The University of South Bohemia

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Mitochondrial energy metabolism in *Trypanosoma brucei*

Complex I and the others.

Ph. D. Thesis

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The thesis summarizes data gathered on various components of respiratory chain of Trypanosoma brucei. Namely, NADH:ubiquinone oxidoreductase (complex I), alternative NADH:ubiquinone oxidoreductase (NDH2) and mitochondrial glycerol-3-phosphate dehydrogenase are discussed themselves and in broader context of energy metabolism. Also, a work done using RNA interference library is described.

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1/4/2011

Declaration of contribution

We, the authors of the listed publications, hereby declare that Zdeněk Verner contributed to the mentioned publications as follows:

Mitochondrial membrane potential-based genome-wide RNAi screen of *Trypanosoma brucei* (Verner Z., Paris Z., Lukeš J.)

- Performed evaluation of selected clones, re-cloned the identified RNAi fragments into p2T7-177 and performed re-evaluation of the newly selected clones.

The Remarkable Mitochondrion of Trypanosomes and Related Flagellates (Lukeš J., Hashimi H., Verner Z., Číčová Z.) – section 6 (Energy Metabolism of *T. brucei* Mitochondrion)

- Wrote the section dealing with mitochondrial proteins and energy metabolism.

Complex I (NADH:ubiquinone oxidoreductase) is active in but non-essential for procyclic *Trypanosoma brucei* (Verner Z., Čermáková P., Škodová I., Kriegová E., Horváth A., Lukeš J.)

- Performed molecular biology part and testing of buffers for biochemical assays. Prepared draft text and pictures.

Characterization of the NADH:ubiquinone oxidoreductase (complex I) in the trypanosomatid *Phytomonas serpens* (Kinetoplastida) (Čermáková P., Verner Z., Man P., Lukeš J., Horváth A.)

- Prepared samples for biochemical and molecular measurements.

Alternative NADH dehydrogenase (NDH2) in procyclic *Trypanosoma brucei* (Verner Z., Škodová I., Ďurišová-Benkovičová V., Horváth A., Lukeš J.) (in preparation)

- Performed molecular biology assays and part of the biochemical testing. Prepared draft text and pictures.

Biochemical characterization of FAD-dependent glycerol-3-phosphate dehydrogenases in trypanosomatids with emphasis on *Trypanosoma brucei* (Škodová I., Verner Z., Fabián P., Lukeš J., Horváth A.) (in preparation)

- Suggested experimental design, supervised I. Škodová and contributed to manuscript writing.

Insights into the functioal organization of cytochrome c oxidase in Trypanosoma brucei (Gnipová A., Paris Z., Verner Z., Horváth A., Lukeš J., Zíková A.)

- Performed pilot experiments with mitochondrial membrane potential.

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1 Abstract

Mitochondrial energy metabolism of *Trypanosoma brucei* has several features distinguishing it from other eukaryotes. Since the mechanism of RNA interference (RNAi) is functional in *T. brucei* and its genome has been sequenced, we have used a genome-wide RNAi-based screen to track down proteins involved in the maintenance of mitochondrial membrane potential. In another study, complex I, the place of NAD regeneration and the largest proton pump in most mitochondria, was studied in procyclic *T. brucei*. Reverse genetic studies combined with biochemical approaches allowed the dissection of the contribution of complex I as well as of an alternative single-protein dehydrogenase capable of performing the same biochemical function as complex I, yet without the capability to translocate protons. In frame of our dissection of the respiratory chain of procyclic trypanosomes, we have studied cytochrome c oxidase complex that seems to be composed of at least 17 nuclear encoded subunits, most of which do not have significant homology to any known eukaryotic proteins. To establish if all or some of the novel subunits are important for function, assembly and/or structural integrity of the complex, we selected three novel subunits and silenced them by RNAi. Importantly, all were shown to be critical for cell viability and proper mitochondrial function, as they play a crucial role in the structural integrity and activity of complex IV. These results may suggest that *T. brucei* cytochrome *c* oxidase is a highly diverged complex possessing novel subunits and it may represent the largest complex of its kind known up to date. Finally, another pair of enzymes was studied by the same means; mitochondrial FAD-dependent glycerol-3-phosphate dehydrogenase and its homologue with an unknown cellular location were both shown in vitro to be capable of performing the same reaction in different organelles. All the data gathered are then discussed in a wider context of mitochondrial metabolism.

2 Preface

This thesis is a result of my stay in the laboratory of molecular parasitology headed by Julius Lukeš. From a simple enthusiams of doing a research in molecular biology I ended up in the neighboring field of biochemistry. A definition told by Paul Michels during my stay in his laboratory fits perfectly: molecular biologist is a biochemist without a diploma. I tried hard to get a grasp of biochemistry while still working in a purely molecular biology laboratory. With the help of Anton Horváth, I might became a hybrid or you may call it half-biochemist. It is up to you, the reader of this thesis, to decide who I became in the end.

As is probably usual, the thesis summarizes official part of my work. The unofficial one including negative results, helping others with experiments, teaching young undergraduate students, giving lectures at high-schools or performing basic molecular biology experiments during freshmen field training were not included for obvious reasons. They are not the pure, peer-reviewable scientific data. This may be used as my defense of relatively modest contribution. But I would like to conclude with another quotation of Paul Michels: Defending student is never convinced that he presented enough results.

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3 Introduction - an organism of interest - *Trypanosoma brucei*

Trypanosoma brucei is a unicellular parasite of mammals. It is spread in sub-Saharan Africa, a place called the tse-tse belt (Figure 1). This geographic designation derives its name from a fly latinized *Glossina*, commonly known as the tse-tse fly. African trypanosomes alternate between this vector and a mammalian host, whose identity depends on the subspecies of *Trypanosoma*.

Figure 1 Distribution of Glossina spp. in Africa.

African trypanosomes constitute a complex comprised of *Trypanosoma congolense, T. vivax* and *T. brucei*. T. brucei itself is subdivided into at least three members: *T. brucei brucei*, a parasite of livestock causing the disease called nagana; *T. brucei gambiense*, a human parasite causing chronic sleeping sickness present mainly in Western and Central Africa; and *T. brucei rhodesiense*, a (sub)species causing acute sleeping sickness in humans and occurring in Eastern and Southern Africa. Members of this complex differ in their susceptibility to the so-called "trypanosome lytic factor" (TLF) in blood serum. In general, TLF protects humans and some primates from most trypanosomes (Lugli, et al., 2004). TLF is composed of proteins and apolipoproteins (for review see Vanhollebeke, et al., 2010). Thus, humans are resistant to *T. b. brucei* and susceptible to *T. b. rhodesiense* and *T. b. gambiense*.

T. b. rhodesiense owes its infectiousness to a single gene – serum resistance-associated gene (SRA) (Xong, et al., 1998). *T. b. gambiense* is resistant to human TLF thanks to a decreased expression of a receptor mediating TLF transport (Kieft, et al., 2010).

Outside of Africa, one can find *T. cruzi* in Latin America and *T. (b.) evansi* and *T. (b.) equiperdum* in Asia, Europe and South America. *T. cruzi* is an intracellular human parasite causing Chagas disease. It is spread by feces of triatomids known as kissing bugs (Reduviidae) (for review see (Lescure, et al., 2010 and Junqueira, et al., 2010). *T. equiperdum* and *T. evansi* originate from Africa and were able to spread outside of it in form of the bloodstream stage (BS) (Lun, et al., 2010), perhaps even being a subspecies of *T. brucei* (Lai, et al., 2008; Lun, et al., 2010). By doing this they disconnected from their invertebrate host thus losing sexual reproduction and recombination as well as a convenient way of spreading. Nowadays, both parasites rely on physical means of transmission from one mammal to another. *T. equiperdum* infects new host during coitus directly via blood from small injuries, while *T. evansi* is transmitted mechanically by blood feeding insects. However, instead of establishing an infection within this insect it relies on a fast acquisition of new bloodmeal and remains on/in the insects' rostrum or proboscis used for penetration into the victim's bloodstream (for general review see Brun, et al., 1998 and Lai, et al., 2008).

Life cycle of *T. brucei* entails two different hosts and multiple different stages (Figure 2). Starting with infection of the tse-tse fly midgut, the parasite multiplies using haemolymph and gut's content for feeding (see Chapter 5 for details on metabolism). This stage is called a procyclic one (PS) according to the mutual position of the flagellum, the nucleus and the kinetoplast. Then trypanosomes migrate thru the insect's intestinal tract into the salivary glands. Here the so-called metacyclic stage stops dividing and await blood feeding by the vector (Sharma, et al., 2009). Upon this event, the metacyclic stage enters mammalian bloodstream and transforms into a fast dividing long-slender stage (bloodstream stage – BS). These trypanosomes depend on glycolysis since they literally swim in a glucose solution. If untreated, this stage establishes a permanent infection thanks to unique feature – their surface is covered with a variable surface glycoprotein (VSG) coat (see below). Part of the offspring of the long slender BS is transformed into non-dividing short stumpy BS, which are pre-adapted for new round of colonization of the insect gut. Upon ingestion, long-slender forms die due to an abrupt change in temperature, pH and low supply of glucose; meanwhile

short-stumpy forms transform into PS and the life cycle is completed (for review see Hammarton, 2007).

