaluate features of N-MTSs in this flagellate, the enzyme responsible for their processing and the character of the core proteins. We predicted 336 N-MTSs that appeared to have standard properties when compared with other eukaryotes. We identified a complete set of genes coding for a/b-MPP subunits as well as for cp1/cp2 proteins. Both MPP subunits were expressed in PF and BF, and we demonstrated an ability of the recombinant MPP to catalyze processing of N-MTS in the predicted cleavage site. The cp1 protein was expressed only in PF, which is consistent with the independent regulation of respiration and protein import in T. brucei. To obtain insight into the evolutionary history of this interesting family of essential eukaryotic proteins, we subjected the T. brucei a/b-MPP and cp1/cp2 proteins to an extensive phylogenetic analysis that included members of all known eukaryotic supergroups.

Materials and Methods

Organisms

Trypanosoma brucei procyclic (PF) strain 29–13 with a T7 RNA polymerase under neomycin resistance and tetracycline (TET) repressor under hygromycin resistance was grown in SDM-79 medium with 10% fetal calf serum at 27 °C (Wirtz and Clayton 1995). BF strain STIB 920 of the same species was used for infection of rats and isolated from their blood using diethyl amino-ethyl cellulose chromatography (Chaudhuri et al. 1995). The BF single marker strain 427 (Wirtz et al. 1999) with a T7 RNA polymerase and TET repressor under neomycin resistance was grown in HMI-9 medium (Hirumi and Hirumi 1984) under 5% CO2 at 37 °C.

Preparation of Recombinant Proteins and Antibodies

Genes for the T. brucei Tbx-MPP (Tb927.2.4110) and Tbβ-MPP (Tb09.160.3110), Tbcp1 (Tb927.5.1060) and iron–sulfur cluster assembly protein (TbIscU) were amplified from genomic DNA of the 29–13 strain, cloned into pET42b (Novagen, Germany) and expressed in E. coli Rosetta (DE3) pLysS with the chaperonins GroEL/ES (Du Pont) in the presence of 1 mM ZnCl2 and 0.1 mM IPTG at 20 °C for 16 h. Tbx-MPP with a carboxy-terminal 6x histidine tag was expressed using a pET28a plasmid (Novagen, Germany) with ampicillin resistance. Tbβ-MPP without a tag was produced using the pET42b plasmid (Novagen, Germany) with kanamycin resistance. The heterodimeric MPP was isolated under native conditions using a nickel column (Ni-NTA Agarose, Qiagen, Netherlands) that binds the polyhistidine tag of the Tbx-MPP subunit. Cells were resuspended in lysis buffer (50 mM HEPES, 20 mM NaCl, 10 mM imidazole, 10% glycerol, pH 8) supplemented with complete ethylenediaminetetraacetic acid (EDTA)-free protease inhibitor cocktail (Roche, Switzerland) and disrupted twice by a French press (1,400 bar; Thermo Scientific, USA). The lysate was centrifuged at 200,000 × g for 10 min at 4 °C, and the resulting supernatant (cleared lysate) was loaded onto the nickel column. Unbound proteins were removed by two washes with 5 ml of lysis buffer containing 40 mM imidazole. Proteins retained on the column were stepwise eluted with lysis buffer containing increasing concentrations of imidazole (100-250 mM). Alternatively, both proteins were expressed separately in E. coli Rosetta (DE3) pLysS with the chaperonins GroEL/ES (Novagen, Germany). Histidine-tagged Tbx-MPP was isolated under native conditions on a nickel column (Ni-NTA Agarose, Qiagen, Netherlands) following the manufacturer’s instructions. Isolated Tbx-MPP (~30 μg) was added to the cleared lysate (20 ml) from the E. coli cells expressing Tbβ-MPP and incubated in dialysis tubing (Membra-Cel; MWCO 3500; Serva, Germany) for 16 h at 4 °C in 51 of the lysis buffer supplemented with 1 mM ZnCl2. The heterodimer of Tbx-MPP and Tbβ-MPP was then isolated on the nickel column as described earlier.

Cloning and Expression of TbMPP in T. brucei

Genes for Tbx-MPP and Tbβ-MPP subunits were cloned into the pJH54 plasmid (kindly provided by Christine Clayton, University of Heidelberg, Germany), which allows for the expression of recombinant preproteins with a carboxy-terminal HA-tag (Wirtz and Clayton 1995). The plasmids were linearized by the NotI restriction enzyme and electroporated into
the PF *T. brucei* by Gene Pulser Xcell (Bio-Rad, USA). The protein expressing cells were selected using 2.5 μg/ml phleomycin.

Plasmid pT7-3V5-PAC (Flaspholer et al. 2010) was used for the expression of V5-tagged Tbx-MPP and Tbjβ-MPP in BF *T. brucei*. Genes were subcloned, and the NotI-linearized plasmids were electroporated by Amaza Nucleofector (Lonza, Switzerland) and transformed cells were selected using 0.1 μg/ml puromycin. Protein expression was triggered by adding 1 μg/ml of tetracycline to the medium.

Immunofluorescent Microscopy

Approximately 1 × 10⁷ PF and BS trypanosomes were incubated with 0.5 μM Mitotracker Red CMXRos (Molecular Probes, USA) for 10 min in SDM-79 and HMI-9, respectively, washed with phosphate-buffered saline (PBS), incubated in corresponding media for another 20 min and washed with PBS. The PF trypanosomes were fixed and permeabilized on slides in −20°C cold methanol for 5 min, followed by a 5-min incubation in −20°C cold acetone. BF trypanosomes were fixed on slides in 4% formaldehyde for 5 min at room temperature, washed with PBS and permeabilized in methanol for 15 min at −20°C. The slides were incubated in blocking solution (0.25% bovine serum albumin, 0.25% gelatin, and 0.05% Tween 20 in PBS) for 1 h at room temperature. Recombinant Tbx-MPP and Tbjβ-MPP were visualized using the mouse monoclonal anti HA-tag (Sigma-Aldrich, USA) and anti V5-tag (Sigma-Aldrich, USA) antibodies, respectively, and donkey anti-rat Alexa Fluor 488 antibody (Invitrogen, USA). The cells were mounted in Vectashield with DAPI (Vector Laboratories, USA) and observed using an Olympus IX81 microscope. Images were captured using a Hamamatsu Orca-AG digital camera and processed using cell^R^ imaging software (Olympus, Japan).

Isolation and Analysis of Mitochondria

The mitochondrion-enriched fraction and cytosolic fraction from *T. brucei* BF and PF were isolated by digitonin fractionation (Smid et al. 2006). The purity of fractions was assessed by measuring the enzymatic activity of mitochondrial and cytosolic marker enzymes threonine dehydrogenase and pyruvate kinase, respectively (Smid et al. 2008). Motifs of N-MTSs were visualized with Weblogo software (Crooks et al. 2004). The reaction mixture contained 0.5 μg of recombinant TbstsU as substrate and 0.2 μg of recombinant TbtMPP heterodimer or 2 μg of mitochondrial lyase in the reaction buffer (50 mM HEPES, 20 mM NaCl, and 1 mM MnCl₂, pH 8.6) in a total volume of 20 μl. The reaction was conducted for 30 min at 27°C and stopped by boiling for 5 min in SD-S-PAGE sample buffer. The cleavage products were separated by SD-S-PAGE and stained with Coomassie brilliant blue. The mitochondrial lyase was prepared by treating the mitochondrion-enriched fraction for 5 min with 0.1% Triton X-100, followed by centrifugation for 5 min at 16,000 × g. The supernatant was used for the cleavage assays.

In Silico Analyses

The N-MTSs and cleavage sites were predicted using Gavel’s consensus patterns search in the PSORT II program (http://psort.hgc.jp/form2.html, last accessed February 19, 2013) (Gavel and von 1990; Braun and Schmitz 1995). The net charge of presequences was estimated as described elsewhere (Smid et al. 2008). Motifs of N-MTSs were visualized with Weblogo software (Crooks et al. 2004).

Phylogenetic analysis was conducted using 80 sequences of core/MPP homologs aligned with the program Muscle 3.8.31 (default parameters) (Edgar 2010) and trimmed with BMGE 1.1 (-b 1 -m BLOSUM30) (Hordijk and Gascuel 2005; Criscuolo and Gribaldo 2010) to a final length of 314 AA. Phylogenetic analysis was performed with PhyML 2.1.0 (topology search: best of NNIs and SPRs, initial tree: BioNJ, Substitution model: WAG, proportion of invariable sites: fixed (0), gamma distribution parameter: estimated; number of categories: 4; bootstrap replicates: 500) (Guindon and Gascuel 2003) and MrBayes 2.0.6 (rate matrix: WAG; rate variation: gamma; gamma categories: 4; chain
length: 11,000,000; heated chains: 4; heated chain temp: 0.2; burn-in length: 100,000 (Huelsenbeck and Ronquist 2001).

Results

Predictions of Mitochondrial-Targeting Sequences

Data set of 402 proteins, identified in the T. brucei mitochondrial proteome and assigned to the organelle with the highest confidence (Panigrahi et al. 2009), were used for the aminoterminal mitochondrial targeting sequences (N-MTS) prediction using PSORT II software. In this set, 336 putative N-MTSs were identified to contain the predicted cleavage site according to the Gavel algorithm (Nakai and Horton 1999). Predicted N-MTSs were 3 to 147 AA in length, with most N-MTSs ranging between 8 and 37 AA (64%), with a median of 26 AA (fig. 1A). The majority of N-MTSs displayed a net charge between +2 and +8, with a median of +5 (fig. 1B) (supplementary table S1, Supplementary Material online). The net charge reflected the number of arginines distal from the cleavage site that were frequently present as doublets or triplets following the initial methionine. The double arginine motifs were found in 114 N-MTSs within the first 5 AA residues (fig. 1C). Longer N-MTS sequences (>16 AA) usually contained glycine or proline between distal and proximal arginines to allow for the flexibility of N-MTSs (Taylor et al. 2001). Negatively charged residues were rarely present in short (3–31 AA) N-MTSs. The analysis of predicted MPP cleavage sites with a conserved arginine residue at position −2 revealed a loosely conserved cleavage site motif RX(L/A/S)(L/S/A/T/P)(S/A/T) (fig. 1D). Altogether, the properties of the T. brucei N-MTSs in terms of length, net charge, and cleavage site motif were comparable with those of other organisms.

Genes-Encoding MPP Subunits and Core Proteins in Genomes of T. brucei

Processing of identified N-MTSs requires an active MPP in the mitochondrion. Thus, the T. brucei orthologs of MPP subunits and core proteins were searched for in the TriTryp database (http://tritrypdb.org, last accessed February 19, 2013) using the protein sequences of Euglena cp1 (P43264) and cp2 (P43265) as queries. Altogether, 5 genes were retrieved with an e value of 0.62–2.5e−16, including a single putative pseudogene (Tb927.10.2140). Sequence alignment of the retrieved sequences with euayctropic orthologs revealed the presence of a glycine-rich loop in the Tb927.2.4110 gene that corresponded with an α-MPP subunit (fig. 2) and a catalytic zinc-binding motif in the Tb09.160.3110 gene that encoded a putative β-MPP subunit (fig. 2). Products of the two other genes possessed none of these MPP-specific motifs; however, Tb927.5.1060 and Tb11.02.1480 displayed 29.5% and 15.8% sequence similarity to β-MPP and α-MPP, respectively. Therefore, we predicted Tb927.5.1060 and Tb11.02.1480 to represent the cp1 and cp2 subunits of the bc1 complex, respectively. These analyses suggest that T. brucei contains a complete set of genes coding for a heterodimeric MPP and two distinct core proteins.

Cellular Localization

Comparison of the T. brucei MPP subunits and core proteins with Euglena orthologs revealed that Euglena cp1 possesses a conserved zinc-binding motif same as the T. brucei β-MPP (Tb09.160.3110) and plant cp1-β-MPP, whereas T. brucei Tb11.02.1480 and Euglena cp2 correspond to typical cp2 orthologs because they lack the glycine-rich loop present in the α-MPP from both organisms (fig. 2). This finding prompted us to establish the cellular localization of the MPP subunits and core proteins in T. brucei. Because key mitochondrial functions, such as the expression of respiratory complexes, dramatically change between the PF and BF cells, we also compared the cellular distribution of the MPP subunits and core proteins between both stages. Polyclonal antibodies were raised against recombinant T. brucei α-MPP, β-MPP, and cp1 and used in a Western blot analysis of the mitochondrial and cytosolic fractions obtained by digitonin fractionation, confirming the association of all these proteins with the organelle (fig. 3A). However, signals for both MPP subunits were approximately 40% lower in BF cells (fig. 3A). The cp1 protein was detected in the PF mitochondria, whereas no signal was observed in the BF mitochondria, which is consistent with the lack of the bc1 complex in this life cycle stage (Oppérdooes and Michels 2008). The mitochondrial outer membrane porin, matrix Cpn60, and cytosolic enolase were used as controls to determine the purity of the cellular fractions. Mitochondrial localization of MPP was further confirmed by immunofluorescence microscopy of the PF and BF trypanosomes expressing the tagged Tbα-MPP and Tbβ-MPP. As shown in figures 3C–F, in both life stages, α and β-MPP subunits co-localized with MitoTracker, which was used here as a mitochondrial marker.