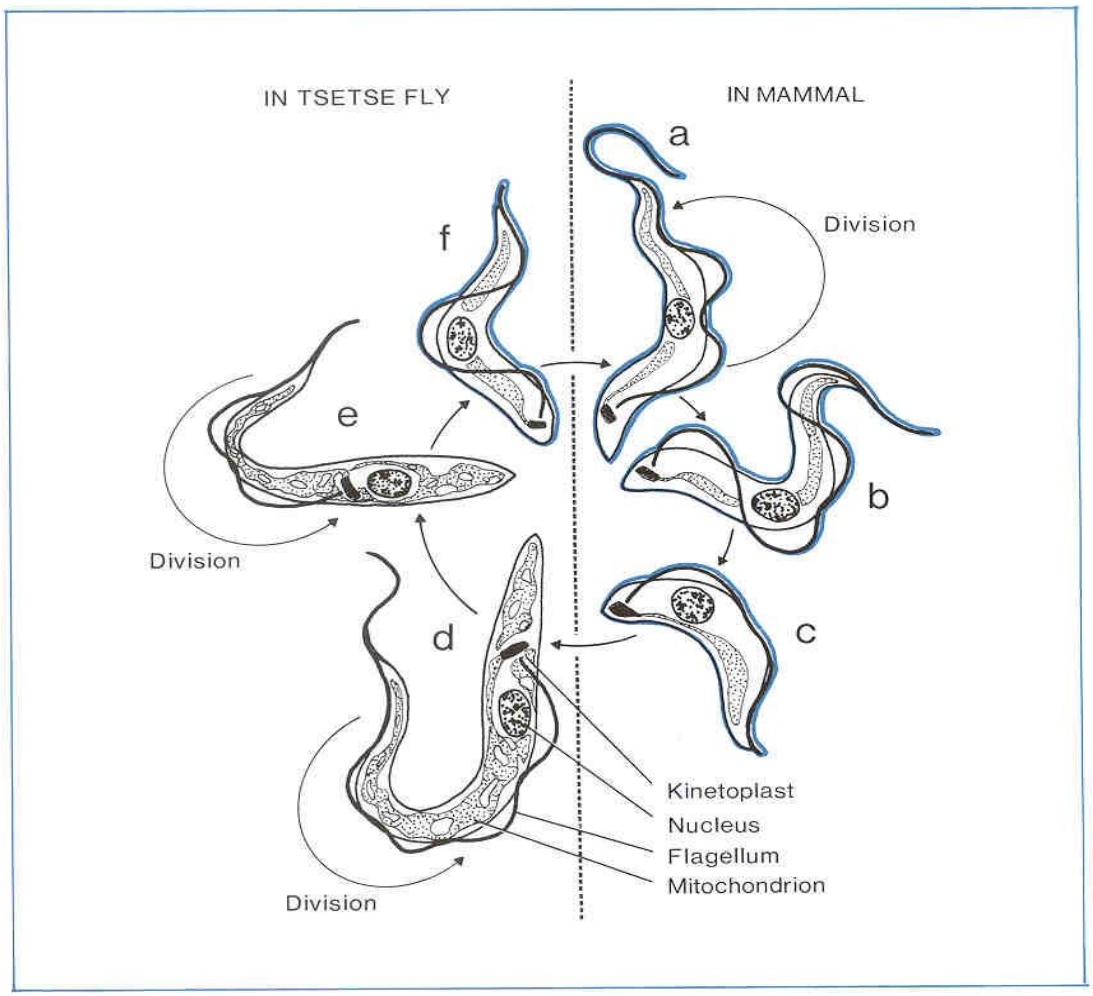

Figure 2 Life cycle of Trypanosoma brucei. A – long slender bloodstream form; b – intermediate bloodstream form; c – bloodstream short stumpy form; d – procyclic form; e – epimastigote form; f – metacyclic form. Picture from http://www.ilri.org/InfoServ/Webpub/fulldocs/Ilrad90/Trypano.htm.

If tse-tse fly ingests two different strains during its bloodmeals, a resulting strain is a combination of its "parents" (Gibson, 2001). Recently, it was described that mating takes place not only between cells originating from different sources but also between cells coming from the same host (Peacock, et al., 2009) making trypanosomes genetically very plastic. The presence of sexual reproduction is in fact a horrific feature. Thru recombination, the parasites possibly multiply the VSG coats and in combination with fast mutation rate and frequent recombination events among VSGs render creation of a vaccine directed against their surface a virtually impossible task (Magez, et al., 2010).

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The disease caused by trypanosomes is fatal if untreated. This is due to the fact that *T. brucei* evolved an excellent feature to avoid mammalian immunity. As mentioned above, surface of bloodstream forms of trypanosomes is covered with VSGs, which the immune cells of the mammal can recognize as foreign proteins and start production of antibodies and subsequent clearance of parasites bearing these VSGs (for recent review on VSG see Black, et al., 2010). However, part of the offspring is coated with novel VSGs thus avoiding the specific immune response. Since the genome of *T. brucei* codes for more than 1600 of these VSGs (Marcello, et al., 2007), they possess enormous palette of possible surfaces. This cycle of defeating one major trypanosome population with concomitant re-establishment of infection by unrecognized sub-population can go virtually forever and leads to the exhaustion of the host. This constitutes a never-ending Red queen-like[1] situation. During later stage of the disease, a fraction of the parasites crosses the blood-brain barrier and establishes parasitaemia in this delicate environment increasing overall host's burden (Rodgers, 2010). Moreover, brain-spine fluid of a healthy human is buffered to pH 7.33. It seems very likely that excreted metabolic end-product(s) of trypanosomes (see chapter 5, Figure 5) do interfere with this value and thus might contribute to clinical symptoms.

According to WHO, there are 36 countries where tse-tse flies can be found. Approximately 60 million people were in direct risk of African trypanosomiasis in 1995 with an estimated 300,000 new cases per year with less than 30,000 cases diagnosed and treated. In 2004, the newly reported cases fell to 17,616, probably due to increased control (http://www.who.int/trypanosomiasis_african/en/index.html); further progress was mirrored by less than 10,000 cases reported in 2009, the least number in 50 years (http://www.who.int/mediacentre/factsheets/fs259/en/). Treatment of human disease is complicated by the fact that the tse-tse belt covers very poor countries with low level health care (Simarro, et al., 2010). Also, classical drugs used for curing the disease contain arsene, which is severely toxic. WHO-supported development of new treatment resulted in the nifurtimox-eflornithine combination treatment (Burri, 2010).

[1] I call this Red queen-like phenomenon because there is a theoretical possibility for mammalian immunity to win this race. Since there is a definite speed by which both, trypanosome can change VSG and immunity adapts to a new one then simply by stochastics the number of parasites might get low enough for total clearance.

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4 Cellular and molecular features of *T. brucei*

T. brucei has been studied since the end of the 19th century. This was the first organism in which an extranuclear DNA was identified (Ziemann, 1898). During 20th century, a whole list of important biological phenomena was discovered in *T. brucei* and/or other kinetoplastids, e.g. membrane protein anchoring via glycosylphosphatidylinositol, changes in telomeric repeats, RNA editing or trans-splicing (Donelson, et al., 1999).

The large amount of extranuclear DNA was coined the name kinetoplast DNA (kDNA) and became the flag character of the whole group of protists (Meyer, 1968)[2]. This unique structure appears as an electron dense band located close to the flagellar pocket (for review see de Souza, et al., 2009). Using electron microscopy, it was shown to be a network of circular DNAs of two types, termed mini- and maxicircles (reviewed in Lukeš, et al., 2002 and Lukeš, et al., 2005). Maxicircles are analogous to typical mitochondrial (mt) DNA, yet numerous stop codons were found throughout most predicted ORFs (Stuart, 1983; Simpson, et al., 1987; Sloof, et al., 1992). The other type of DNA molecules, minicircles, was without precedent in mtDNA from other organisms and was originally considered to have a structural role as a scaffold (Englund, et al., 1982), to be a regulator of transcription of maxicircles (Michelotti, et al., 1987) or to be involved in maxicircles segregation (Hoeijmakers, et al., 1980).

This mystery was solved by the discovery of mt mRNA editing. Benne et al. sequenced mt transcript of coxII and found discrepancies between what is encoded and what is transcribed (Benne, et al., 1986). Subsequent studies showed that for RNA editing several protein complexes composed of more than a hundred proteins are needed. Using small guide RNAs encoded on the minicircles, these protein complexes in co-ordinated action specifically insert and/or delete uridines, leading to the formation of translatable mRNAs (reviewed in Lukeš, et al., 2005 and Stuart, et al., 2005). With this information, most genes encoded in kDNA were identified, the last one being ND2, a component of respiratory complex I, invisible because of pan-editing of its transcript (Kannan, et al., 2008). However, there are

[2] Being limited to the use of internet, I found this article to be the firts to mention kinetoplast; I hereby appologize for not citing the very first work to use the name kinetoplast.

still at least four putative open reading frames with unknown function (MURF2 and 5 and CR3 and 4 – see Figure 3).

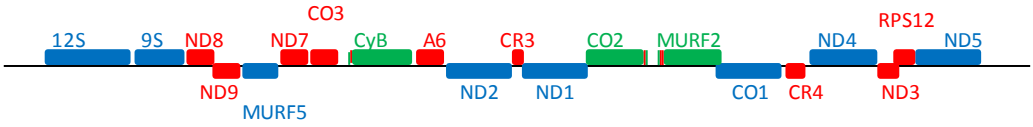

Figure 3 Genes present on mitochondrila maxicircle. Blue boxes – unedited genes; red boxes – edited (part(s)) of genes; green boxes – unedited part(s) of genes.

Outside of the mitochondrion, another unique feature of *T. brucei* is the presence of peroxisome-like organelles called glycosomes (reviewed in Michels, et al., 2006). These microbodies harbor, among others, almost all enzymes of glycolysis (see chapter 5). The relation between glycosomes and peroxisomes is derived from the common origin of these organelles, reflected among others by the same targeting signals (Michels, et al., 2006). Apart from glycolytic enzymes, glycosomes also contain (parts of) pathways related to purine salvage and pyrimydine synthesis, pentose pathway, and ether-lipid metabolism and oxidative stress protection via trypanothion (Michels, et al., 2006).

Let's have a look at the nucleus. *T. brucei* has a genome containing n=11 diploid megabase- (1-6 Mbp), 1-5 intermediate- (200-900 kbp) and approximately 100 mini- (50-150 kbp) chromosomes (for review on nuclear architecture see Navarro, et al., 2007). The minichromosomes contain repetitive sequences with VSG genes found at their subtelomeric regions (El-Sayed, et al., 2000). In several aspects transcription on the chromosomes differs from most eukaryotes and rather resembles prokaryotic transcription. Tens of genes are oriented in one direction and once transcription is initiated, it goes on thru multiple loci generating large polycistronic transcript (reviewed in Martinez-Calvillo, et al., 2010). In contrast to bacterial transcription, the polycistrons do not involve functionally related genes. Polycistrons are subsequently spliced and polyadenylated into classical functional mRNAs. Splicing in *T. brucei* is, however, mostly not in *cis*, as is the case of- exon/intron splicing producing mature mRNAs in the majority of eukaryotes. In *T. brucei*, only two genes are known to be *cis*-spliced (polyA polymerase Tb927.3.3160 and a putative RNA helicase Tb927.8.1510) (Gunzl, 2010). On the contrary, the extent of *trans*-splicing in trypanosomes is massive. The long polycistronic molecule is cut by a mechanism similar to regular splicing. The 5'-end of all nuclear-encoded mRNAs is capped with the so-called splice leader RNA, a

39 bp-long molecule present in multiple copies in the genome (Martinez-Calvillo, et al., 2010). This, together with the added polyA tail allows subsequent translation of the transcript. Without splice-leader, the RNA molecule is degraded (Campbell, et al., 2003).

In general, trypanosomes do not regulate their gene expression at the level of transcription. Instead, their expression is regulated via RNA stability and translation (Campbell, et al., 2003). However, comparison of transcriptome in different stages revealed the presence of regulation, as was shown recently in *T. brucei* (Nilsson, et al., 2010). A single protein expression is regulated by a concert of different RNA-binding proteins and mRNA sequence. Moreover, as shown in the relatives of trypanosomes - *Leishmania tarentolae* and *Phytomonas serpens*, protein expression also depends on pre-ATG triplet of the mRNA (Lukeš, et al., 2006).

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5 Use of RNA interference library in *T. brucei*

Very fruitful field of research opened with the discovery of RNA interference (RNAi) in Caenorhabditis in 1998 (Fire, et al., 1998). Almost instantly, this phenomenon was uncovered also in *T. brucei* (Ngo, et al., 1998). Unfortunately, it seems to be lacking in most other trypanosomatids, *Leishmania braziliensis* being another exception (Lye, et al., 2010). RNAi-based approach allowed reverse genetics to be successfully applied on a large scale (Morris, et al., 2002; Motyka, et al., 2004).

RNAi is an ancient mechanism of RNA degradation that has important roles in eukaryotic cells. It takes place in cellular defense against dsRNA viruses and transposones (for review, see Obbard, et al., 2009), yet its greatest role likely lies in fine tuning of gene expression (Grewal, 2010). It is now being shown that non-coding regions of the genome are transcribed into small dsRNAs that subsequently regulate a vast number of different cistrons.

In general, dsRNA is recognized, cut to small pieces that are subsequently used as templates for ssRNA degradation (Fire, et al., 1998). The innate ability of cells to degrade specific RNAs was exploited for the purpose of reverse genetics. Usually after the cell encounters any dsRNA, it triggers RNAi no matter what the final target is (Hardy, et al., 2010). We can thus introduce dsRNA homologous to mRNA of a studied protein and subsequently study a phenotype in cell depleted for the target.

Discovery of RNAi in *T. brucei* led to the development of an inducible system that might be used for reverse genetics and/or overexpression of genes of interest (Wirtz, et al., 1999). Briefly, viral T7 polymerase and a bacterial tetracycline (tet) represor were introduced into the genome of *T. brucei* by homologous recombination. These new genes are under selection markers (Hyg and Neo) ensuring their transmission into the offspring generations. From a vector bearing T7 promotor, tet-regulated transcription occurs (Wirtz, et al., 1999).

Nowadays, this tet-inducible system is used for RNAi as well as for homologous recombination or overexpression of proteins. In the former case, the cells are transfected with linear DNA bearing either two T7 promotors opposing each other with a fragment of

target gene cloned in between or the construct contains only one T7 promotor with a gene fragment cloned twice so that its transcript forms a hairpin. Double T7 promotor system is used for its ease in cloning while the single promotor strategy is reported to be more efficiently regulated.

In homologous recombination, the inducible system is used for generation of conditional knock-outs. If a targeted gene is essential for cell survival, regular knock-out cannot be obtained, since all successful null mutants die. Workaround is either using RNAi or preparing an overexpressing vector that is used as a second step in homologous recombination. Then cells bear one intact allele in a genome, one knocked-out allele and one tet-regulated ectopic copy. Addition of tet ensures its transcription while the second allele is being knocked-out. Then, after a selection of clonal cell line, the tet is removed from medium switching off the ectopic copy and one can start studying the phenotype (Wirtz, et al., 1999).

A powerful tool of RNAi brought an idea of high throughput screening for novel genes participating in the phenomena of interest (reviewed in Mohr, et al., 2010). This led to the creation of RNAi library (Morris, et al., 2002). Briefly, whole genome of *T. brucei* was cut to pieces by sonication and resultant DNA fragments were subsequently cloned into modified pZJMβ vector (Wang, et al., 2000). This way a whole genetic content of *T. brucei* was covered. The most difficult part of using this powerful tool appeared to be choosing the right screening method that would still be high throughput. The simplest method is to check growth of tet-induced cell lines for a couple of days. However, this can be used just for tens of cell lines at once, given one researcher is doing such a screening. If one would like to cover a significant part of the genome, thousands of cell lines would require this screening thus disqualifying growth curve from being a candidate for screening. Moreover, affected growth is really a general feature saying nothing about what caused it.

RNAi library was successfully used to search for genes associated with various cellular features (Motyka, et al., 2004). The following study of kDNA represents an example of use of the library (Englund, et al., 2005). After tet-induction, cells were screened “manually” by staining of kinetoplast and those with anomalous signal were picked for further analysis. In our project with this library we have experienced two obstacles: difficulties with choosing

the screening method and the lack of automated post-screening identification of genes of interest.

Our laboratory is interested in various aspects of trypanosome mitochondrion. We decided to use RNAi library in a search for novel proteins involved one way or another in mitochondrial respiratory chain. Apart from proteins directly involved in respiration itself, there must be tens or maybe even hundreds of proteins involved in respiratory chain assembly and regulation. At the beginning of this project, we focused at oxygen consumption. Any cell line with affected respiratory chain should either have altered oxygen consumption or have shifted its sensitivity to various inhibitors of respiration. In a pilot study, cyanide, an inhibitor of complex IV, was used as a selectable drug.

The study was designed as follow: culture of RNAi library growing in a glucose-depleted medium (see chapter 5) would be tet-induced for 5 days; then a previously-tested concentration of cyanide would be added, so that all cells with regular respiration are killed, while cells that had switched to cyanide-insensitive trypanosomal alternative oxidase (TAO) would survive. A sensitivity of remaining cells to salicylhydroxamic acid, an inhibitor of TAO served as a cross-control. Final step would be cloning of selected culture using limited dilution and sequencing integrated RNAi vectors.

Unfortunately, this straightforward design was proven non-functional. The key assumption, that the cells with affected cyanide-sensitive pathway would be advantaged over those without such an affliction, was not confirmed. For testing, cell lines generated during complex III and IV study (Horváth, et al., 2005) were cultivated with different cyanide concentrations and their growth was compared with the parental cell line. None of the concentration was advantageous for RNAi KDs and so this design was abandoned (Figure 4). Here, I am speculating on possible explanation of these observations.