To investigate the mitochondrial topology of the MPP subunits and core proteins, soluble (matrix) and sedimentable (membrane) fractions were prepared from the T. brucei PF mitochondria. As expected, both MPP subunits together with matrix marker Cpn60 were associated predominantly with the matrix (fig. 3B), whereas cp1 and the Rieske protein (associated with the bc1 complex from the intermembrane space-facing side, used here as a control) were both present predominantly in the membrane fraction (fig. 3B).

Enzymatic Activity of the MPP Heterodimer and Complex Substrate Processing by the Mitochondrial Lysate

To investigate the function of the identified MPP subunits, recombinant histidine-tagged Tbα-MPP and non-tagged Tbβ-MPP were co-expressed in E. coli and isolated from the bacterial lysate using a nickel column via the tagged subunit. Affinity purification of the TbMPP complex from bacteria cultivated in standard LB media led to the isolation of a tagged
Tbα-MPP subunit, whereas Tbβ-MPP was conspicuously absent (data not shown). However, when E. coli were cultivated in a medium supplemented with Zn\(^{2+}\), which is known to be required for formation of a functional MPP heterodimer (Luciano et al. 1998), MPP was indeed purified in an active form. The same results were obtained when isolated tagged Tbα–MPP was mixed with an E. coli lysate expressing only nontagged Tbβ–MPP in the buffer supplemented with Zn\(^{2+}\). Presence of both subunits in the MPP heterodimer was confirmed by Western blot analysis (supplementary fig. S1, Supplementary Material online) and mass spectrometry (data not shown).

The processing activity of the purified TbMPP heterodimer was tested on a substrate represented by a recombinant TbIscU that has been shown to reside in the mitochondria. (Smid et al. 2006). According to PSORT II predictions, this protein possesses a 20 AA-long N-MTS with arginine in the -2 position from the putative MPP cleavage site. Incubation of TbIscU with the recombinant MPP heterodimer purified from E. coli resulted in a partial cleavage of the substrate. Determination of the cleavage site by Edman degradation of the processed product confirmed that TbIscU was cleaved exactly at the site RS/LY predicted by PSORT II (figs. 4A and B).

Next, we tested the processing of TbIscU in the mitochondrial fractions isolated from PF and BF cells. As in the case of recombinant MPP, incubation of recombinant TbIscU with the mitochondrial lysate resulted in the appearance of a single band of lower molecular weight (fig. 4C). Comparison of the processivity of the BF and PF mitochondrial lysate revealed an approximately 3-fold lower cleavage activity in the former lysate. However, Edman degradation of the cleavage product revealed the presence of two peptides. The first peptide commenced with serine, corresponding with the LR/SL cleavage, which is unlikely to be catalyzed by MPP (fig. 4D). The second peptide started with the second serine next to the proximal arginine, indicating cleavage between LY and SP (fig. 4D). In this case, TbIscU was most likely cleaved by MPP between RS and LY as observed with recombinant MPP and subsequently two following amino acid residues L and Y were removed by thus far unknown protease. These results were reproducible using organelles isolated from both life cycle stages and

**Fig. 1.**—Characteristics of amino-terminal MTSs identified in 336 proteins of *Trypanosoma brucei* mitochondria. (A) Length of N-MTSs predicted by PSORT II and (B) calculated net charge. (C) Sequence logo analysis of the N-MTS revealed the presence of a double arginine motif in 181 N-MTSs within the first 20 AA. (C) Sequence logo analysis of the MPP cleavage site in the 366 predicted N-MTSs.
MPP Is Essential for *T. brucei*

To test whether MPP is an indispensable protein for *T. brucei*, we silenced the gene for Tb\(\alpha\)-MPP by RNAi. The Tb\(\alpha\)-MPP subunit became virtually undetectable within 4 days following RNAi induction, arresting the growth of the cells (fig. 5A and B). Western blot analysis of the mitochondria isolated from the PF cells ablated for Tb\(\alpha\)-MPP revealed the accumulation of unprocessed TbIscU at the expense of its fully processed form, which is consistent with Tb\(\alpha\)-MPP-dependent processing (fig. 5B). Taken together, these results demonstrate that MPP activity is essential for *T. brucei* and that both soluble MPP subunits are required for the processing activity within its mitochondria.

Distribution and Phylogenetic Analysis of MPP and Core Proteins in Eukaryotes

To perform phylogenetic analysis, we searched for the *T. brucei* \(\alpha/\beta\)-MPP and the cp1/cp2 orthologs across main eukaryotic groups. Interestingly, we were able to identify the complete set of \(\alpha/\beta\)-MPP subunits and cp1/cp2, present in *T. brucei* and closely related *Leishmania* spp., only in metazoans and some fungi (table 1). In all major eukaryotic groups, we found lineages that possess only three components, namely \(\alpha\)-MPP, \(\beta\)-MPP, and cp2. Only two components (bc\(1\)-\(\alpha\)-MPP and bc\(1\)-\(\beta\)-MPP) were present in the majority of plants; however, the green algae *Chlamydomonas* and *Volvox* also possess cp2, in addition to both MPP subunits. The same as in higher plants, the cp1/cp2 pair is absent from the apicomplexans. Protists with functionally and/or morphologically reduced mitochondria that lack the bc\(1\) complex either retain only the soluble MPP (e.g., *Trichomonas*, *Giardia*) or lost both MPP and the core proteins (e.g., *Enthamoeba*, *Encephalitozoon*) (table 1).

Phylogenetic analysis was performed with 80 selected eukaryotic orthologs of MPP and cp1/cp2 proteins (fig. 6). As expected, the analysis revealed 2 major, well-supported clades corresponding to \(\alpha\)-MPP/cp2 and \(\beta\)-MPP/cp1. The \(\alpha\)-MPP subunits and cp2 proteins were separated into two subclades, revealing a moderately supported monophyletic origin of cp2. The \(\alpha\)-MPP group includes the bc\(1\)-\(\alpha\)-MPP subunits of plants. Surprisingly, the topology of cp1 suggests that this \(\beta\)-MPP-related core protein evolved independently at least three times, namely in the kinetoplastid, fungal, and metazoan lineages (fig. 6). In each of these three groups,
the cp1 protein is closely related to the corresponding β-MPP from the same taxon. Multiple origins of cp1 in kinetochores, fungi and metazoa, together with the conspicuous absence of cp1 in other lineages, suggests that the 3-component system (α-MPP, β-MPP, and cp2) represents an ancestral stage, whereas the acquisition of the fourth component, as in the case of T. brucei and other organisms, represent a more evolutionarily derived stage.

**Discussion**

Here, we investigated a MPP/core protein family in T. brucei to contribute to the understanding of the evolutionary history of trypanosomatid mitochondria, which is in many features highly divergent from the mitochondria of other eukaryotes (Madison-Antenucci et al. 2002; Besteiro et al. 2005; Allen et al. 2008). These features include a substantially simplified mitochondrial protein import machinery that might be
considered either as a primitive feature of an ancient mito-
chondrion (Cavalier-Smith 2010; Pusnik et al. 2011) or the
result of specific adaptation, as was proposed for the reduced
import machinery in the mitochondria-derived organelles of
Giardia and Trichomonas (Smid et al. 2008; Dagley et al. 2009;
Rada et al. 2011). Our investigation revealed that
T. brucei possesses a complete set of proteins consisting of soluble
\( \alpha \)-and \( \beta \)-MPP subunits and cp1 and cp2 subunits associated
with the inner membrane respiratory complex bc1. We
demonstrated that the matrix-located heterodimeric MPP is
expressed in both stages of T. brucei. It apparently catalyzed
processing of the mitochondrial preprotein TbIscU in vitro, and
the substrate processing was impaired when Tb\( \beta \)-MPP expres-
sion was silenced by RNAi in vivo. During preparation of this
manuscript, another group reported the presence of canonical
MPP in T. brucei (Desy et al. 2012). In addition to MPP, we
identified cp1 and cp2 genes that are paralogs of \( \beta \)-MPP and
\( \alpha \)-MPP, respectively. We were able to show that cp1 is asso-
ciated with the mitochondrial membrane fraction and that in
BF cells, its expression is downregulated together with the bc1
complex. Accordingly, recent proteomic analysis of the T. brucei
mitochondrial respirasome revealed the presence of the Tb927.5.1060 and Tb11.02.1480 gene products that
correspond to the cp1 and cp2 proteins in the bc1 complex
(Acestor et al. 2011). Unlike the MPP subunits, the T. brucei
cp1/cp2 proteins lack the domains required for the processing
activity. It is therefore likely that in T. brucei, these proteins are
proteolytically inactive and serve as structural bc1 compo-
nents, a situation reminiscent of that described for S. cerevisiae
(Zara et al. 2004).

It has been proposed that plants represent an ancient stage
of MPP evolution, with active \( \alpha \)- and \( \beta \)-MPP subunits

![Processing of TbIsCU preproteins by Trypanosoma brucei recombinant MPP activity and mitochondrial lysate isolated from PF and BF trypanosomes.](image)

**Fig. 4.** Processing of TbIsCU preproteins by Trypanosoma brucei recombinant MPP activity and mitochondrial lysate isolated from PF and BF trypanosomes. (A) The TbIsCU preprotein was treated with recombinant TbMPP and analyzed by SDS-PAGE. S, substrate (TbIsCU); p, preprotein; m, mature protein. (B) Amino-terminal sequences of the preprotein and mature proteins were determined by Edman degradation. The peptide sequences of preprotein and mature protein are underlined by thin line and heavy line, respectively. Arrows indicate the TbIsCU cleavage site. N-MTS was predicted by the PSORT II program (broken line). (C) The TbIsCU preprotein was treated with mitochondrial lysates and analyzed by SDS-PAGE. Control, recombinant TbIsCU without mitochondrial lysate. (D) Edman sequencing of mature TbIsCU produced by PF and BF mitochondrial lysates revealed the presence of two amino-terminal peptides under both conditions (heavy line).

![Effect of \( \beta \)-MPP silencing on Trypanosoma brucei growth and TbIsCU maturation.](image)

**Fig. 5.** Effect of \( \beta \)-MPP silencing on Trypanosoma brucei growth and TbIsCU maturation. (A) Growth curve of wild-type cells (29–13) strain (filled diamonds) and strain with induced (filled triangles) and noninduced (filled squares) RNAi against \( \beta \)-MPP. (B) Immunoblot analysis of mitochondrial fractions isolated from transformed T. brucei before and after induction of RNAi against \( \beta \)-MPP. The \( \beta \)-MPP depletion after a 4-day induction corresponded with an accumulation of TbIsCU preprotein. Enolase was used as a loading control.
peripherally associated with the bc1 complex, whereas meta-
zaoans and yeast represent a more recent evolutionary stage in
which MPP subunits were duplicated and formed membrane-
associated cp1/cp2 proteins and soluble α/β-MPP (Braun and
Schmitz 1995, 1997; Glaser and Derni 1999). Neurospora
was considered to represent an intermediate stage in which a
single gene encodes a bi-functional α-MPP that is shared be-
tween α-MPP to constitute a soluble MPP heterodimer and
and cp2 proteins differ in the confinement of the glycine-
rich loop, which is important for substrate recognition, to
the former protein (Nagao et al. 2000). However, it has
been shown that bovine cp2 forms with cp1 a proteolytically
active heterodimer capable of processing substrates such as
the Rieske protein (Deng et al. 2001). Therefore, we can
hypothesize that the original processing machinery consisted
of an enzymatically active cp2-like/β-MPP enzyme associated
with the bc1 complex that processed a limited number of
imported proteins (fig. 7). As a consequence of growing
mitochondrial complexity, the gene encoding a cp2-like
protein was duplicated: One of the copies gained the glycine-rich
loop (α-MPP) and formed a soluble heterodimer with β-MPP.
It has been shown that the association of α-MPP with β-MPP
is essential for the enzyme’s stability in solution (Janata et al.
2004). At this stage, β-MPP has acquired a dual function, as
observed in Neurospora, while in plants, cp2 was likely
replaced with the α-MPP to increase substrate specificity of
the membrane-associated complex. Indeed, a more stringent
recognition system was needed in plants to distinguish pre-
proteins targeted into the mitochondria from those aimed for
the plastids, as both systems used a similar targeting mecha-
nism (Teixeira and Glaser 2012). In support of the secondary
loss of cp2 in plants, we identified cp2 orthologs in the green
algae Chlamydomonas and Volvox that displayed monophony
with other eukaryotic cp2 proteins. Similar to plants, apicom-
plexans also possess exclusively α- and β-MPP subunits.
Although their cellular localization remains to be established,
the presence of the bc1 complex and a remnant plastid sug-
gests that the situation in these parasitic protists might be
similar to plants. In several organisms lacking bc1, the MPP/
core protein family is limited to a soluble MPP (Giardia
and Trichomonas) (Smid et al. 2008), or MPP is completely absent
as a result of reductive evolution, as is the case of
Encephalitozoon and Entamoeba (Burri et al. 2006; Burri
and Keeling 2007). On the other side of the spectrum, the
Neurospora-type 3-component MPP/core family gained an-
other member, cp1, by gene duplication and specialization of
β-MPP. According to our phylogenetic analysis, cp1
emerged independently in metazoans, fungi, and higher kine-
toplastids, indicating significant evolutionary pressure for this
event. The cp1 proteins either possess a degenerate zinc-bind-
ing motif and retain enzymatic activity, such as in metazoans,
or this motif has been lost, as is the case of yeasts and kine-
toplastid flagellates. Evolution of the cp1 subunit allowed for
separate respiration and mitochondrial biogenesis; for exam-
ple, in yeast, the acquisition of the cp1 system allowed for the
downregulation of the respiratory chain during anaerobic fer-
mentation when mitochondrial protein import still occurs.
Similarly, the cytochrome c-dependent respiration is silenced in
the BF T. brucei; however, the respective mitochondrial still
needs to import proteins to maintain processes such as Fe–S
cluster assembly and fatty acid synthesis. Indeed, we demon-
strated that cp1, along with the bc1 complex, is absent from