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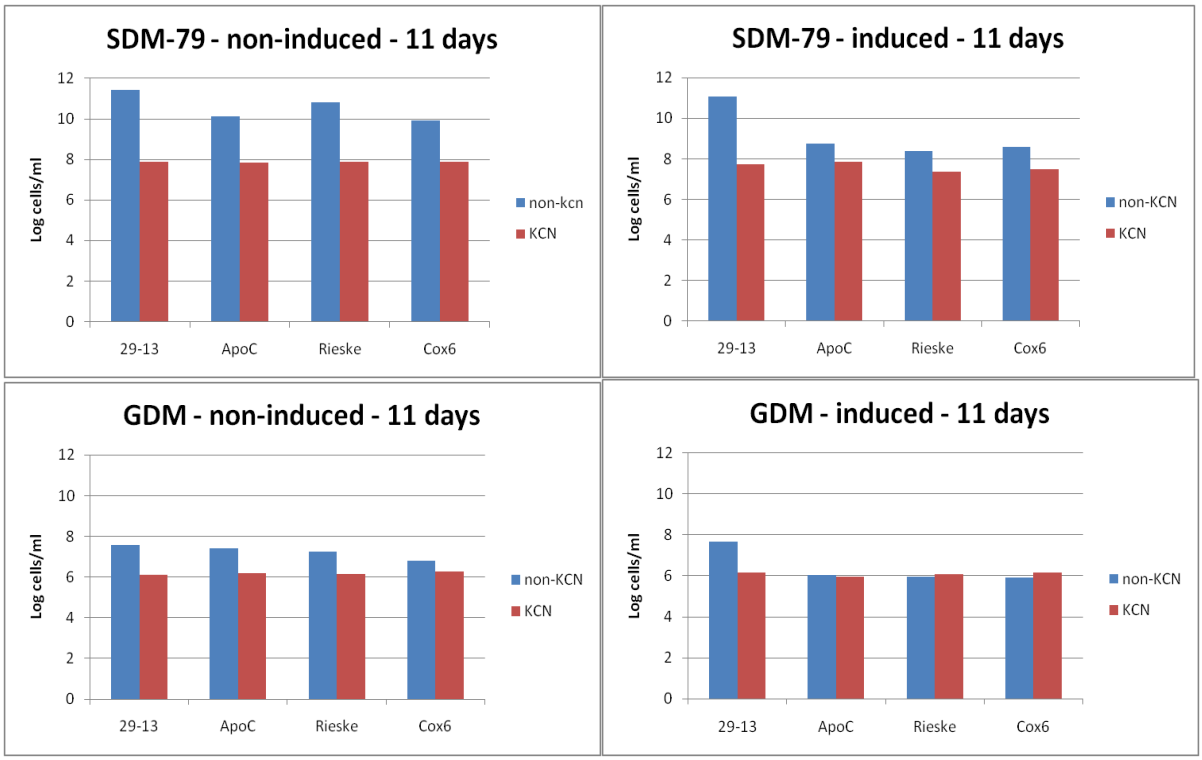

Figure 4 Comparison of cell concentration of various RNAi cell lines grown under different conditions on day 11. Cell concentration in log scale of either non-induced cell lines (left part) or tet-induced cell lines (right part). Effect of cyanide (red columns) compared to non-treated cells (blue columns). Cyanide has no advantageous effect on any cell line. SDM-79 is a standard cultivation medium containing both, glucose and amino acids. GDM stands for glucose-depleted medium or SDM-80 (Bringaud, et al., 2006). N. B. that no matter what cell line was used, they grew significantly slower in GDM than in SDM-79.

Complexes III and IV are both proton pumps and the tested knock downs had affected either the ApoC or Rieske Fe-S protein (subunits of complex III) or cox6 (subunit of complex IV). Both subunits were essential in the SDM-79 medium, which contains glucose and amino acids. In these knock-downs respiration shifted from cyanide-sensitive cytochrome pathway to TAO (Horváth, et al., 2005). In a pilot study, these same cells were cultivated in a glucose-depleted medium thus forcing them to use respiratory chain for energy production. Missing of the two major proton pumps, the cells were unable to generate ATP using oxidative phosphorylation. Also, any ATP generated by substrate level phosphorylation was used to cope with this situation, e.g. for maintaining of mitochondrial membrane potential by complex V (F1F0 ATP synthase) in a manner similar to the BS (Schnaufer, et al., 2005). A collapsed membrane potential means a protein import into mitochondrion is affected. This consequently leads to the disruption of many essential processes including FeS cluster synthesis. Use of sensitivity to the inhibitor was an option, however, such an approach would probably not be possible in a high throughput study.

Since it turned out that cyanide is unsuitable for RNAi library screening, mitochondrial membrane potential was used as a means of selection. In the mentioned study (Horváth, et al., 2005) depletion of subunits of complexes III or IV affected membrane potential significantly. In the new experiment design cells were induced for 6 days, stained for membrane potential and then analyzed by FACS to choose knock down cells with lower potential. This led to the selection of 75 clones. Upon successful cultivation, these clones were again tested for stable decrease of mitochondrial membrane potential. Approximately half of the tested cell-lines retained the ability to decrease membrane potential upon induction. We sequenced three randomly chosen clones and re-cloned the obtained fragments into p2T7-177 RNAi vector. Then, we transfected *T. brucei* 29-13 strain and confirmed the phenotype in this vector. As expected at the beginning of the study, we found 2 proteins previously not associated with membrane potential (Tb927.7.830 and Tb09.160.1350). The third sequence does not belong directly to ORF. Instead, this region lies in between of two genes. RNAi in this case affected either one of 3'-UTRs or an intrinsic regulatory non-coding sequence (see appendix 10.1 for Verner, et al., 2010).

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6 Energy metabolism of *T. brucei*

Energy metabolism of *T. brucei* dramatically differs between the BS and PS cells. While the BS literally swim in a glucose solution, the PS have to survive mostly by anabolism of amino acids (see review by Tielens, et al., 2009).

Surplus of glucose available to the BS allows this stage to cover all ATP demands by glycolysis. Mitochondrion of this stage is transformed into a tubular structure lacking the Krebs cycle enzymes. This is mirrored by the absence of some components of mitochondrial respiratory chain. The most notable is the lack of cytochromes meaning that any electrons coming from ubiquinone do not have a natural sink such as complex III, soluble cytochrome c and complex IV (Tielens, et al., 2009).

Nevertheless, the BS trypanosomes do need oxygen and convert it into water. Already in 1977 Opperdoes and colleagues showed that oxygen is indispensable for this stage and the mitochondrion is the place of oxygen consumption (Opperdoes, et al., 1977). An alternative oxidase (TAO) was later identified to be the enzyme responsible for this reaction (Clarkson, et al., 1989; Chaudhuri, et al., 1995). Using glycerol-3-phosphate dehydrogenase (G3PDH) bound to the mitochondrial inner membrane and facing intermembrane space (Figure 5), trypanosomes feed glycolysis with dihydroxyacetone phosphate (DHAP) thus avoiding a branch of glycolysis leading to glycerol; by employing G3PDH, trypanosomes save one net-produced ATP molecule that would otherwise be lost since only “half” glucose would reach a glycolytical pay-off phase (Michels, et al., 2006). Electrons from glycerol-3-phosphate (G3P) are passed onto FAD cofactor and then to ubiquinole. This electron carrier passes them onto TAO. This single-protein enzyme grounds electrons to oxygen without any work being done. This is a crucial difference from complex IV that uses energy of electron transport thru the complex to pump protons across mitochondrial membrane (Chaudhuri, et al., 2006).

For the BS, glycolysis is thus the most important ATP generator. As mentioned above, most glycolytic enzymes reside inside glycosomes (Michels, et al., 2006). Impermeability of the single membrane of this organelle allows substrates to enter only via transporters and enzymes are sequestered there during its biogenesis. The enzymatic content then remains

stable but may differ from organelle to organelle reflecting changing environment and metabolic requirements. The impermeability of the glycosomal membrane requires a careful redox balance. Concerning glycolysis, current models show perfect balance of all co-factors needed (Figure 5) (Michels, et al., 2006). Two molecules of ATP per molecule of glucose consumed at the phosphorylation stage of glycolysis are returned at the first ATP producing step, while the net ATP produced is available outside of the glycosome in BS; PS covers ATP expenses via a glycosomal pathway leading to succinate. In a model, redox balance of glycolysis is maintained by two means: i/ under anoxic conditions, the pathway is split after carbon ring cut; glyceraldehyde-3-phosphate branch goes on regularly, while dihydroxyacetone phosphate is converted to glycerol via glycerol-3-phosphate; this set up keeps ATP and NAD/H ratio inside the organelle, meanwhile lowering cell's ATP net production; ii/ under standard oxygen conditions, the glycerol-3-phosphate is exported out of the glycosome and converted back to dihydroxyacetone phosphate that returns into the glycosome at G3PDH dehydrogenase residing at the inner mitochondrial membrane; this avoids the irreversible conversion of G3P into glycerol and boosts ATP net production. In BS, pyruvate created via glycolysis is not used any longer and is excreted out of the cell (Michels, et al., 2006).

The PS is in general similar to other eukaryotes, as it can utilize either glucose or amino acids as energy source. If glucose is used, it is converted into pyruvate the same way as in the BS, the only exception being localization of phosphoglycerol kinase. The glycosomal isoform expressed in the BS provides glycosomal ATP that is consumed in the phosphorylation of glucose. As mentioned above, in the PS, this ATP requirement is met in a glycosomal succinate-producing pathway. Phosphoenolpyruvate can re-enter the glycosome, where it is converted into succinate in the subsequent oxaloacetate-malate-fumarate-succinate steps (Figure 5). However, part of phosphoenolpyruvate is converted into pyruvate within the glycosome and this pyruvate is then transported out into the cytosol (Michels, et al., 2006). Next, pyruvate enters the mitochondrion and follows standard reactions to acetyl co-enzyme A (AcCoA).

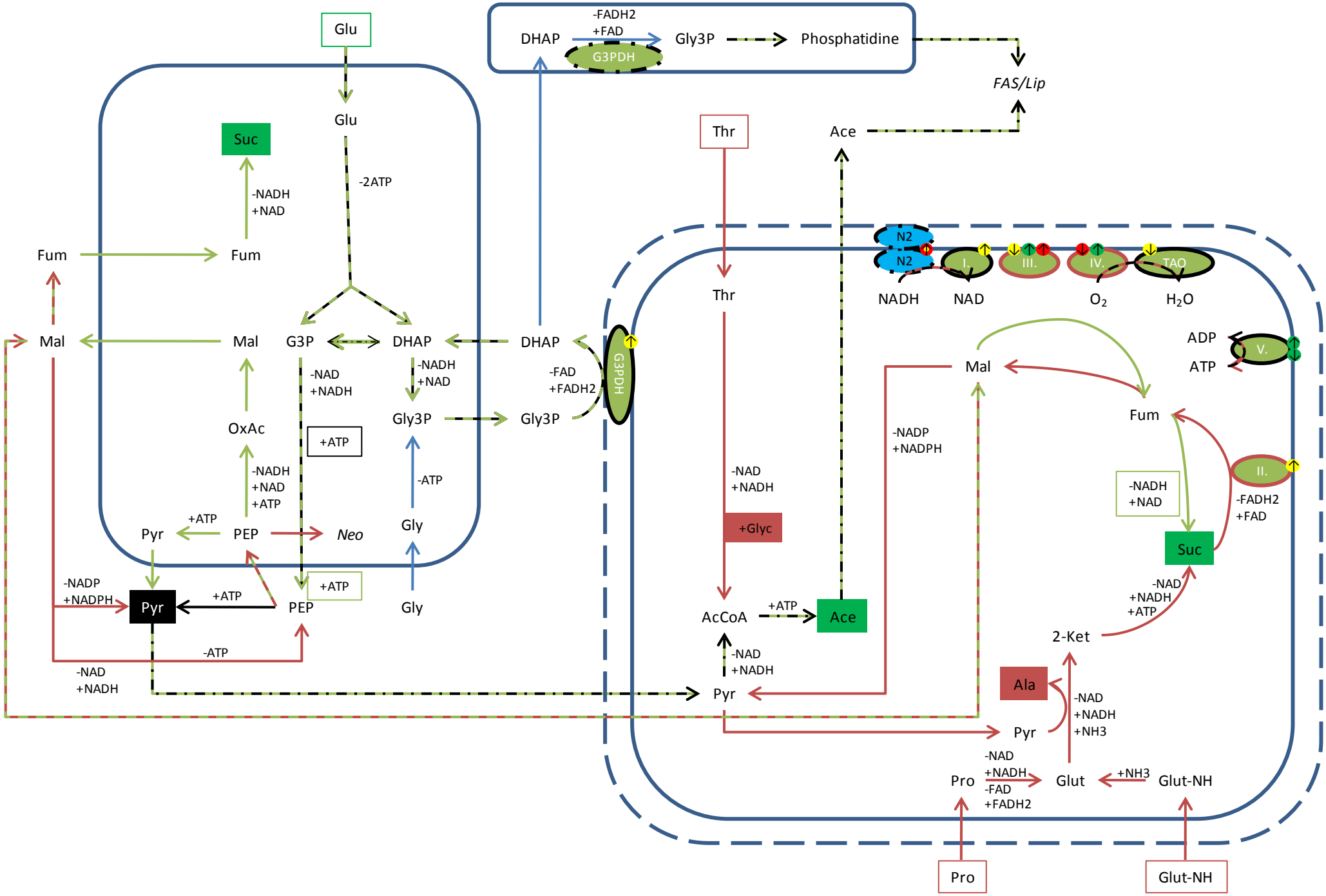
Glu
DHAP
-FADH2
+FAD
G3PDH
Gly3P
Phosphatidine
FAS/Lip
Glu
Suc
-NADH
+NAD
-2ATP
Thr
Ace
Fum
Fum
N2
N2
I.
III.
IV.
TAO
NADH
NAD
O2
H2O
Mal
Mal
G3P
DHAP
DHAP
-NAD
+NADH
-NADH
+NAD
-FAD
+FADH2
G3PDH
Thr
ADP
ATP
V.
OxAc
+ATP
Gly3P
Gly3P
-NADP
+NADPH
Mal
Fum
-NADH
+NAD
+ATP
-ATP
-NAD
+NADH
+Glyc
-NADH
+NAD
II.
-FADH2
+FAD
+ATP
Pyr
PEP
Neo
Gly
Suc
-NADP
+NADPH
Pyr
+ATP
+ATP
PEP
Gly
AcCoA
+ATP
Ace
-NAD
+NADH
2-Ket
-ATP
-NAD
+NADH
Pyr
Ala
-NAD
+NADH
+NH3
Pyr
-NAD
+NADH
-FAD
+FADH2
Pro
Glut
+NH3
Glut-NH
Pro
Glut-NH

Figure 5 (previous page) Simplified schematic representation of energy metabolism of *T. brucei*. For bloodstream stages, glucose (Glu) is the only energy source and its fate is depicted in full black or dashed black arrows. For procyclic stage, Glu can be used as well as proline (Pro), threonine (Thr) and glutamine (Glut-NH); metabolic pathways depicted either in full or dashed green (Glu) or red or dashed red (Pro, Thr, Glut-NH) arrows. Blue arrows represent metabolic pathways that we speculate to be running in both life stages as depicted. Dot-and-dashed black lines represent metabolic pathways that we speculate to be running in bloodstream forms. Filled boxes show metabolic end-products with coloring following carbon source as follows: green – procyclics grown on glucose; red – procyclics grown on amino acids; black – bloodstream forms. Outlined ATP and cofactors represent production/consumption of boxed items in a given life stage: green – procyclic grown on glucose; black – bloodstream form. Enzymes are represented as ovals. Full black outlines denote enzymes present in both life stages; dash-and-dotted outlines represent enzymes speculated to be present in bloodstream stages; full red outlines represent enzymes to be present in procyclic form only. Blue filling denotes uncertain localization. Yellow balls represent ubiquinone, red balls represent cytochrome C, red outlined yellow ball depicts uncertain electron carrier; arrows in balls represent electron flow: up – onto, down – out of electron transporter. Green balls represent proton flow either into intermembrane space (arrow up) or into mitochondrial matrix (arrow down). Italics refers to another metabolic processes: NEO – gluconeogenesis; FAS – fatty acids synthesis; Lip – lipid metabolism. ATP and cofactors are either used (-) or produced (+). Abbreviations used: I.-V. – respiratory complexes I to V; 2-Ket – 2-ketoglutarate; AcCoA – acetyl-coenzyme A; Ace – acetate; Ala – alanine; DHAP – dihydroxyacetone phosphate; Fum – fumarate; G3P – glyceraldehyde-3-phosphate; G3PDH – glycerol-3-phosphate dehydrogenase; Glu – glucose; Glut – glutamate; Glut-NH – glutamine; Glyc – glycine; Gly – glycerol; Gly3P – glycerole-3-phosphate; Mal – malate; N2 – alternative rotenone-insensitive NADH dehydrogenase; OxAc – oxaloacetate; PEP – phosphoenolpyruvate; Pro – proline; Pyr – pyruvate; Suc – succinate; TAO – trypanosomal alternative oxidase; Thr – threonine.

AcCoA is a crossroad of various metabolic processes. In trypanosomes cultivated under laboratory conditions with high level of glucose available, AcCoA is converted into acetate with concomitant production of ATP thru succinyl-CoA, a reaction similar to the one present in the Krebs cycle (Coustou, et al., 2008). Next, acetate exits the mitochondrion and is used either for de novo synthesis of lipids or is excreted (Riviére, et al., 2009). Interestingly, AcCoA does not seem to ever enter the Krebs cycle (Bringaud, et al., 2006), which is instead fed with various intermediates (Figure 5). When glucose is available, then malate is used in part of the cycle forming succinate from fumarate (Coustou, et al., 2008; Bringaud, et al., 2010).

When *T. brucei* grows on amino acids, a situation mimicking the environment in the tse-tse fly, ATP is produced using the mitochondrial respiratory chain (Bringaud, et al., 2006). Threonine is metabolized into AcCoA in a pathway producing NADH. This is re-oxidized at respiration, while AcCoA gives rise to ATP the same way as mentioned above, during glucose degradation. The other amino acid proline, requires pyruvate to be present in the cell. Together they produce 2-ketoglutarate that enters the Krebs cycle and L-alanine that is used in biosynthesis or is excreted. 2-ketoglutarate goes thru Krebs cycle up to malate, another metabolic crossroad. Malate either exits the mitochondrion or stays inside it and is used for

pyruvate formation via cytosolic/mitochondrial malic enzyme or for gluconeogenesis (Bringaud, et al., 2006; Coustou, et al., 2008).

NADH and FADH2 are re-oxidized at complex I (NADH:ubiquinone oxidoreductase) or rotenone-insensitive NADH dehydrogenase and at succinate dehydrogenase, respectively. Electrons then go to ubiquinol and subsequently either to TAO or to the classical cytochrome pathway – complex III (cytochrome bc1 complex/cytochrome c reductase), soluble cytochrome c and complex IV (cytochrome c oxidase) (Bringaud, et al., 2006).

In typical eukaryotes, complexes III and IV are proton pumps generating membrane potential across the inner mitochondrial membrane. We showed previously, that complexes III and IV are indispensable for the PS of strain 29-13, even in the presence of glucose (Horváth, et al., 2005). This is somehow conflicting with an idea of the respiratory chain not being used when glucose is present (Bringaud, et al., 2006). We speculate that functional complexes III and IV are not indispensable because of the ATP demand but due to the requirement of mitochondrial membrane potential for protein import (Williams, et al., 2008).