### Table 1
Presence of the αβ-MPP and the cp1/cp2 Orthologs across Main Eukaryotic Groups

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aPartial sequence.
bPresence of soluble αβ-MPP was inferred based on absence of bc1 complex.
cPartial sequence.
dIncorporation of αβ-MPP within bc complex was inferred based on presence of bc complex and absence of cp1 and cp2 proteins as in plants.
Phylogenetic analysis was performed with PhyML 2.1.0 and MrBayes 2.0.6. The numbers at the nodes represent the bootstrap values for 500 replicates (PhyML) and Bayesian posterior probability. Branches with bootstrap support lower than 50 are shown as polytomy. The scale bar indicates the substitution rate. Accession numbers for protein sequences that were used in phylogenetic analyses are shown in supplementary table S2, Supplementary Material online.
Fig. 7.—Model of MPP/core protein family evolution. MPP/core proteins most likely evolved from an α-proteobacterial protease present in the endosymbiotic ancestor of mitochondria. (A) The genes coding for the original bacterial protease were inherited from the mitochondrial ancestor, and the active cp2-like/β-MPP enzyme became associated with a respiratory bc1 complex in the inner mitochondrial membrane. (B) Subsequently, the gene encoding the cp2-like protein was duplicated. One paralog (cp2) was retained as the bc1 complex subunit, and the second paralog (α-MPP) gained the glycine-rich loop and formed a soluble heterodimer with β-MPP. At this stage, a single β-MPP has dual function as a subunit of soluble MPP and bc1 complex. (C) In three eukaryotic lineages (metazoa, fungi, and kinetoplastea), the β-MPP was also duplicated, forming core protein cp1 and soluble β-MPP. This event allows for the functional separation of respiration and protein processing. (D) In plants, we propose that cp2 was replaced by α-MPP. Consequently, enzymatically active α-MPP and β-MPP subunits were associated with the bc1 complex (bc1-αMPP, and bc1-βMPP). In this case, core proteins and MPP subunits are identical, and the bc1 complex facilitates a dual function: respiration and protein processing. Similar systems might be present in apicomplexans. In some anaerobic organisms and intracellular parasites with reduced mitochondria, the core proteins were lost together with the bc1 complex, and they possess only soluble MPP heterodimers (e.g., Trichomonas) (E), a single subunit MPP (e.g., Giardia) (F), or the MPP is absent (e.g., Entamoeba) (G). IM, inner mitochondrial membrane; IMS, intermembrane space; OM, outer mitochondrial membrane.
the BF trypanosomes, whereas a soluble MPP heterodimer remains active. Interestingly, isolation and characterization of the bc\textsubscript{1} complex in Euglena identified cp2 and \(\beta\)-MPP as its genuine subunits (Cui et al. 1994). Although the complete genome sequence for Euglena is not yet available, identification of \(\beta\)-MPP in the bc\textsubscript{1} complex suggests that unlike T. brucei, Euglena contains the Neurospora-type system. Altogether, identification, functional characterization, and phylogenetic analysis of the MPP/core protein family in T. brucei did not support the ancient character of its mitochondrial biogenesis.

We further analyzed the T. brucei N-MTSs, which are substrates for MPP. It has been noticed that T. brucei MTSs are often shorter compared with those in metazoans and plants (Clayton et al. 1995). Until recently, only a limited number of T. brucei proteins were available with experimentally confirmed mitochondrial localization. Since the T. brucei mitochondrial proteome is now available (Panigrahi et al. 2009), we were able to analyze a set of 402 proteins assigned to the organelle with high confidence, in which 336 N-MTSs were predicted by PSORTII. The length of in silico predicted that T. brucei N-MTSs widely vary from 3 to 147 AA, with a median length of 26 AA. Similar results were recently obtained by a bioinformatic analysis of 38 putative T. brucei mitochondrial proteins using two other programs, MITOPROT and TargetP, which predicted N-MTSs with median lengths of 25 and 30 AA, respectively (Krnacova et al. 2012). Taken together, these analyses determined that mitochondrial proteins of this model flagellate are furnished with N-MTSs comparable in size with those in yeast and mammals (Schneider et al. 1998). Furthermore, other features of the T. brucei N-MTSs, such as net charge, AA composition and cleavage site motif did not show any remarkable deviation from the usual pattern in other eukaryotic lineages, with the sole exception of a frequent occurrence of double and triple arginines close to the amino terminus. Therefore, the analyzed N-MTSs do not share the simplicity of the Giardia and Trichomonas presequences as suggested previously (Pusnik et al. 2011; Schneider et al. 2009). On the contrary, T. brucei is able to import and process mitochondrial preproteins from a large spectrum of evolutionary distant organisms, including the excavate Trichomonas, the model plant Arabidopsis, the stramenopile Blastocystis and humans (Long, Jirku, et al. 2008; Long et al. 2008; Tsoumis et al. 2012). Moreover, it has been shown that the expression of the human frataxin preprotein with a 55 AA long N-MTS in T. brucei resulted in its correct import and processing as in human mitochondria (Long, Jirku, et al. 2008).

Production of the active recombinant MPP allowed us to demonstrate in vitro that T. brucei MPP recognizes the typical cleavage site in N-MTS of TblscU with arginine in the \(-2\) position, as it was predicted by the PSORT II program. However, the protein processing within mitochondria seems to be a more complex process (Teixeira and Glaser 2012). The treatment of TblscU with mitochondrial lytase resulted in the production of two peptides with arginine in \(-1\) and \(-4\) positions from the cleavage sites, respectively. It is not clear which process is responsible for the peptide cleavage in the R-1 position. The second (R-4) peptide is most likely a product of multistep processing in which the first step is carried out by MPP, which catalyses the cleavage between RS and LYS. Subsequently, L and Y might be removed by another mitochondrial protease, such as Icp55 (Teixeira and Glaser 2012). Indeed, the Icp55 typically removes L, Y, or F from the cleavage motif F(L/Y)\(\alpha\)(ISA), and we found several candidates for Icp55-like peptidase in T. brucei genome (e.g., Tb09.211.4330). However, Icp55 is known to cleave off a single amino acid residue at the R-3 position (Naamati et al. 2009), while two amino acids were removed following MPP cleavage site in TblscU. Similar R-4 cleavage was also observed in the case of hydrogenosomal flavodiiron protein in T. vaginalis (Smutna et al. 2009). We can speculate that the observed R-4 trimming of TblscU is performed either in a single step in which L\(\gamma\) dipeptide is removed or L and Y are removed in two successive steps. Future studies of putative N-MTS trimming enzymes in T. brucei will be needed to understand their possible role in the processing of N-MTSs.

Recently, important support for an ancient character of the trypanosomatid import system came from the identification of a putative ATOM as the main gate of the T. brucei outer mitochondrial membrane, whereas Tom40 was considered to be absent (Pusnik et al. 2011). It has been proposed that unlike Tom40, ATOM is related to the YtfM subgroup of the bacterial \(\beta\) barrel Omp85 protein family. The ATOM-like translocase was suggested to mediate mitochondrial protein import in the last common eukaryotic ancestor that was in the course of eukaryotic evolution replaced by Tom40 in and is present in all extant eukaryotes except trypanosomes (Pusnik et al. 2011). In addition, several other scenarios were considered, including a possibility that ATOM replaced Tom40 in trypanosomatids during excavate evolution by a lateral gene transfer from bacteria or that Tom40 might have evolved from the ATOM. However, later bioinformatic analysis questioned the relationship between the ATOM and bacterial Omp85-like proteins and showed that the former protein shares a Porin_3 domain with Tom40 and VDAC sequences and thus more likely represents a divergent ortholog of Tom40 (Zarsky et al. 2012).

Taken together, we believe that the features of the T. brucei MPP/core protein family described herein, together with the known features of its mitochondrial import machinery, can be best explained as adaptations to a complex life cycle. The T. brucei MPP/core proteins seem to represent a highly evolved form that allows a separate regulation of respiration and protein import in insect- and vertebrate-dwelling stages of the parasite. Our extensive analysis of N-MTSs revealed standard properties encountered in mitochondrial preproteins across main eukaryotic lineages. Although the TOM
and TIM complexes of *T. brucei* show apparent simplicity compared with *S. cerevisiae* and other eukaryotes, currently there is insufficient evidence to distinguish whether this simplicity reflects a primitive evolutionary trait or resulted from an extensive reductive evolution, which occurred independently in anaerobic *Giardia* and *Trichomonas* that belong to the same eukaryotic supergroup.

**Supplementary Material**

Supplementary tables S1 and S2 and figure S1 is available at Genome Biology and Evolution online (http://www.gbe.oxfordjournals.org).

**Acknowledgments**

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**Associate editor**: Martin Embley
7.2. Publication 2: Mach et al., 2013

Efficient Iron Uptake via a Reductive Mechanism in Procyclic *Trypanosoma brucei*

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**ABSTRACT:** The bloodstream form of *Trypanosoma brucei* acquires iron from transferrin by receptor-mediated endocytosis. However, it is unknown how procyclic forms that cannot bind transferrin acquire iron. Here, we show that the procyclic form of *T. brucei* efficiently takes up iron from ferric complexes via a reductive mechanism and that iron obtained using this mechanism is transported to, and used in, the mitochondria. The affinity of the transport system is comparable to that of *Saccharomyces cerevisiae*, with an apparent *Km* of 0.85 μM.

Pathogenic microorganisms must acquire iron from the host environment to establish infection. However, mammals maintain minimal concentrations of extracellular iron, and the accessibility of this iron is limited due to iron being bound to transport proteins such as transferrin and lactoferrin. Pathogenic microorganisms have developed various mechanisms to satisfy their high demand for iron. These mechanisms include reducing a broad variety of ferric complexes followed by importing free ferrous iron or producing siderophores with high affinities for iron, followed by uptake via specific receptors (Sutak et al., 2008).

In bloodstream forms of *Trypanosoma brucei*, iron acquisition is mediated by host transferrin that binds to a unique heterodimeric receptor encoded by 2 expression site–associated genes (expression site–associated genes 6 and 7). After the binding of transferrin to the receptor in the flagellar pocket, the receptor-transferrin complex is endocytosed, and iron is released by acidification in endosomes (Taylor and Kelly, 2010). However, it is unknown how iron uptake is achieved by procyclical forms of *T. brucei* despite the fact that these forms likely have a higher demand for iron than bloodstream forms of *T. brucei*. Procyclic *T. brucei*, unlike the bloodstream forms, possess fully active mitochondria with iron-sulfur cluster-dependent enzymes (Durieux et al., 1991). Transferrin uptake does not occur in insect forms of *T. brucei* (Ligtenberg et al., 1994), and the use of iron from heme is unlikely because no ortholog of heme oxidase, an enzyme required for releasing iron from the porphyrin ring, has been identified in trypanosomatids.