It is very interesting that even in the PS TAO is still present. Thus, electrons from ubiquinone can flow either to the above-mentioned classical cytochrome depending pathway generating membrane potential or to a single protein alternative oxidase. We speculate that fate of an electron depends on its origin and hence its energy. Then, an electron coming to the system from G3PDH would be channeled to TAO while electrons from other sources would be headed to complexes III and IV. This would be facilitated by the presence of multiple classes of ubiquinone, every class shuttling electrons from a different source. However, only one class of ubiquinon was identified so far in the BS *T. brucei* mitochondrion, confirming the presence of CoQ9 and excluding CoQ10 (Clarkson, et al., 1989). Since lower classes of CoQ were not checked for, presence of CoQ8 or lower ones cannot be excluded without experimental evaluation.

7 FAD-dependent glycerol-3-phosphate dehydrogenase

Glycerol-3-phosphate dehydrogenase (G3PDH) is a component of glycerol-3-phosphate:dihydroxyaceton phosphate shuttle (G3P:DHAP) (Figure 5). This shuttle transfers reducing equivalents from cytosolic (glycosomal in *T. brucei*) NADH to mitochondrial FADH2. G3PDH is located in the inner membrane of mitochondria facing the intermembrane space. Bound FAD co-factor accepts two protons producing FADH2; reduced flavine shuffles two electrons onto ubiquinon thus participating in the respiratory chain. The protons are released back into the intermembrane space.

In *T. brucei* the part of glycolysis producing DHAP is located in the glycosomes (Michels, et al., 2006). To keep NAD/NADH ratio in a state allowing the pay-off phase, part of DHAP molecules is converted into G3P in a reaction that uses NADH (see Figure 5). Glycerol kinase can use G3P for production of intraglycosomal ATP and glycerol. This reaction is hypothesized to be used by trypanosomes under the anoxic conditions (Michels, et al., 2006). Unless such condition is met, G3P is transported out of the glycosomes and converted back into DHAP by mitochondrial G3PDH. DHAP is then transported back to the glycosome where it is either converted to glyceraldehyde-3-phosphate or again to G3P. Thus, trypanosomal G3P:DHAP shuttle regenerates not a cytosolic NAD^+ but a glycosomal one.

The reaction is extremely important for the BS that rely on glycolysis for their energy generation. Since the glycosomal membrane is impermeable for ATP, the favorable branch of glycolysis is of course the one leading to pyruvate. Along this branch, four ATP molecules are generated per molecule of glucose: two inside of the glycosome and the other two in the cytosol (Figure 5). Under above-mentioned anoxic conditions, the net production is halved due to the glycerol branch producing only intraglycosomal ATP. The importance of G3PDH is obvious when enzymatic activities for this enzyme are compared in the BS and PS cells. Our data show that the difference in activities measured between both forms is more than 10-fold.

Genome of *T. brucei* contains three different genes coding for G3PDH. One encodes NAD-dependent glycosomal protein that catalyzes DHAP:G3P reaction thus regenerating

intraglycosomal NAD. The other two code FAD-dependent proteins. Tb11.02.5281 (mtG3PDH) is a stage-regulated protein with 22-fold increas of transcription in the BS as compared to the PS cells. Its homologue from *Leishmania major* was shown to be a mitochondrial enzyme (Guerra, et al., 2006). The other one, Tb927927.1.1130 (putG3PDH), is not regulated its transcription being the same infor both stages. Its localization was confirmed to be extramitochondrial by study done on mitochondrial proteome (see database http://www.trypsproteome.org/Default.aspx, (Panigrahi, et al., 2009)). In silico prediction done by Opperdoes targeted this protein into the endoplasmic reticulum (see database entry at www.genedb.org).

The increase of G3PDH activity in the BS mitochondrion correlates with accelerated transcription of mtG3PDH. RNAi against this protein led to growth phenotype in the BS while growth of the other stage remained unaffected (data not shown). Despite this lack of growth alteration, a specific mitochondrial G3PDH activity was eliminated in both stages. For the BS, the putG3PDH apparently was not able to compensate for the loss of mitochondrial homologue. In the PS, the situation might mirror the overall lower activity of the mitochondrial enzyme. Thus, it was predicted that the growth phenotype would be unaltered thanks to the remaining activity of putG3PDH.

i) To confirm this hypothesis we prepared RNAi cell lines in which either mtG3PDH, exG3PDH or both are targeted. While confirmed results for mtG3PDH knock-downs are presented here, the other two cell lines are currently tested for efficient mRNA degradation (data not shown). Since key experiments are still ongoing in the time of writing, they will not be described here. Instead, I will speculate on possible role of putG3PDH. G3P is known to be a precursor for phosphatidic acid synthesis. putG3PDH could then be used for generation of G3P from DHAP. A scheme would look like this (Figure 5): i/ G3P is generated inside the glycosomes and exported into the cytosol; ii/ there, it enters the mitochondrial intermembrane space where it is converted into DHAP; iii/ DHAP is then partly transported back to the glycosome and partly into another compartment with the endoplasmic reticulum being the best candidate.

To test this scenario, total lipid composition in cells depleted for both mt- and putG3PDH must be compared. Also, identification of DHAP transporters would be beneficial.

Their affinity towards DHAP would support the proposed scheme. Both extraglycosomal G3PDHs should be characterized in terms of kinetic properties. Do they prefer one of the reactants (G3P or DHAP)? Is their Km the same or does it differ? Can they substitute each other thus rescuing a loss of one of them? Finally, where exactly is the extramitochondrial enzyme located? Answering at least some of these questions is a topic of a manuscript in preparation (see appendix 10.6).

8 Complex I (NADH:ubiquinone oxidoreductase) and rotenone-insensitive NADH dehydrogenase

Complex I (NADH:ubiquinone oxidoreductase) is the largest component of the respiratory chain; in bacteria it is comprised of 13-14 subunits while in eukaryotes even more than 40 subunits were reported. The best characterized NADH:ubiquinone oxidoreductase is from bovine heart where it is composed of 45 different subunits (Carroll, et al., 2006), out of which only 7 are present in the mitochondrial genome (Attardi, et al., 1986). Together with 7 nuclear-encoded subunits, this establishes the so-called core that corresponds to the bacterial complex (Bai, et al., 2004). Integral parts of complex I also include FMN and 9 Fe-S clusters (Zickermann, et al., 2008).

Complex I homolog from a bacterium has been already crystallized (Arteni, et al., 2006; Sazanov, et al., 2006) confirming its overall L-shape. Nowadays, methods for purification of the bacterial complex for large scale studies are available (Pohl, et al., 2007), while the purification of complex I from bovine heart showed that it tends to fell apart into several sub-complexes (Lenaz, et al., 2006). Recently, general characterization of the mitochondrial proteome led to the discovery of partial complex I in the PS *T. brucei* (Panigrahi, et al., 2009).

When dealing with complex I it is neccessary to mention the alternative NADH dehydrogenase, which is present and active in the PS *T. brucei*. It is a monomeric enzyme with a molecular mass of 54 kDa insensitive to rotenone, a classical inhibitor of complex I, but sensitive to diphenyl iodonium (DPI) (Fang, et al., 2002; Fang, et al., 2003); DPI is a potent inhibitor of flavine enzymes (Majander, et al., 1994; Ratz, et al., 2000). We speculate that this enzyme might be sensitive to 1-hydroxy-2-dodecyl-4(1H)quinolone, a specific inhibitor of the alternative dehydrogenase from *Toxoplasma gondii* and *Plasmodium falciparum* (Saleh, et al., 2007).

In *T. brucei* and in Kinetoplastida in general complex I has been a contentious issue for a long time. Not only its importance for these parasites was questioned, but even its very presence in these organisms remains controversial. On one hand there are papers by Fang and Beattie describing specific activity, purification and specific inhibition, on the other hand

studies exist that question their findings and/or methodology (Denicola-Seoane, et al., 1992; Hernandez, et al., 1998; Bochud-Allemann, et al., 2002). Since the whole genome was sequenced (Berriman, et al., 2005), the evidence for the existence of complex I is further supported by the detection of its numerous putative subunits (Opperdoes, et al., 2008). To evaluate the essentiality and function of complex I in the PS cells, three cell lines each interfered against a different subunit of complex I were prepared, as well as a knock-downs in which alternative NADH dehydrogenase (NDH2) was inducibly silenced. Also, a catalytic subunit (NUBM) was prepared in a tet-inducible expression vector bearing a tandem affinity purification (TAP) tag. A pilot experiment with TAP-tagged subunit revealed that other proteins homologous to the complex I subunits were associated with overexpressed NUBM. However, since a recent work described the same results with even more complex I homologues (Panigrahi, et al., 2008; Panigrahi, et al., 2009), these results will not be discussed and I will focus on the functional study.

The elusive existence and function of complex I are evaluated in three knock-downs of complex I subunits. Upon successful RNAi confirmed by Northern blot analysis, we got stuck with no observable phenotype. Cell proliferation was not affected in either standard SDM-79 or the glucose-depleted medium (see appendix 10.3). Based on our previous experience with knock-downs of complexes III and IV where growth was affected yet not arrested with concomitant reproducible decrease of membrane potential (Horváth, et al., 2005), we tested this mitochondrial feature in these cells as well. Unexpectedly, mitochondrial membrane potential was not showing any traces of change. Three explanations have been put forward:

i) complex I is not involved in proton translocation; lack of growth retardation and the ability of NDH2 to take over all NADH re-oxidation with complexes III and IV doing the proton pumping qualifies this as the most plausible scenario.
ii) complex I is involved in proton translocation but its disruption by RNAi was balanced by compensatory mutation(s); this possibility cannot be excluded at present, however, one would expect at least a slight change in cells doubling time; also, admitting this to be true is very difficult without raising the questions as to why this happens for complex I only – disruption of either complex III or IV rendered each of

them inactive (electrons cannot enter complex III at all or remain there not having a sink when complex IV is affected) without any traces of a mutation is hard to imagine.

iii) complex I is involved in proton translocation but the targeted subunits do not disrupt the complex as whole; not having a reliable antibody against a complex I subunit, we cannot exclude this possibility; however, it is highly unlikely that none of the three targeted subunit would caused destabilization of the complex.

To extend our insight into the functions of the protein complex under study, NDH2 was knocked-down too. After confirmation of RNAi by Northern blot, a growth curve analysis was performed (see appendix 10.5). In contrast to complex I KDs, on day four a growth retardation was observed. Subsequently, the same essays as in the complex I study have been performed, which showed that mitochondrial membrane potential and ROS production were decreased. Analysis of glycerol gradient fractions showed a different distribution of NADH:ubiquinone oxidoreductase activity. Surprisingly, overall NADH:Q2 activity did not change while mtG3PDH activity increased by 40 %. Analysis of respiration in vivo showed decreased sensitivity to KCN, an inhibitor of complex IV (cytochrome c oxidase).

Taken together the available data lead us to conclude that complex I and NDH2 probably physically interact with each other. This is the simplest explanation of different distribution of NADH:Q2 activity observed in all knock-downs. Next, cells ablated for complex I subunits show mild decrease of NADH:Q2 activity with an increased sensitivity of various glycerol fractions to DPI, a specific inhibitor of flavine enzymes. However, this is not observed in the case of NDH2. Thus, loss of complex I leads to an upregulation of a different flavine-containing NADH:Q2 oxidoreducase; also, NDH2 is probably not capable of using Q2 as an effective electron acceptor. Finally, NDH2 is a major contributor of electrons into a classical, cytochrome-containing respiratory pathway. Knock-down leads to the loss of sensitivity to KCN suggesting also that electrons coming to the system from mtG3PDH are grounded at TAO. However, we cannot exclude that in vitro-observed enzymatic increase is not mirrored in in vivo respiration due to complex metabolic interactions and regulations.

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Verner, Z. 2011. Mitochondrial energy metabolism in *Trypanosoma brucei:* Complex I and the others.

10 Appendices

10.1 Mitochondrial membrane potential-based genome-wide RNAi screen of *Trypanosoma brucei* (Verner Z., Paris Z., Lukeš J.)

Mitochondrial membrane potential-based genome-wide RNAi screen of Trypanosoma brucei.

Verner, Z.; Paris, Z.; Lukeš, J.

Parasitol. Res. 2010. 106(5): 1241-4

We have screened the *Trypanosoma brucei* genome-wide RNAi library by staining the procyclics with the dye JC-1 followed by sorting the differentially stained cells by flow cytometry. This allowed us to highly enrich for cells in which mitochondrial membrane potential was decreased. We have further validated a subset of selected clones by a reverse approach in which we showed that cloning the selected genomic regions into another RNAi vector also results in a drop in mitochondrial membrane potential.

Provedli jsme skrínink celogenomové RNAi knihovny pomocí barvení procyklických buněk Trypanosoma brucei barvivem JC-1 a následně jsme různě obarvené buňky rozřadili pomocí průtokové cytometrie. To nám umožnilo získat frakci obohacenou o buňky, které měly snížený mitochondriální membránový potenciál. Dále jsme validovali část vybraných klonů opačným přístupem, ve kterém jsme ukázali, že klonování vybraných genomových oblastí do jiného RNAi vektoru rovněž vyústilo v pokles mitochondriálního membránového potenciálu.

Contribution: 50 %

10.2 The Remarkable Mitochondrion of Trypanosomes and Related Flagellates (Lukeš J., Hashimi H., Verner Z., Číčová Z.) – section 5 and 6 (Mitochondrial-Encoded Proteins and Energy Metabolism of *T. brucei* Mitochondrion)

The Remarkable Mitochondrion of Trypanosomes and Related Flagellates (Section 5 – Mitochondrial-Encoded Proteins and 6 – Energy Metabolism of *T. brucei* Mitochondrion).

Lukeš J.; Hashimi H.; Verner Z.; Číčová Z.

In: Structures and organelles in pathogenic protists (ed. De Souza W.). 2010. Springer-Verlag Berlin, Heidelberg; pp. 228-252

The single mitochondrion of trypanosomes and their relatives is a remarkable organelle, containing almost all of its hallmarks as well as some unique features. Among the latter are exceedingly complex mitochondrial DNA and RNA editing. Furthermore, the organelle is very different in the two principal stages of the life cycle, as it is metabolically active in the procyclic stage transmitted by the insect vector (Fig. 1d), while its morphology and metabolism are highly reduced in the bloodstream stage (Fig. 1e). Thanks to initiatives such as description of the mitochondrial proteome of the procyclic stage, our knowledge of the organelle of *Trypanosoma brucei* and *Leishmania* species increased substantially within the last decade. In this review we will focus on mitochondrial DNA, its transcription and translation, and conclude with a brief description of the function of mitochondrial-encoded proteins and energy metabolism. Within the last decade, these topics were subject to several authoritative reviews (Besteiro et al., 2005; Bringaud et al., 2006; Liu et al., 2005; Lukeš et al., 2002, 2005; Rubio and Alfonzo 2005; Shlomai 2004; Schnaufer et al., 2002; Schneider 2001; Simpson et al., 2004, 2006; Stuart et al., 2005). Here, we will briefly summarize our present knowledge with somewhat more detailed treatise of findings obtained mostly within the last five years.

Jediná mitochondrie trypanosom a jejich příbuzných je pozoruhodnou organelou, která obsahuje jak téměř všechny klíčové mitochondriální komponenty tak rovněž některé unikátní

vlastnosti. Mezi tyto patří neuvěřitelně komplexní mitochondriální DNA a editování RNA. Dále se tato organela velmi liší ve dvou hlavních stádiích životního cyklu, jelikož je metabolicky aktivní v procyklickém stádiu přenášeném hmyzím vektorem (Fig. 1d), zatímco její morfologie a metabolismus jsou vysoce redukovány ve stádiu krevním (Fig. 1e). Díky iniciativám, jakými jsou popis mitochondriálního proteomu procyklického stádia, se naše znalosti o této organele v Trypanosoma brucei a a drhu Leishmania během posledního desetiletí zásadně rozšířily. V tomto přehledu (review) se zaměříme na mitochondriální DNA, její transkripci a translaci a kapitolu uzavřeme stručným popisem funkce mitochondriálně-kódovaných proteinů a energetického metabolismu. V minulé dekádě byla tato témata předmětem mnoha směrodatných přehledů (Besteiro et al., 2005; Bringaud et al., 2006; Liu et al., 2005; Lukeš et al., 2002, 2005; Rubio and Alfonzo 2005; Shlomai 2004; Schnaufer et al., 2002; Schneider 2001; Simpson et al., 2004, 2006; Stuart et al., 2005). My zde shrneme naši současnou znalost s poněkud větším důrazem na objevy učiněné většinou v posledních pěti letech.

Contribution: 30 %

10.3 Complex I (NADH:ubiquinone oxidoreductase) is active in but non-essential for procyclic *Trypanosoma brucei* (Verner Z., Čermáková P., Škodová I., Kriegová E., Horváth A., Lukeš J.)

Complex I (NADH:ubiquinone oxidoreductase) is active in but non-essential for procyclic *Trypanosoma brucei.*

Verner Z.; Čermáková P.; Škodová I.; Kriegová E.; Horváth A.; Lukeš J.

Mol. Biochem. Parasitol. 2011. 175(2): 196-200.

The requirement of complex I (NADH:ubiquionone oxidoreductase) for respiration in *Trypanosoma brucei* is controversial. Recent identification of homologues of its subunits in mitochondrial proteome resolved a question of its presence or absence. However, with one exception, no data has been available concerning the function(s) of complex I or its subunits. Here we present a functional RNAi study of three (NUBM, NUKM, NUEM) putative subunits of this complex. Although no changes were detected in growth, mitochondrial membrane potential or reactive oxygen species production in cell lines depleted for target protein, the NUBM and NUKM RNAi knock-downs showed decreased specific NADH:ubiquinone oxidoreductase activity. Moreover, glycerol gradients of all cell lines revealed the presence of two distinct peaks of NADH dehydrogenase activity, with shifted sensitivity to inhibitors of complex I upon RNAi induction. Thus complex I is not only present in the procyclic stage of *T. brucei* 29-13 strain, but it does participate in electron transport chain.

Potřeba komplexu I (NADH:ubichinon oxidoreduktázy) pro dýchání u Trypanosoma brucei je sporná. Nedávný objev homologů jeho podjednotek v mitochondriálním proteomu odpověděl na otázku jeho přítomnosti či absence. Nicméně pouze s jedinou výjimkou nejsou k dispozici žádná data popisující funkci komplexu I nebo jeho podjednotek. My zde předkládáme RNAi studii funkce tří (NUBM, NUKM, NUEM) možných podjednotek tohoto komplexu. Ačkoli jsme nezaznamenali žádné změny v růstu, mitochondriálním

membránovém potenciálu nebo produkci kyslíkových radikálů v buněčných liniích depletovaných pro cílové proteiny, linie NUBM a NUKM vykazovaly sníženou NADH:ubichinonovou oxidoreduktázovou aktivitu. Navíc jsme ukázali, že glycerolové gradienty všech buněčných linií obashují dva odlišné vrcholy NADH dehydrogenázové aktivity, jejichž citlivost k inhibitorům komplexu I se měnila po indukci RNAi. Komplex I tedy není v procyklickém stádiu kmene 29-13 *T. brucei* pouze přítomen, ale rovněž se podílí na přenosu elektronů v dýchacím řetězci.