Here, we aimed to test whether procyclical forms of *T. brucei* use a yeast-like reductive mechanism to take up iron from ferric complexes. A procyclical form of *T. brucei* (strain 29-13) was grown in SDM-79 medium supplemented with 15% fetal bovine serum. In total, 1 × 10⁸ cells were incubated in 1 ml of incubation buffer (10 mM HEPES, 150 mM NaCl, 3 mM KCl, 24 mM NaHCO₃, and 11 mM glucose) with 1 μM ⁵⁷Fe(III)-citrate at 27°C for 1 hr and washed 3 times in the same buffer by centrifugation. The level of cell-associated radioactivity was determined by liquid scintillation counting. The uptake rate was 9.4 ± 0.9 nmol/min per 10⁶ cells (the rate of iron uptake by iron-sufficient *Saccharomyces cerevisiae* was 11.5 ± 0.7 nmol/min per 10⁶ cells). In cells incubated on ice, the uptake rate decreased to 18 ± 4% of that observed at 27°C, demonstrating that iron uptake is an active process. Further experiments were conducted to determine whether, or ferrous, iron is transported into the cells. Iron uptake was inhibited by 81 ± 7% when Fe(II)-trapping agent bathophenanthroline disulfonate (BPS) (0.2 mM) was added to the cells immediately before addition of ⁵⁷Fe(III)-citrate, whereas the addition of 1 mM ascorbate (a reductant) increased iron uptake by 218 ± 74%.

These results indicate that iron is reduced before transport. The cells also were able to take up iron from another iron-chelating agent, nitrolotriacetate, although with lower efficiency (data not shown).

In *S. cerevisiae*, reduction of ferric iron to ferrous iron before iron transport across the cell membrane is mediated by specific ferric reductase activity. Thus, we tested ferric reductase activity by using intact trypanosomes, as described previously (Lesuisse et al., 1987). The cells (1 × 10⁶ cells/ml) were incubated at 27°C in the dark in the presence of 0.5 mM Fe(III)-citrate and 1 mM BPS for various periods and then centrifuged before measuring absorbancy (535 nm) of the supernatant (ε =19.5 mM/cm) as a negative control, we measured changes in absorbance of cell-free BPS with Fe(III)-citrate as well as BPS with the cells without addition of iron. The cells exhibited a ferric reductase activity of 1.1 ± 0.17 pmol/min per 10⁶ cells when Fe(III)-citrate was the iron source (ferric reductase activity of iron-sufficient *S. cerevisiae* was 11.4 ± 0.45 pmol/min per 10⁶ cells). To estimate the affinity of the uptake system for ferrous iron, we measured the concentration-dependent uptake of ascorbate-reduced iron (Fig. 1A). The apparent *Km* value of the Fe(II) uptake system was 0.85 ± 0.13 μM, a value that is comparable to the *Km* for the high-affinity Fe(II) uptake system in yeast (0.15 μM) (Eide et al., 1992).

To determine whether iron acquired from Fe(III)-citrate is metabolized intracellularly, we performed blue native polyacrylamide gel electrophoresis (BN-PAGE) of mitochondrial proteins isolated from Fe(III)-citrate-incubated *T. brucei* cells incubated with 1 μM ⁵⁷Fe(III)-citrate (1:20). This method is non-dissociative, allowing identification of different metal-containing protein complexes (Babuška et al., 2007). The radiograph (Fig. 1B) showed 2 distinct high-molecular-weight bands, indicating that iron is imported to the mitochondria and incorporated into protein complexes.

**Figure 1.** (A) Concentration dependence of Fe(II) uptake by *Trypanosoma brucei*. Cells were incubated for 1 hr at 27°C with different concentrations of iron reduced with 1 mM ascorbate. After washing, the level of cell-associated radioactivity was determined by liquid scintillation counting. The means ± SD from 3 experiments are shown. (B) BN-PAGE of mitochondrial proteins from ⁵⁷Fe-labeled *T. brucei*. Mitochondria from 1 × 10⁸ cells were isolated by digitonin fractionation as described previously (Smid et al., 2006). The purity of the fraction was verified by enzymatic activities and western blots. BN-PAGE (4–16%) was performed as described previously (Schamel, 2008) by using 1% n-dodecyl β-maltoside as a detergent. The gel was vacuum-dried and subjected to autoradiography.

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Taken together, our results show that procyclic \textit{T. brucei} takes up iron from ferric complexes via a 2-step mechanism in which ferric iron is reduced to ferrous iron and is subsequently transported. This strategy allows acquisition of iron from diverse ferric complexes and may be advantageous in the insect gut and salivary glands where host transferrin is not present.

Involvement of ferric reduction in iron acquisition has already been described in the promastigote of \textit{Leishmania chagasi} (Wilson et al., 2002). The gene that encodes surface-associated ferric reductase required for the differentiation into infective forms was identified in \textit{L. amazonensis} (Flannery et al., 2011). Further studies are required to elucidate the molecular basis of these processes \textit{T. brucei}, in particular to identify the ferric reductase and iron transporters. A ferric reductase homolog is present in the genome of \textit{T. brucei} (Tb11.02.1990) as well as several putative divalent metal transporters with ZIP domain (Tb11.01.0720, Tb11.01.0770, Tb11.01.0760, Tb11.01.0730, and Tb11.01.0725).

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LITERATURE CITED


7.3. Publication 3: Štáfková et al., 2016

Mitochondrial pyruvate carrier in Trypanosoma brucei

Introduction

Pyruvate is a central intermediate metabolite involved in many cellular catabolic and anabolic pathways. This compound is the cytosolic product of glycolysis, and in most eukaryotic cell types, pyruvate enters the mitochondrion for further oxidation to acetyl-CoA to fuel the tricarboxylic acid cycle. Thus, pyruvate represents an important branching point in cellular metabolism for balancing glycolysis and oxidative phosphorylation.

The availability of pyruvate in the mitochondrion is determined through a specific carrier located in the inner mitochondrial membrane. The principal biochemical features of mitochondrial pyruvate carrier (MPC) were characterized in the 1970s. Pyruvate is symported with one proton, and this transport is driven by ΔpH (Papa et al., 1971; Halestrap, 1978). However, the molecular identity of MPC has only recently been revealed, and currently MPCs have been characterized in Saccharomyces cerevisiae, Drosophila melanogaster, Homo sapiens (Bricker et al., 2012; Herzig et al., 2012) and Arabidopsis thaliana (Li et al., 2014).

The mitochondrial pyruvate carrier comprises two small hydrophobic paralogous proteins, MPC1 and MPC2, which are essential and sufficient for the transport of pyruvate into mitochondria (Bricker et al., 2012; Herzig et al., 2012). In S. cerevisiae, a third paralog, MPC3, exists, sharing 71% amino acid sequence identity with MPC2, and MPC3 expression is induced upon growth on nonfermentable carbon sources (Herzig et al., 2012; Timon-Gomez et al., 2013; Bender et al., 2015).

Structural predictions have revealed 2-3 transmembrane (TM) helices in all MPC homologs (Bricker et al., 2012; Herzig et al., 2012). Pfam lists MPC proteins as members of the MtN3-like clan, together with SWEET transporters (‘Sugars Will Eventually be Exported Transporters’) and the PQ-loop protein family (Finn et al., 2014). A previous study suggested that the structure of prokaryotic SemiSWEET transporters, members of the same diverse clan, is similar to that of MPC (Vanderperre et al., 2014).

Trypanosoma brucei is a pathogen of livestock and humans transmitted through tsetse flies in sub-Saharan Africa. The different life-cycle stages of trypanosomes present specific adaptations to their environments. For the bloodstream (BSF) and procyclic (PCF) forms of...
T. brucei, these adaptations include changes in mitochondrial morphology, function, and overall metabolic rearrangements reflected by different spectra of metabolic end products. In BSF, ATP is primarily generated through glycolysis, and pyruvate is the predominant excreted end product of metabolism (Creek et al., 2015). In contrast, PCF that live in the midgut of the insect vector where nutrients are scarce, depend on the mitochondrial catabolic pathway for ATP production. Pyruvate is utilized for substrate level phosphorylation, resulting in the production of acetate and ATP. In addition, proline and threonine are important carbon sources for these stages when glucose is limited. In glucose-depleted media, proline is metabolized to alanine, glutathione, proline and threonine are important carbon sources as acetate and glycine (Linstead et al., 1977; Lamour et al., 2005).

The regulation of pyruvate availability in the mitochondrion is one of the mechanisms for balancing oxidative phosphorylation and glycolysis (Vanderperre et al., 2014; Bender et al., 2015). Similarly to yeast grown on fermentable carbon substrates, in BSF T. brucei, this balance is predominantly shifted towards glycolysis. Recently, a plasma membrane pyruvate transporter has been characterized in T. brucei (Sanchez, 2013); however, there is no information regarding the molecular characteristics of pyruvate transporters in mitochondria. Therefore, the aim of the present study was to determine whether T. brucei transports pyruvate into the mitochondrion using a putative MPC homolog and address the relative importance of pyruvate and the pyruvate transporter in PCF and BSF trypanosomes.

Results
MPC homologs in T. brucei

Two genes encoding putative MPC proteins were identified in the T. brucei genomic database (www.tritrypdb.org) after a BLAST search of MPC1, MPC2 and MPC3 from S. cerevisiae: Tb927.9.3780, annotated as ‘hypothetical protein, conserved’ was the only result for the ScMPC1 query, while Tb927.7.3520, annotated as ‘mitochondrial pyruvate carrier protein 2, putative’, resulted from BLAST searches using both ScMPC2 and ScMPC3 as queries. Multiple protein alignment was generated using homologs from different organisms and edited by BMGE (Supporting Information Fig. S1), and an unrooted phylogenetic tree was reconstructed using PhyML and MrBayes (Fig. 1A). The tree shows that Tb927.9.3780 and Tb927.7.3520 clustered with MPC1 and MPC2/3 orthologs, respectively, with high statistical support. Based on this analysis, the corresponding T. brucei proteins were designated as TbMPC1 and TbMPC2. Subsequently, a series of in silico analyses was performed. The results from MitoProt II and PSORTII, which predict mitochondrial targeting sequences and subcellular protein locations, indicated the mitochondrial localization of both TbMPC1 and TbMPC2 (the calculated probability of mitochondrial import was 0.85 and 0.83, respectively, according to MitoProt II; the PSORTII k-NN prediction was 47.8% mitochondrial for TbMPC1 and 43.5% cytosolic for TbMPC2). TMpred and TMHMM were used to predict the location of putative transmembrane helices in TbMPCs and all eukaryotic MPC homologs described thus far (Fig. 1B). Experimental evidence for the membrane topology of ScMPC1 and ScMPC2 according to Bender et al. (2015) was considered. Similar to other analyzed MPCs, TbMPC1 and TbMPC2 contain two and three transmembrane domains, respectively. Notably, in all MPC sequences, a tryptophan residue is conserved in the putative binding pocket in MPC2 (W89 in TbMPC2), and this amino acid position is substituted with phenylalanine, another aromatic amino acid, in MPC1 (F52 in TbMPC1). The proline forming the hinge region of the SemiSWEET transporter is conserved in all selected MPC2 and most MPC1 homologs, while trypanosomatid MPC1 revealed a proline-alanine substitution (P50 in TbMPC2, A15 in TbMPC1; Fig. 1B).

Based on the results from the in silico analysis, we concluded that the predicted T. brucei MPCs are evolutionary and structurally related to known MPC proteins.

TbMPC1 and TbMPC2 localization

To determine the cellular localization of MPC paralogs in the PCF and BSF T. brucei, we prepared constructs for expression of TbMPC1 and TbMPC2 with a C-terminal epitope tag. We established PCF cell lines expressing both epitope-tagged TbMPC1 and TbMPC2 and an epitope-tagged TbMPC1 BSF cell line. Despite multiple attempts, we were not successful in obtaining a BSF cell line expressing epitope-tagged TbMPC2.

The localization of TbMPC1 and TbMPC2 was investigated using immunofluorescence microscopy and Western blotting of subcellular fractions (Fig. 1C, Supporting Information Fig. S2). In PCF, both TbMPC1 and TbMPC2 were observed in reticular structures that colocalized with mitochondrial MitoTracker staining (Fig. 1C, panels I, II). To prove the presence of TbMPCs in the mitochondrial membrane, we fractionated crude mitochondrial preparations to obtain mitochondrial matrix-enriched and membrane-enriched fractions. Western blot analysis confirmed the presence of both TbMPC1 and TbMPC2 only in the membrane fractions (Supporting Information Fig. S2). Mitochondrial membrane localization of TbMPC1 was demonstrated also in BSF (Fig. 1C, panel III and Supporting Information Fig. S2).

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Generation of TbMPC1 and TbMPC2 PCF null mutants and pyruvate uptake analysis

Deletion of the tbmpc1 or tbmpc2 gene was accomplished in wild type PCF (strain 427) by two rounds of transformation in which the ORFs were replaced by an antibiotic resistance gene. Deletion of both alleles was confirmed in several clones by PCR, and clones B6 (Δtbmpc1-B6) and 2C4 (Δtbmpc2-2C4) were selected for further experiments (Fig. 2A). Growth of both KO clones in glucose-containing SDM-79 was not significantly affected (Fig. 2B). Thus,
Fig. 2. Analysis of the genotype and growth phenotype of Δtbmpc1-B6 and Δtbmpc2-2C4 in PCF cell lines, and Δtbmpc1-2 and knock-in cell lines expressing TbMPC1-HA in BSF.

A. PCR analysis of genomic regions flanking tbmpc-coding sequences in wild type and null mutant cell lines. Primers in the mpc coding region and external to the cassette insertion were used (scheme and Supporting Information Table S1). Water instead of DNA was used in the negative control. M: molecular weight marker (*, 500 bp).

B. In vitro growth of the wild type PCF strain (open circles), Δtbmpc1-B6 (triangles) and Δtbmpc2-2C4 (diamonds) cell lines. Average values of three independent experiments are shown; relative standard deviation was consistently below 10%.

C. Verification of the tbmpc1 deletion in the selected Δtbmpc1 clone 2 using Southern blot analysis. The membranes were probed against TbMPC1 and blasticidin deaminase coding regions.

D. Expression of TbMPC1-HA determined by Western blotting in wild type, Δtbmpc1-2, and knock-in BSF cell lines in wild type and Δtbmpc1-2 background. HSP60 expression was visualized as loading control.

E, F. Growth of BSF wild type (circles), Δtbmpc1-2 (triangles) and knock-in cell lines (wild type + TbMPC1-HA, full squares; Δtbmpc1-2 + TbMPC1-HA, open squares) in HMI-9 (E) or CMM (F). Average values of three independent experiments are shown; relative standard deviation was consistently below 10%.

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neither TbMPC1 nor TbMPC2 is essential under standard culture conditions.

The role of TbMPC1 and TbMPC2 in pyruvate import to mitochondria of PCF trypanosomes was assessed by measurement of $^{14}$C-pyruvate uptake by mitochondrial fractions of wild type cells and respective null mutants ($\Delta$tbmpc1-B6, $\Delta$tbmpc2-2C4). Incorporation of radioactivity in mitochondrial preparations from both mutant cell lines was decreased by approximately 60% compared with wild type samples. A similar decrease in pyruvate uptake was observed in wild type mitochondria incubated with UK-5099, an inhibitor of MPC and monocarboxylate transporters (Halestrap, 1975). On the contrary, UK-5099 did not affect pyruvate uptake by mitochondrial preparations from either mutant cell line (Fig. 3). These results indicate that both TbMPC1 and TbMPC2 are needed for inhibitor-sensitive pyruvate import into mitochondria and confirm that MPC-mediated pyruvate uptake in the mitochondrion is abolished in both mutant cell lines.

**Analysis of metabolic end products in $\Delta$tbmpc1-B6 and $\Delta$tbmpc2-2C4 PCF cell lines**

In PCF trypanosomes, phosphoenol pyruvate derived from glycolysis is further metabolized to succinate in the glycosome, or in the form of pyruvate, it enters mitochondria, where it is metabolized to acetate and succinate (Fig. 4). Therefore, we investigated the effect of $tbmpc1$ and $tbmpc2$ gene knockouts on the glucose-dependent formation of metabolic end products. In PCF, pyruvate production was increased in both $\Delta$tbmpc1-B6 and $\Delta$tbmpc2-2C4 cell lines compared with wild type samples, consistent with defective pyruvate transport into the mitochondrion. In addition, a significant decrease in acetate and succinate production was observed in these mutant cell lines (Table 1). Reduced acetate generation in the mitochondrion is consistent with a reduction in mitochondrial pyruvate metabolism. Null mutants also displayed a trend towards diminished lactate production ($P = 0.07$). It should be noted that pyruvate cannot be reduced to lactate in *T. brucei* due to the lack of lactate dehydrogenase activity. Lactate detected in our assays is expected to be a product of detoxification of methylglyoxal which arises spontaneously in the course of glycolysis (Greig *et al*., 2009). Changes in pyruvate, acetate, succinate and lactate levels in wild type samples incubated with UK-5099 showed patterns similar to those observed in mutant cell lines, albeit these effects were less pronounced. As expected, the presence of the inhibitor did not affect acetate, succinate and lactate levels in mutant cell lines. Together, these results provide further evidence for the involvement of TbMPC1 and TbMPC2 in mitochondrial pyruvate transport.

To confirm these data, the end products of glucose and/or threonine metabolism in wild type and TbMPC null cell lines were investigated using $^1$H-NMR spectroscopy, which facilitates a quantitative comparison, as previously described (Millerioux *et al*., 2012; Bringaud *et al*., 2015). PCF were incubated in PBS containing 4 mM [U-$^{13}$C]glucose in the presence or absence of 4 mM threonine prior to $^1$H-NMR quantification of the end products excreted from these carbon sources. In this experiment, threonine-derived acetate production was used as a reference to estimate the impact of $tbmpc$ gene deletion on pyruvate-dependent acetate production, as threonine in the incubation buffer served as a substrate for the pyruvate-independent acetate production by threonine degradation pathway (Millerioux *et al*., 2013). The amount of glucose consumed during the incubation was determined under these conditions and was found to be similar in all three cell lines: $3.33 \pm 0.36$, $3.44 \pm 0.63$ and $3.89 \pm 0.11 \mu$mol in wild type, $\Delta$tbmpc1-B6 and $\Delta$tbmpc2-2C4, respectively [mean of 4 or 5 biological replicates ± standard deviation (SD)].

Direct visual comparison of cells incubated with and without threonine, irrespective of the cell line, revealed slightly lower motility in samples without threonine, although the cells in all samples were viable at the end of the incubation. Production of $^{13}$C-enriched acetate, pyruvate and alanine from [U-$^{13}$C]glucose and production of nonenriched acetate from threonine were quantified for the three cell lines studied (wild type, $\Delta$tbmpc1-B6 and $\Delta$tbmpc2-2C4; Table 2). No succinate was
using the Kruskal–Wallis test with a Metabolic end products were determined by HPLC. Parental produced elevated levels of pyruvate and decreased levels results of the HPLC analysis, both mutant cell lines pro-
succinate fermentation branch. Consistent with the expense of the downregulation or suppression of the
branch-point intermediate that can be converted to pyruvate (Pyr) or to fumarate (Fum) in the cytosol, leading to the formation of succinate (Suc) in G, or transported to the mitochondrion (M), where this molecule enters the incomplete tricarboxylic acid cycle (TCA) or is converted to Pyr. The remaining Pyr is converted to Pyr, which is either excreted or transported through MPC (full circle) into M. In the mitochondrial matrix, Pyr and Thr are metabolized to acetyl CoA (ACoA), which is subsequently catabolized to acetate (Ace). Both Pro and Gln are converted to glutamate (Glu), which enters the TCA cycle upon transamination with Pyr, yielding alanine (Ala) and 2-oxoglutarate (2-oxo). 2-Oxo is metabolized to mitochondrial succinate (Suc) in G, or transported to the mitochondrion (M), where this molecule enters the incomplete tricarboxylic acid cycle (TCA) or is converted to Lac. The following enzymes are shown: dihydroxyacetone phosphate (DHAP) is converted to glyceraldehyde phosphate (GAP), while a minor part of DHAP is spontaneously dephosphorylated to methylglyoxal (Mgx). This harmful compound is then detoxified to L-lactate (Lac). The following enzymes are shown: 1 – triose phosphate isomerase; 2 – glyceraldehyde phosphate dehydrogenase; 3 – phosphoglycerate kinase; 4 – phosphoglyceromutase; 5 – enolase; 6 – phosphoenolpyruvate carboxykinase; 7 – malate dehydrogenase; 8 – cytosolic fumarase; 9 – glycosomal fumarate reductase; 10 – mitochondrial fumarase; 11 – mitochondrial fumarate reductase; 12 – mitochondrial malic enzyme; 13 – pyruvate kinase; 14 – pyruvate dehydrogenase; 15 – threonine dehydrogenase; 16 – 2-amino-3-ketobutyrate coenzyme A ligase; 17 – acetate:succinate CoA-transferase; 18 – L-proline dehydrogenase; 19 – pyrroline-5-carboxylate dehydrogenase; 20 – glutaminase; 21 – L-alanine aminotransferase; 22 – nonenzymatic phosphate elimination; 23 – methylglyoxal reductase; 24 – lactaldehyde dehydrogenase; 25 – cytosolic malic enzyme.

detected, likely reflecting the absence of NaHCO₃ in the incubation buffer, which was used to support the flux towards the mitochondrial oxidation of pyruvate at the expense of the downregulation or suppression of the succinate fermentation branch. Consistent with the results of the HPLC analysis, both mutant cell lines produced elevated levels of pyruvate and decreased levels of glucose-derived acetate compared to wild type cells, regardless of the presence of threonine (Table 2). In the presence of threonine, alanine production from glucose was observed in wild type and mutant cell lines, whereas in the absence of threonine, relatively small amounts of alanine were only detected in TbMPC null mutants.

Table 1. Effect of mpc1 and mpc2 gene deletion, PEPCK downregulation and presence of the pyruvate transporter inhibitor (UK-5099) on the production of metabolic end products in PCF cell lines.

<table>
<thead>
<tr>
<th>Metabolic end product</th>
<th>Cell line</th>
<th>n</th>
<th>Pyruvate</th>
<th>Succinate</th>
<th>Lactate</th>
<th>Acetate</th>
<th>Fumarate</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Parental</td>
<td>6</td>
<td>32 ± 5 (4%)</td>
<td>325 ± 19 (45%)</td>
<td>42 ± 2 (6%)</td>
<td>325 ± 10 (45%)</td>
<td>1.25 ± 0.24 (0.17%)</td>
<td>726 ± 36</td>
</tr>
<tr>
<td></td>
<td>Parental + UK5099</td>
<td>3</td>
<td>74 ± 1 (13%)</td>
<td>256 ± 3 (44%)</td>
<td>21 ± 2 (4%)</td>
<td>235 ± 7 (40%)</td>
<td>1.10 ± 0.03 (0.19%)</td>
<td>588 ± 12</td>
</tr>
<tr>
<td>mmpc1PEPCK</td>
<td>3</td>
<td>6 ± 0 (2%)</td>
<td>56 ± 5 (22%)</td>
<td>38 ± 4 (15%)</td>
<td>155 ± 23 (61%)</td>
<td>0.18 ± 0.02 (0.07%)</td>
<td>255 ± 32</td>
<td></td>
</tr>
<tr>
<td>Δmpc1B6</td>
<td>6</td>
<td>122 ± 13 (23%)</td>
<td>236 ± 28 (45%)</td>
<td>14 ± 3 (3%)</td>
<td>153 ± 17 (29%)</td>
<td>1.06 ± 0.10 (0.20%)</td>
<td>527 ± 51</td>
<td></td>
</tr>
<tr>
<td>Δmpc1B6 + UK5099</td>
<td>3</td>
<td>95 ± 3 (19%)</td>
<td>225 ± 16 (44%)</td>
<td>17 ± 1 (3%)</td>
<td>173 ± 8 (34%)</td>
<td>0.86 ± 0.09 (0.17%)</td>
<td>511 ± 28</td>
<td></td>
</tr>
<tr>
<td>Δmpc2C4</td>
<td>6</td>
<td>135 ± 16 (24%)</td>
<td>247 ± 12 (44%)</td>
<td>14 ± 2 (2%)</td>
<td>169 ± 8 (30%)</td>
<td>1.50 ± 0.27 (0.27%)</td>
<td>565 ± 29</td>
<td></td>
</tr>
<tr>
<td>Δmpc2C4 + UK5099</td>
<td>3</td>
<td>105 ± 2 (20%)</td>
<td>240 ± 2 (44%)</td>
<td>15 ± 1 (3%)</td>
<td>178 ± 1 (34%)</td>
<td>1.28 ± 0.07 (0.24%)</td>
<td>541 ± 2</td>
<td></td>
</tr>
<tr>
<td>Δmpc1B6mmpc2C4PEPCK</td>
<td>3</td>
<td>53 ± 4 (28%)</td>
<td>57 ± 8 (31%)</td>
<td>15 ± 3 (8%)</td>
<td>62 ± 6 (33%)</td>
<td>0.27 ± 0.00 (0.15%)</td>
<td>187 ± 21</td>
<td></td>
</tr>
</tbody>
</table>

Metabolic end products were determined by HPLC. n = 3; The detected differences in metabolites are statistically significant as evaluated using the Kruskal–Wallis test with α = 0.05.
Table 2. NMR determination of metabolic end products from glucose (Glc) and l-threonine (Thr) in PCF with deleted mpc1 and mpc2 genes.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Glucose</th>
<th>Glucose + Threonine (Glc + Thr)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pyruvate</td>
<td>Alanine</td>
</tr>
<tr>
<td>Parental</td>
<td>127 ± 48</td>
<td>0</td>
</tr>
<tr>
<td>(\Delta\text{tbmpc1-B6})</td>
<td>1064 ± 62</td>
<td>67 ± 48</td>
</tr>
<tr>
<td>(\Delta\text{tbmpc2-2C4})</td>
<td>1037 ± 40</td>
<td>108 ± 30</td>
</tr>
</tbody>
</table>

\(n = 5\). The detected differences in metabolites are statistically significant as evaluated using the Kruskal–Wallis test with \(x = 0.05\).

The preference for threonine over glucose for the generation of acetate (approximately 2.6:1) was documented in wild type samples. This ratio was further increased in both null mutants [approximately 7.9:1 and 7.0:1 in \(\Delta\text{tbmpc1-B6}\) and \(\Delta\text{tbmpc2-2C4}\) cells, respectively (Table 2)], consistent with the expected decrease in pyruvate import into the mitochondrion. The NMR analysis showed that acetate production from glucose, through the pyruvate-dependent pathway, was reduced approximately threefold and fivefold in the TbMPC null mutants in the presence and absence of threonine, respectively. Clearly TbMPC is involved in acetate production from pyruvate; however, the significant residual acetate production from glucose implies that an alternative route is used in PCF trypanosomes.

Rearrangement of metabolism in \(\Delta\text{tbmpc1-B6}\) PCF upon knockdown of PEPCK

Glucose-derived malate enters the mitochondrion and serves as a substrate for the mitochondrial malic enzyme (mitME) in a reaction yielding pyruvate (Allmann et al., 2013). We suppressed this pathway in PCF wild type and \(\Delta\text{tbmpc1-B6}\) cell lines by RNAi targeting phosphoenolpyruvate carboxykinase (PEPCK) to distinguish the contribution of TbMPC1-dependent pyruvate transport and malate-dependent pyruvate production to the formation of acetate. Downregulation of PEPCK expression in \(\text{RNA}\text{APEPCK}\) and \(\Delta\text{tbmpc1-B6}\text{RNA}\text{APEPCK}\) samples was examined using Western blotting with an anti-PEPCK antibody (Supporting Information Fig. S3). Clones A11 (\(\text{RNA}\text{APEPCK}\)) and C5 (\(\Delta\text{tbmpc1-B6}\text{RNA}\text{APEPCK}\)) were selected for further experiments. No PEPCK signal was detected in either clone, allowing us to assume that PEPCK downregulation was efficient. To avoid the appearance of revertants of constitutive RNAi, freshly selected cells were used in the assays. The growth of PCF 427, \(\Delta\text{tbmpc1-B6}\), \(\text{RNA}\text{APEPCK}\) and \(\Delta\text{tbmpc1-B6}\text{RNA}\text{APEPCK}\) cell lines in standard SDM79 was comparable (Supporting Information Fig. S3). The end products of glucose metabolism were analyzed using HPLC in knockdown, parental and the \(\Delta\text{tbmpc2-2C4}\) cell lines (Table 1). The production of succinate and fumarate was reduced in both RNAi cell lines, reflecting the partial inhibition of the succinate fermentation pathway. Acetate production was decreased in the \(\text{RNA}\text{APEPCK}\) and \(\Delta\text{tbmpc1-B6}\text{RNA}\text{APEPCK}\) cell lines compared with the parental wild type and \(\Delta\text{tbmpc1-B6}\) cell lines, respectively, as previously described for the \(\text{pepck}\) mutant (Ebikeme et al., 2010). However, relative acetate production was similar in \(\Delta\text{tbmpc1-B6}\), \(\Delta\text{tbmpc2-2C4}\) and \(\Delta\text{tbmpc1-B6}\text{RNA}\text{APEPCK}\) cell lines (29–33% of the excreted end products from glucose metabolism; Table 1). The same relative production of acetate, regardless of PEPCK expression, strongly suggests that the contribution of malate to mitochondrial pyruvate metabolism is not significant. Alternatively, the activation of a third unknown adaptive route in the double mutant cannot be excluded.

Analysis of the \(\Delta\text{tbmpc1}\) BSF mutant cell line

The \(\text{tbmpc1}\) gene was deleted in the 427 BSF strain. The deletion of both alleles in several clones was confirmed by Southern blot analysis, and clone 2 (\(\Delta\text{tbmpc1-2}\)) was selected for further experiments (Fig. 2C). Knock-in BSF strains expressing epitope-tagged TbMPC1 in \(\Delta\text{tbmpc1-2}\) as well as wild type background were generated and the expression of TbMPC1-HA was confirmed by Western blotting (Fig. 2D). Interestingly, the \(\Delta\text{tbmpc1-2}\) BSF cell line did not exhibit any growth defect when cultured in complex HMI-9 medium (Fig. 2E), whereas the growth of this mutant was not supported in the recently developed Creek’s minimal culture medium (CMM) (Creek et al., 2013) (Fig. 2F). Growth rates were calculated from data within the linear sections of the curves (96–168 h post introduction to CMM), showing that the growth rate of the deletion mutant reached 36% (relative SD = 1.6%, \(n = 3\)) of the wild type cell line (100%, SD = 6.2%, \(n = 3\)). Partial complementation of the growth phenotype was observed in the knock-in cell line in \(\Delta\text{tbmpc1-2}\) background (75%,
SD = 2.2%, n = 3; Fig. 2F). Following the observation of \( \Delta tbmpc1-2 \) growth defect in CMM, we checked whether \( \Delta tbmpc1-2 \) showed altered mitochondrial membrane potential. Parental and \( \Delta tbmpc1-2 \) cells were stained using tetramethylrhodamine ethyl ester and analyzed by flow cytometry using a published protocol (see Subrtova et al., 2015, and Supporting Information). No change in the potential was observed in cells cultured in either media (Mann–Whitney U-test = 4; \( P = 1.0; n = 3 \) for both HMI-9 and CMM). Next, we attempted to supplement CMM with all individual components of HMI-9 which are missing in CMM (see Supporting Information Table S5 in Creek et al., 2015) at concentrations present in HMI-9. All individual supplements were tested in a pilot screen aimed at identifying a supplement which (i) would restore the growth of \( \Delta tbmpc1-2 \) cell line in CMM and (ii) would not affect the growth of parental \( T. brucei \) Lister 427 BSF. The cells were diluted to \( 2 \times 10^5 \) cells per ml, aliquoted at 2 ml in culture plates and counted for 3 days after inoculation. HMI-9 and CMM were used as controls. No tested component of HMI-9 matched both requirements, leading us to the conclusion that the growth defect of \( \Delta tbmpc1-2 \) in CMM is not caused by the lack of a single missing component present in HMI-9.

Because the in vitro growth of the \( \Delta tbmpc1-2 \) BSF mutant in CMM was affected, we compared the development of infection in mice. All mice infected with wild type parasites died within 5 days of inoculation, whereas only two out of five \( \Delta tbmpc1-2 \)-infected mice died within 10 days according to Kaplan–Meier survival curves (log rank test = 9.29; \( P < 0.01 \); Fig. 5). Mice infected with the wild type strain also showed high parasitemia, with a maximum of \( 1 \times 10^{11} \) trypanosomes per ml at 72 h post-inoculation of the parasites. In contrast, the parasitemia in mice infected with \( \Delta tbmpc1-2 \) was considerably lower, with a maximum of approximately \( 2 \times 10^{10} \) trypanosomes per ml at 96 h post-infection, followed by a subsequent decline. Taken together, these data suggest that in BSF \( T. brucei \) TbMPC1 plays an important role in vivo and under specific conditions in vitro.

To study acetate production from glucose, the \( \Delta tbmpc1-2, tbmpc1^{-/+} \) (single allele mutant) and wild type 427 BSF trypanosomes were incubated in PBS containing \( [U-^{13}C] \) glucose and threonine for the \( ^1H-NMR \) quantification of excreted end products, as previously described (Mazet et al., 2013; Creek et al., 2015). Apart from pyruvate, which represents ~85% of the end products excreted from glucose, alanine, acetate and lactate were detected using \( ^1H-NMR \). Differences in the levels of acetate produced from glucose and the ratio of threonine/glucose utilization for acetate production were observed. Similar to observations in PCF, the rate of glucose-derived acetate production was significantly decreased in the \( \Delta tbmpc1-2 \) cell line (4.6-fold) compared to wild type cells. In addition, an intermediate situation was observed for the \( tbmpc1^{-/+} \) cell line with a 1.5-fold reduction of glucose-derived acetate production compared to wild type (Table 3). This effect shifted the ratio of threonine to glucose utilization for acetate production from approximately 0.8 in wild type cells to 1.3 and 3.7 in the \( tbmpc1^{-/+} \) and \( \Delta tbmpc1-2 \) cell lines, respectively. As observed for the PCF mutants, the results of the NMR analyses are consistent with a role for TbMPC in pyruvate transport to the mitochondria in BSF trypanosomes.

### Discussion

In this study, we assign a pyruvate transport function to two MPC proteins (TbMPC1 and TbMPC2) in the parasitic protist \( T. brucei \). We demonstrated that both MPC subunits localized in the mitochondrial membrane of PCF \( T. brucei \), and we showed the importance of both subunits for pyruvate uptake using null mutant cell lines. We also addressed the adaptations of metabolic fluxes in MPC deletion mutants in both PCF and BSF trypanosomes and investigated the phenotype of BSF \( tbmpc1 \) null mutants in vivo in mouse infections.

In the genome of \( T. brucei \), we identified two MPC paralogs. MPC proteins are members of the MtN3-like clan, bearing structural similarity to bacterial SemiSWEET proteins that mediate sugar transport (Vanderperre et al., 2014). Whereas the SemiSWEET transporter functions as a symmetrical dimer of triple-helix units, the pyruvate carrier is asymmetrical, comprising two TM helices of MPC1 and three TM helices of MPC2 (Bender et al., 2015). We analyzed the primary sequences of MPCs described thus far and TbMPCs, focusing on conserved amino acids implicated in SemiSWEET transport function. In all analyzed MPC proteins, a conserved tryptophan typical of the SemiSWEET binding pocket is present in MPC2 and substituted for pheryllalanine in MPC1 proteins. However, it is unlikely
that this substitution affects the transport function of MPCs, as a corresponding tryptophan to phenylalanine mutation in the SemiSWEET transporter did not affect sucrose uptake through SemiSWEET proteins in a liposome assay (Lee et al., 2015). The different number of transmembrane helices in SemiSWEET and MPC transporters results in differences in the binding pocket, which can explain the specificity of the transported substrates (sucrose uniport vs. pyruvate-proton symport). A rather surprising finding concerns the expected hinge region of TbMPC1. All other analyzed MPC1 subunits presented a conserved proline in the second TM helix, consistent with Lee et al. (2015), which serves as an important molecular hinge for the binder clip-like transition between open-outward and open inward states of the transporter in SemiSWEET proteins and proteins from the PQ-loop family. A proline-to-alanine substitution in SemiSWEET at this site strongly diminishes sucrose transport (Lee et al., 2015). The same substitution is present in TbMPC1. We propose that for the opening of TbMPC pore, it is sufficient for proline-induced intramolecular conformational changes to occur in only one protomer.

Both TbMPC1 and TbMPC2 proteins were localized to the mitochondria of PCF trypanosomes by indirect immunofluorescence, and the expression of both proteins was detected in mitochondrial membrane fractions. This result is consistent with previous finding of these proteins in the PCF mitochondrial proteome (Panigrahi et al., 2009). The mitochondrial localization of TbMPC1 was also determined in BSF. No staining for TbMPC1 was detected at the BSF plasma membrane, precluding the involvement of MPC in the export of pyruvate as a metabolic end product. It has recently been reported that pyruvate export is mediated through distinct TbPT transporters (Sanchez, 2013). Although we were unable to express TbMPC2 in BSF, the localization of TbMPC2 in the BSF mitochondrion is conceivable based on the localization in PCF and the reported association of MPC1 with MPC2 in other species (Bricker et al., 2012; Herzig et al., 2012).

The essential function of both TbMPC1 and TbMPC2 for mitochondrial pyruvate transport was directly demonstrated by monitoring 14C-pyruvate uptake in mitochondria isolated from PCF wild type and TbMPC null mutants. TbMPC-dependent pyruvate import accounted for the total inhibitor-sensitive import, consistent with observations in S. cerevisiae (Herzig et al., 2012). Functional transport complexes were not detected in either ∆tbmpc1-B6 or ∆tbmpc2-2C4 cell lines, consistent with available data from yeast (Herzig et al., 2012; Bender et al., 2015).

The analysis of the metabolic end products in cell lines lacking either TbMPC subunit strengthened the notion that TbMPCs represent pyruvate transporters in PCF and BSF trypanosomes. Specifically, the decreased production of glucose-derived acetate in both BSF and PCF lacking one tbmpc gene most likely reflects a decreased intramitochondrial pyruvate concentration. However, some glucose-derived acetate is still produced in both PCF and BSF mutant cell lines, while acetate production from glucose is abolished in both BSF and PCF cell lines upon knock down of the subunit E2 of pyruvate dehydrogenase (RNAiPDH-E2) (Mazet et al., 2013; Millerioux et al., 2013). This finding supports the view that an alternative route is used in both trypanosome stages to produce/import glucose-derived pyruvate in the mitochondrion. According to the current model of PCF central metabolism, a significant part of the flux from glucose diverted to the glycosome for malate production is exchanged with the mitochondrion to produce pyruvate through mitME inside the mitochondrion (Allmann et al., 2013). This hypothesis is consistent with the observed decrease in succinate production based on HPLC analysis in both PCF null mutants, as a higher proportion of malate would be used as a substrate for mitME to compensate for the lack of pyruvate transport through MPC, eventually leading to a decrease in succinate levels. To further address this hypothesis, we analyzed metabolic perturbations or adaptations in ∆tbmpc1-B6/RNAiPEPCK and RNAiPEPCK

### Table 3. Effect of mpc1 gene deletion (−/−) and single allele mpc1 deletion (−/+ ) in BSF on the production of glucose-derived metabolic end products using NMR.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Pyruvate Glc</th>
<th>Alanine Glc</th>
<th>Acetate Glc</th>
<th>Total Glc</th>
<th>Thr</th>
<th>Total Thr</th>
</tr>
</thead>
<tbody>
<tr>
<td>Parental</td>
<td>13,277 ± 2076</td>
<td>1648 ± 252</td>
<td>532 ± 116</td>
<td>410 ± 89</td>
<td>15,686 ± 2384</td>
<td>410 ± 89</td>
</tr>
<tr>
<td>∆tbmpc1−/+</td>
<td>11,307 ± 2351</td>
<td>1381 ± 292</td>
<td>354 ± 93</td>
<td>435 ± 134</td>
<td>13,117 ± 2581</td>
<td>435 ± 134</td>
</tr>
<tr>
<td>∆tbmpc1−/−</td>
<td>11,280 ± 1358</td>
<td>1240 ± 256</td>
<td>116 ± 70</td>
<td>426 ± 108</td>
<td>12,825 ± 1597</td>
<td>426 ± 108</td>
</tr>
</tbody>
</table>

n = 10. The values indicated in bold represent statistically significant differences in the production of a given metabolite as evaluated using the Kruskal–Wallis test, with α = 0.05.
PCF cell lines in which the first step of the glycosomal succinate branch is downregulated. The control RNAi-PEPCK cell line showed a 2-fold reduction of acetate production compared to the wild type parasite, consistent with previous analyses of the Δpepck mutant (Ebi-keme et al., 2010). A similar reduction of the rate of acetate production was also observed in Δtbmpc1-B6RNAiPEPCK compared to Δtbmpc1 (2.5-fold). These data suggest that either the pathway comprising PEPCK, malate dehydrogenase and mitME contributes poorly to pyruvate/acetate production from glucose in the mitochondrion, or a third route is used in the Δtbmpc1-B6RNAiPEPCK cell line, that is, MPC-independent mitochondrial pyruvate transport. Indeed, approximately 40% of the total pyruvate uptake was observed in mitochondria treated with UK-5099 and MPC knock-out cell lines, which is in favour of an alternative pyruvate transporter, such as a member of the monocarboxylate transporter family with broader specificity. Monocarboxylate transporters are typically present at the cell surface, but the mitochondrial localization of these proteins has been reported in rats and baker’s yeast (Nalecz et al., 1991; Butz et al., 2004). However, it cannot be excluded that the mitochondrial membrane integrity of digitonin-permeabilized cells was partially affected in our experiments, facilitating the passive diffusion of 14C-pyruvate into the mitochondrion. Clearly, additional experiments are required to understand how glucose-derived acetate is produced in Δtbmpc mutant cell lines.

The upregulation of TbMPC proteins in PCF was observed in two proteomic studies, with a fivefold upregulation for TbMPC1 according to Butter et al. (2013) and twofold upregulation for TbMPC1 according to Urbaniak et al. (2012). This finding is consistent with a higher proportion of glucose-derived pyruvate converted into acetate in PCF compared to BSF, representing up to 70% and 5% of the end products excreted from glucose metabolism, respectively (Mazet et al., 2013; Bringaud et al., 2015). As expected, no growth defect was apparent in either PCF Δtbmpc1-B6 or Δtbmpc2-2C4 cells in vitro, as the acetate production capacity was not impaired, that is, both mutant cell lines still produced acetate from glucose and threonine, the other source of acetate present in the growth medium.

In contrast to PCF trypanosomes, the MPC gene deletion seems to affect BSF metabolism because (i) the growth of the Δtbmpc1-2 BSF cells was not supported in CMM minimal medium compared with the wild type cells, (ii) we were unable to either express or knock out TbMPC2 in BSF and (iii) the Δtbmpc1-2 BSF mutant showed reduced lethality of infection in mice. Although pyruvate is the principal metabolic end product excreted from glucose metabolism in BSF trypanosomes, the diminished pyruvate transport into the mitochondrion strongly affected this parasite, as an 80% reduction in acetate production was observed in the Δtbmpc1-2 mutant (Table 3). In the mitochondrion, pyruvate is further oxidized by PDH, generating acetyl-CoA and eventually acetate. It was recently shown that PDH and threonine dehydrogenase (TDH) are synergistically essential for the growth of BSF in rich medium because of acetate production; both RNAiPDH-E2 and Δtdh single mutants remain viable under these conditions. Accordingly, the RNAiPDH-E2 cell line is lethal in the absence of threonine (Mazet et al., 2013). Interestingly, the growth of the ΔTbMPC1 BSF mutant in CMM could not be rescued by threonine, suggesting that the observed reduction in acetyl-CoA or acetate production is not responsible for Δtbmpc1-2 death. Alternatively, we hypothesize that MPC is necessary in BSF for supplying the substrate for mitochondrial alanine aminotransferase (AAT). It has been suggested that AAT catalyzing the transamination of pyruvate and glutamate into alanine and α-ketoglutarate is essential in both PCF and BSF (Spitznagel et al., 2009). No activity of the downstream enzyme, α-ketoglutarate dehydrogenase, could be detected in bloodstream T. brucei (Sykes and Hajduk, 2013), suggesting that the essential function of AAT might not be directly connected to cellular metabolism. In this context, the question of availability of alanine for mitochondrial translation should be addressed: while it is well documented that mitochondrial translation is essential in both procyclic and bloodstream T. brucei (Cristodero et al., 2010), information on amino acid import into the mitochondrion is very limited in T. brucei (de Macedo et al., 2015) and surprisingly scarce in general (King, 2007).

In summary, we identified a mitochondrial pyruvate transporter comprising two subunits and described the properties and function of this protein in the metabolism of a human parasite and an important model organism, T. brucei. Furthermore, these data support the recently emerging picture of BSF functioning well beyond glycolysis, with unexpectedly active mitochondrial metabolic pathways (Roldan et al., 2011; Mazet et al., 2013; Creek et al., 2015).

**Experimental procedures**

In silico analyses

Putative mpc1 and mpc2 genes were identified using BLAST in the T. brucei genome database (www.tritrypdb.org) (Aslett et al., 2010). Both genes were aligned to 29 selected MPC homologs obtained by a BLAST search using S. cerevisiae MPCs as queries using Muscle 3.8.425
software (default parameters) (Edgar, 2004) and trimmed with BIMSA 1.12 (BLOSUM30) (Criscuolo and Gribaldo, 2010). The protein evolution model was selected using ProtTest 3.2 (Darriba et al., 2011). PhyML 2.2.0 (topology search: best of NNIs and SPRs, initial tree: BioNJ; Substitution model: LG, proportion of invariable sites: fixed (0), gamma distribution parameter: estimated; number of categories: 4; bootstrap replicates: 500) (Guindon and Gascuel, 2003) and MrBayes 3.2.2 (rate matrix: LG; rate variation: gamma; gamma categories: 4; chain length: 2,000,000; heated chains: 4; heated chain temp: 0.2; burn-in length: 500,000) (Huelsenbeck and Ronquist, 2001) were used to reconstruct the phylogenetic tree. Transmembrane domains were predicted using the TMHMM 2.0 (Krogh et al., 2001) and TMPred (Hofmann and Stoffel, 1993). MitoProt (Claros and Vincens, 1996) and PSORTII (Nakai and Horton, 1999) were used to predict subcellular localization.

**Cell cultivation**

*T. brucei* PCF strains 427 and 29-13 (expressing TetR and T7RNAP) (Wirtz et al., 1999) were grown at 27°C in SDM-79 medium (Brun and Schonenberger, 1979) supplemented with 10% (v/v) fetal calf serum. *T. brucei* BSF 427 and New York Single Marker (SM, expressing TetR and T7RNAP) (Wirtz et al., 1999) strains of the same species were grown in HMI-9 medium supplemented with 10% (v/v) fetal calf serum (Hirumi and Hirumi, 1989) or CMM supplemented in HMI-9 medium supplemented with 10% (v/v) fetal calf serum (Creek et al., 1993) at 37°C in 5% CO₂. PCF 29-13 *T. brucei* were grown in the presence of hygromycin (25 μg/ml) and G418 (15 μg/ml). BSF SM cells were grown in the presence of G418 (1.5 μg/ml). The following concentrations were used for additional antibiotics in cultures of PCF cell lines after transformation: 1 μg/ml of puromycin, 2.5 μg/ml of phleomycin and 10 μg/ml of blasticidin. For BSF mutant cell lines, puromycin at 0.1 μg/ml, blasticidin at 5 μg/ml and phleomycin at 2.5 μg/ml were used. A Z2 cell counter (Beckman Coulter, USA) was used to count the growing trypanosome cultures. The cells were maintained at the exponential growth phase (PCF and BSF were diluted daily to 2 to 3 x 10⁶ and 1 x 10⁶, respectively), and cumulative cell numbers were calculated.

**Generation of mutant cell lines**

Tagged TbMPC1 and TbMPC2 were expressed in PCF 29-13 and BSF SM trypanosomes. The entire *tbmpc1* ORF was PCR-amplified from PCF Tb427 gDNA using the appropriate primers (see Supporting Information Table S1) and subcloned into plasmid pT7-3V5-PAC (Flaspohler et al., 2010) with a C-terminal V5 tag and a puromycin resistance marker. Plasmid pJH54, with a triple HA tag bearing the phleomycin resistance marker (derived from pLEW100; a kind gift from C. Clayton, University of Heidelberg, Germany), was used for expression of whole *tbmpc2* ORF.

To generate *tbmpc1* and *tbmpc2* null mutants, the 5′- and 3′-flanking regions of *tbmpc1* and *tbmpc2* ORFs were PCR amplified (see Supporting Information Table S1 for primers) and sequentially inserted into the plasmids pBS-blast and pBS-phleo (Ruepp et al., 1997), resulting in four plasmids in which the genes encoding blasticidin deaminase or bleomycin binding protein were flanked by 5′ and 3′UTRs of TbMPC1 or TbMPC2. Two rounds of transformation and selection were required to obtain Δtbmpc1 and Δtbmpc2 clonal cell lines.

Kock-in BSF strains expressing TbMPC1-HA in Δtbmpc1-2 as well as wild type background were generated using the plasmid pHDI034 (Quijada et al., 2002). Puromycin was used to select stable transformants and the ectopic expression of tagged TbMPC1 driven by an rRNA promoter was checked by Western blotting with a mouse monoclonal anti-HA-tag antibody (Sigma-Aldrich, USA).

To generate cell lines showing the constitutively downregulated expression of PEPCK, a sense-antisense fragment, comprising the 3′ portion of PEPCK (Tb927.2.4210) coding sequence and the beginning of the 3′UTR of PEPCK, was excised from an existing vector (Coustou et al., 2008) and inserted in pHDI034 to generate the PEPCK RNAi vector. Wild type and Δtbmpc1-68 PCF cell lines were used as parental cell lines for the transformations. The downregulation of PEPCK expression in both resulting cell lines was evaluated through Western blotting with an anti-PEPCK antibody (a kind gift from Thomas Seebeck, University of Bern, Switzerland).

Linearized plasmids were electroporated into parental PCF *T. brucei* using two subsequent pulses (1500 V and 1700 V) with a Gene Pulser Xcell (Bio-Rad, USA) (Vondruskova et al., 2005), or parental BSF was transfected using an Amaxa Nucleofector (Lonza, Switzerland) (Burkard et al., 2011), respectively. The transformed cells were subjected to limiting dilution. The growth of PCF clones was facilitated using parental feeder cells in conditioned medium and a 5% CO₂ atmosphere (pouches with CO₂ gen compact, Oxoid).

**Southern blotting**

Genomic DNA was isolated from cultures of BSF *T. brucei* 427 and Δtbmpc1-2 strains using the TELT method (Medina-Acosta and Cross, 1993) and digested with BamHI or EcoRV enzymes. Approximately 10 μg of digested gDNA was loaded per well. Gel electrophoresis and Southern blotting was performed using standard procedures (Southern, 2006). Digoxigenin-labeled probes for the complete coding sequences of TbMPC1 and blasticidin deaminase were prepared using the PCR DIG Probe Synthesis Kit (Roche) with the primers listed in Supporting Information Table S1. Hybridization was conducted using DIG Easy Hyb buffer at 42°C. Washing and blocking buffers and anti-digoxigenin-alkaline phosphatase with CSPD as substrate were purchased from Roche. Chemiluminiscent signal was detected on ImageQuant LAS4000 (GE Healthcare).

**Fluorescence microscopy**

Approximately 2 x 10⁶ PCF and BSF trypanosomes were used per slide. The PCF cells were incubated with 0.5 μM Mitotracker Red CMXRos (Life Technologies, USA) for 10 min in SDM-79 at 27°C, washed with phosphate-buffered...
saline (PBS), incubated in SDM-79 media for another 20 min and subsequently washed with PBS. The cells were fixed and permeabilized on the slides using -20°C cold methanol for 5 min, followed by 5 min incubation in -20°C cold acetone.

BSF trypanosomes were incubated with 25 nM Mitotracker Red CMXRos for 30 min in HMI-9 in 37°C, collected by centrifugation and incubated again for 10 min in HMI-9, followed by washing with PBS. The cells were fixed on slides using 3.6% formaldehyde for 15 min at room temperature, washed with PBS and permeabilized using 0.1% Triton X-100 for 10 min.

The slides were incubated in blocking solution (0.25% bovine serum albumin, 0.25% gelatin and 0.05% Tween 20 in PBS) for 1 h at room temperature. Expressed TbMPC1-V5 and TbMPC2-HA were visualized using mouse monoclonal anti-V5-tag and anti-HA-tag antibodies, respectively (both from Sigma-Aldrich, USA), and a secondary donkey anti-mouse Alexa Fluor 488 antibody (Life Technologies, USA). The cells were mounted in Vectashield with DAPI (Vector Laboratories, USA) and observed using an Olympus IX81 microscope. The images were captured using a Hamamatsu Orca-AG digital camera and processed using cell®R imaging software (Olympus, Japan).

Infection of mice

Female 8-week-old Balb/c mice (n = 5) were intraperitoneally infected with 5 x 10⁶ BSF trypanosomes. Prior to inoculation, the cells were harvested in mid-log phase from HMI-9 medium and washed once in PBS. Parasitemia was counted in Diff-Quik-stained (Medion Diagnostics, USA) smears prepared from the tail blood of infected mice. Animal handling was approved by the Czech Ministry of Agriculture (53407/ENV/13-2300/630/13). The acquired data were analyzed by Kaplan-Meier survival analysis in MedCalc (MedCalc Software).

Cell fractionation

Crude mitochondrial fractions were obtained through digitonin solubilization according to Smid et al. (2006). The integrity of the mitochondria and purity of the fractions were assessed after measuring activities of the cytosolic enzyme pyruvate kinase and the mitochondrial enzyme threonine dehydrogenase as markers for cytosolic and mitochondrial fractions, respectively. The extent of cross-contamination of the fractions was consistently below 2%. The mitochondrial membrane and matrix fractions were isolated using digitonin according to Mach et al. (2013).

To localize expressed recombinant TbMPC1 and TbMPC2 proteins, individual cell fractions were separated using SDS-PAGE, followed by Western blotting and visualization using mouse monoclonal anti V5-tag and anti HA-tag antibodies, respectively, and peroxidase-conjugated goat anti-mouse IgG (Sigma-Aldrich, USA). The purity of fractions was evaluated using antibodies against mitochondrial matrix (HSP60), mitochondrial membrane (porin) and cytosolic (enolase) marker proteins, kind gifts of S.L. Hajduk (University of Georgia, USA), M. Chaudhuri (Meharry Medical College, USA) and P. Michels (Catholic University of Louvain, Belgium), respectively.

Pyruvate uptake

Uptake of radioactively labeled pyruvate was performed with digitonin-solubilized cells. Pyruvate stock solution was prepared after mixing nine volumes of 2 mM cold pyruvate with one volume of 2 mM [2-¹⁴C]-labeled pyruvate (ARC, USA). Following solubilization, the cell pellets (3.5 mg; equivalent of 5–6 x 10⁶ cells) were stored on ice. For pyruvate import, the pellet was resuspended in 200 μl of mannitol buffer, pH 7.4 (650 mM mannitol, 50 mM potassium phosphate, 1 mM EGTA, 0.1% BSA, 10 mM MgSO4 and 1 mM ATP), and incubated on ice for 5 min. Next, the samples were pelleted and resuspended in 200 μl of mannitol buffer, pH 6.3, containing 200 μM UK-5099 [alpha-cyano-beta-(2-phenylindol-3-yl)acrylate] or the same volume of DMSO (2 μl) for 2 min at 27°C. Subsequently, 100 μM pyruvate diluted from the stock solution was added, and the samples were incubated at 27°C for 15 min. The reaction was quenched after the addition of 1 ml ice-cold mannitol buffer, pH 7.4, containing 10 mM pyruvate. The permeabilized cells were washed four times in quenching buffer and resuspended in 1 ml AquaLuma (Lumac Systems, USA) for scintillation counting.

Analysis of excreted end products from metabolism of carbon sources

High-performance liquid chromatography (HPLC) and nuclear magnetic resonance (NMR) were used to identify and quantify the end products of glucose or L-threonine metabolism.

HPLC analysis was performed using on a Hi-Plex H column (300 x 7.7 mm, 8 μm) (Polymer laboratories, USA) at 65°C and a flow rate of 0.4 ml/min, using 5 mM H₂SO₄ as eluent. The amount of the metabolite was quantified as absorbance at 205 nm. The system was calibrated by 5-point external calibration curves of different concentrations of metabolites expected to be present in the samples. The samples were prepared using the following method: 10⁸ trypanosomes per sample were collected through centrifugation, washed and resuspended in glucose incubation buffer (PBS with 24 mM NaHCO₃ and 10 mM glucose, pH 7.3) with 10 μM UK-5099 or the same volume of DMSO (2 μl) to final volume of 200 μl and incubated for 2 h at 27°C. Subsequently, the cells were centrifuged, and the supernatant was filtered through a 0.22-μm filter and 30 μl was loaded onto the HPLC column. The output was visualized and analyzed using Clarity 5 software (DataApex). NMR analyses of end products excreted from glucose and/or threonine metabolism were performed according to Millerioux et al. (2013) for PCF and Mazet et al. (2013) for BSF. T. brucei PCF (5 x 10⁷) or BSF (2.5 x 10⁷) cells were collected after centrifugation at 1400 g for 10 min, washed once with phosphate-buffered saline (PBS) containing 4 mM glucose (BSF) or no glucose (PCF) and incubated for 6 h at 27°C (PCF) or 5 h at 37°C (BSF) in 2.5 ml of PBS buffer (pH 7.4) containing 4 mM [U-¹³C]glucose in the

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presence or absence of 4 mM threonine. The integrity of the cells during the incubation was assessed through microscopic observation. The supernatant was collected, and 50 µl of maleate solution in D$_2$O (20 mM) was added as an internal reference. $^1$H-NMR spectra were performed at 125.77 MHz using a Bruker DPX500 spectrometer equipped with a 5 mm broadband probe head. Measurements were recorded at 25°C using the EREATIC method. This method provides an electronically synthesized reference signal. The following acquisition conditions were used: 90° flip angle, 5000 Hz spectral width, 32 K memory size and 9.3 sec total recycle time. Measurements were performed with 256 scans for a total time close to 40 min. Prior to each experiment, the phase of the EREATIC peak was precisely adjusted. Resonances of the obtained spectra were integrated, and the results were expressed relative to the $^1$H-NMR quantification of the end products pyruvate and acetate) throughout the experiment was confirmed with 256 scans for a total time close to 40 min. Prior to each experiment, the phase of the EREATIC peak was precisely adjusted. Resonances of the obtained spectra were integrated, and the results were expressed relative to the $^1$H-NMR quantification of the end products pyruvate and acetate) throughout the experiment was confirmed with 256 scans for a total time close to 40 min. Prior to each experiment, the phase of the EREATIC peak was precisely adjusted. Resonances of the obtained spectra were integrated, and the results were expressed relative to

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References


Supporting information

Additional supporting information may be found in the online version of this article at the publisher’s web-site.
8. Conclusions

8.1. Processing of N-terminal mitochondrial targeting sequences

Evolutionary origin of trypanosomal mitochondrion is a long discussed issue. There are two contradictory opinions, whether it is an ancient or highly divergent organelle. Initial identifications of short N-terminal mitochondrial targeting sequences (N-MTS) and distinct mitochondrial translocase machinery were interpreted as an ancient feature; on the contrary, highly derived and missing machineries may reflect diversification and adaptation to parasitism.

In the genome of *Trypanosoma brucei*, we found canonical α and β subunits of mitochondrial processing peptidase (MPP) with glycine rich loop and zinc binding domain, respectively and two subunits of MPP paralogs, the core proteins (cp1 and cp2) that are associated with respiratory complex III (bc1). As expected, MPP subunits were localized within mitochondrial matrix. In bloodstream form (BSF), MPP expression was decreased, which corresponds to downregulation of mitochondrial pathways in this stage. Consistently with lack of bc1 complex in BSF, core proteins were not detectable in BSF, whereas in the procyclic form (PCF) cp1 was associated with the mitochondrial membrane. Unlike the MPP, core proteins lack domain that are required for the processing activity and thus we hypothesize that they are inactive and functions as a structural subunit of bc1.

Analysis of *T. brucei* N-MTS that were predicted in 336 mitochondrial proteins shows no significant difference in net charge, length and cleavage site motif in comparison to canonical N-MTSs of *Saccharomyces cerevisiae*.

Phylogenetic analysis revealed that all eukaryotic lineages include members of the same type MPP/core protein family, whereas cp1 subunit evolved independently from β-MPP duplication in metazoans, some fungi and kinetoplastids. We propose that evolution of cp1 allows the independent regulation of mitochondrial respiration and protein import. These results support hypothesis that trypanosomal mitochondrion is not ancient, but rather highly evolved and adapted for the parasitic lifestyle.
8.2. Mitochondrial pyruvate transporter

Pyruvate is a key intermediate of energetic metabolism of PCF and end-product of BSF. For many years it was known, that pyruvate is actively transported across mitochondrial membrane and that transport is driven by pH, co-transports one proton, and the pyruvate transport is inhibited by compound UK-5099. However, molecular character of putative pyruvate transporter was not known until recently. The mitochondrial pyruvate carrier (MPC) appeared to comprise of two paralogous proteins MPC1 and MPC2. We found homologs of both proteins in \textit{T. brucei} genome. They are both localized within mitochondrial membrane and we demonstrated that they are indeed responsible for import of pyruvate to mitochondria. Surprisingly, decrease of pyruvate import in the cells without MPC is only partial, which raises the question what is responsible for the rest of the transported pyruvate. MPC is not essential for the trypanosomal growth in the glucose-rich cultivation medium, but in the glucose-depleted medium, the growth of BSF was significantly slower. Experimental infection of mice revealed that the strain with impaired MPC function caused lower parasitemia as well as mortality in comparison with the wild type \textit{T. brucei} strain.

Within the MPC ablated PCF and BSF cells, we observed dramatic changes in the glucose metabolism. Due to impaired MCP system, pyruvate is excreted from PCF cells in higher amounts. However, some pyruvate is able to reach mitochondria via alternative route, possibly through malate import that could be converted to pyruvate by activity of mitochondrial malic enzyme or by MPC-independent mitochondrial pyruvate transport. In BSF cells, we were unable to overexpress or knock-out MPC2, which may reflect considerably different situation in this stage. Although pyruvate is the main end-product of BSF trypanosomes, the reduction of pyruvate import has high impact on glucose-derived acetate production. Moreover, threonine was unable to rescue the growth of MPC ablated cells in the absence of glucose, suggesting that pyruvate has other function than acetate-production. We hypothesize that pyruvate may be necessary for mitochondrial alanine production by glutamate dehydrogenase.
8.3. Iron uptake in procyclic \textit{T. brucei}

Mechanism of iron acquisition by BSF has been elucidated many years ago, however this process remain unknown for PCF cells. BSFs utilize a specific receptor for transferrin to acquire sufficient amount of iron. We found that PCF are able to utilize different source of iron in the environment than the BSF in mammalian host. Within the insect host, the uptake of transferrin is not involved in iron acquisition. Many parasites can acquire iron by the uptake of heme followed by heme oxidase processing. However, heme oxidase was not found in the \textit{T. brucei} genome, thus iron acquisition from heme is unlikely. We show that PCF \textit{T. brucei} takes up iron via reduction of low molecular weight ferric complexes and subsequent transport of ferrous iron. Moreover, we show that iron imported by this pathway can be subsequently used in the mitochondria. We found possible ferric reductase in the genome of \textit{T. brucei}, as well as several putative metal transporters that can participate in iron uptake. The efficiency of ferrous iron import is comparable to process previously described in yeast. Different iron acquisition pathway in each life cycle stage may be advantageous. Reduction of the ferric complexes and subsequent iron import can be essential especially in the insect environments without mammalian host transferrin and with particular emphasis to full development of PCF mitochondrion.