Contribution: 70 %

10.4 Characterization of the NADH:ubiquinone oxidoreductase (complex I) in the trypanosomatid *Phytomonas serpens* (Kinetoplastida) (Čermáková P., Verner Z., Man P., Lukeš J., Horváth A.)

Characterization of the NADH:ubiquinone oxidoreductase (complex I) in the trypanosomatid *Phytomonas serpens* (Kinetoplastida)

Čermáková P.; Verner Z.; Man P.; Lukeš J.; Horváth A.

FEBS J. 2007. 274(12): 3150-8.

NADH dehydrogenase activity was characterized in the mitochondrial lysates of Phytomonas serpens, a trypanosomatid flagellate parasitizing plants. Two different high molecular weight NADH dehydrogenases were characterized by native PAGE and detected by direct in-gel activity staining. The association of NADH dehydrogenase activities with two distinct multisubunit complexes was revealed in the second dimension performed under denaturing conditions. One subunit present in both complexes cross-reacted with the antibody against the 39 kDa subunit of bovine complex I. Out of several subunits analyzed by MS, one contained a domain characteristic for the LYR family subunit of the NADH:ubiquinone oxidoreductases. Spectrophotometric measurement of the NADH:ubiquinone 10 and NADH:ferricyanide dehydrogenase activities revealed their different sensitivities to rotenone, piericidin, and diphenyl iodonium.

NADH dehydrogenázová aktivita byla charakterizována v mitochondriálních lyzátech Phytomonas serpens, bičíkovce ze skupiny trypanosomatid, který parazituje v rostlinách. Dvě odlišné vysokomolekulární NADH dehydrogenázy byly charakterizovány nativní PAGE a detekovány přímým barvením aktivity v gelu. Asociace NADH dehydrogenázových aktivit se dvěma odlišnými mnohopodjednotkovými komplexy byla odhalena ve druhé dimenzi, která byla provedena za denaturačních podmínek. Jedna podjednotka přítomná v obou komplexech křížově reagovala s protilátkou proti 39 kDa podjednotce kravího komplexu I. Z několika podjednotek analyzovaných pomocí MS, jedna obsahovala doménu charakteristickou pro LYR rodinu podjednotek NADH:ubichinon oxidoreduktáz. Spektrofotometrické měření

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NADH:ubichinon-2 a NADH:ferrikyanid dehydrogenázové aktivity odkrylo jejich rozdílnou citlivost k rotenonu, piericidinu a difenyliodoniu.

Contribution: 10 %

10.5 Alternative NADH dehydrogenase (NDH2) is the major source of electrons for respiratory chain in procyclic *Trypanosoma brucei* (Verner Z., Škodová I., Ďurišová-Benkovičová V., Horváth A., Lukeš J.) (in preparation)

Alternative NADH dehydrogenase (NDH2) is the major source of electrons for respiratory chain in mitochondrion of procyclic *Trypanosoma brucei*

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Trypanosoma brucei is a unicellular parasite which causes lethal nagana in cattle and sleeping sickness in humans. Due to these mostly lethal diseases, this flagellate renders vast areas of sub-Saharan Africa unsuitable for agriculture.

During its life, *T. brucei* faces two dramatically distinct environments: the bloodstream of its mammalian host and the midgut and salivary glands of its tse-tse fly vector *Glossina* spp. While the blood is an energy-rich environment with a constant level of glucose, the insect represents a glucose-poor environment. This is reflected by a different activity of the parasite's single mitochondrion, which is in the bloodstream stage represented by a reduced tubular organelle lacking classical respiratory chain (1). This stage covers its ATP demands purely by glycolysis and excretes pyruvate as the major end-product. In contrast, in the procyclic stage, mitochondrion is a highly active organelle with all classical respiratory complexes present and substantially contributing to ATP production (reviewed in 2).

A classical respiratory chain consists of four multisubunit complexes: NADH:ubiquinone oxidoreductase (complex I), succinate:ubiquinone oxidoreductase (complex II), ubiquinone:cytochrome *c* reductase (complex III) and cytochrome *c* oxidase (complex IV). Along with large multi-subunit complexes, several single-protein enzymes that contribute to the flow of electron in trypanosome cells are known. In the case of the *T. brucei* respiratory chain, these are represented by glycerol-3-phosphate dehydrogenase (3), alternative rotenone-insensitive NADH:ubiquinone oxidoreductase (4) and trypanosome alternative oxidase (TAO) (5). TAO sinks electrons to oxygen producing water while the other two enzymes supply electrons to ubiquinone. While complexes III and IV pump protons out of the mitochondrial matrix, none of the known single-protein enzymes is capable of such an activity.

In *T. brucei*, all respiratory complexes have been studied to some extent. Complex I was shown to be present in the procyclic stage *T. brucei*, however, the RNAi-based depletion of its three core subunits did not affect viability of cells grown in media either containing or lacking glucose (6). Complex II can be disrupted in a glucose-containing medium without any effect on the growth of these cells. On the contrary, in a medium depleted for glucose medium, disruption of this component of the respiratory chain is lethal (7). Finally, complexes III and IV were shown to be essential for growth and maintenance of membrane potential in the procyclic trypanosomes regardless of the availability or lack of glucose (8).

The non-essentiality of any of the tested core subunits of complex I lead to the prediction that in procyclics its activity can be fully replaced by an alternative dehydrogenase (NDH2). This prediction was corroborated by an increased sensitivity of mitochondrion-enriched glycerol fractions to diphenylen iodonium (DPI), an inhibitor of flavine enzymes (6).

Previously, we showed that this inhibitor was specific for NDH2 in *Phytomonas serpens*, a close relative of *T. brucei* (9). The increased sensitivity to DPI was mirrored by lower sensitivity to rotenon, a specific inhibitor of complex I (6).

To investigate the putative ability of NDH2 to fully compensate for the loss of complex I, we have herein knock its respective gene down using inducible RNAi. Our results indicate that NDH2 can not only fully compensate for loss of the NADH dehydrogenase capacity caused by the inactivation of complex I, but is indispensable for the procyclic *T. brucei*.

NDH2 is required for growth of *T. brucei* procyclics. A 593 bp-long fragment of the rotenone-insensitive NADH:ubiquinone oxidoreductase (NDH2) gene (Tb927.10.9440) was PCR-amplified using primers N2-fw (5'-**GGATCC**GACCCTCACCACTA-3') and N2-rv (5'-C**AAGCTT**GCGGGTAAGTCCAC-3') (added BamHI and HindIII restriction sites are in bold). The amplicon was subcloned into the pGEM-T-Easy vector (Promega), sequenced, excised via the above restriction sites and cloned into the p2T7-177 RNAi vector. The procyclic cells were electroporated and selected as described elsewhere (10). Next, total RNA was isolated from the parental wild type 29-13 cells, non-induced cells and those in which RNAi was induction for 3 days by the addition of 1 μg/ml of tetracycline (tet+) to the medium. Northern blot analysis was performed with the NDH2 gene fragment used as a probe. Hybridization took place overnight at 55 ^{o}C in the Na-Pi solution (11) and was followed by a two-step wash (2 x SSC + 0.1% SDS for 30 min at room temperature and 0.2 x SSC + 0,1% SDS for 30 min at 55 ^{o}C). Massive production of double stranded (ds) RNA followed by efficient elimination of target mRNA proved that RNAi was functional (data not shown).

Following this experiment, the culture was diluted to a concentration of one cell/ml and plated onto a 96-well plate. Six obtained clones were screened by Northern blot analysis and clone labeled N2D1 was selected for further experiments due to efficient ablation of the NDH2 mRNA (Fig. 1A; inset). Growth of this clone in the absence (tet-) and presence of tetracycline (tet+) was measured for six days (Fig. 1A). Upon reaching the concentration of 1 x 10^{7} cells/ml, cells were always diluted 10x. Since a growth difference between the non-induced and RNAi-induced cells started appearing on day 3, all following experiments were performed on day 4. Growth phenotype of NDH2 suggests that while disruption of complex I can be compensated by another dehydrogenase, ablation of NDH2 has probably a more

profound effect. The simple upregulation of complex I, a principal NADH dehydrogenase of respiratory chain in higher eukaryotes, cannot take place probably due to the complexity of the enzyme. A common upregulation of tens subunits would be required (12; 13), a situation difficult to imagine in *T. brucei* due to two facts: i/ nuclear transcription is polycistronic (14) thus upregulation of tens required proteins would be mirrored in production of many unrelated proteins and ii/ complex I requires mitochondrially-encoded subuits for its functionality; these proteins in *T. brucei* udergoes a complex process of editing (15). Cross-talk between nuclear and editing upregulation seems very difficult to take place.

Ablation of NDH2 leads to decrease of mitochondrial membrane potential and lower production of ROS. In most eukaryotes, complex I is a major proton pump of the respiratory chain translocating four protons across the mitochondrial inner membrane at once. Except this proton-pumping function, in the mitochondrion of procyclic *T. brucei* NDH2 performs the same biochemical reaction. RNAi against of the NUBM, NUKM or NUEM subunits of complex I did not result in any change of the mitochondrial membrane potential (6). Surprisingly, when NDH2 knock-down was tested for this feature, a dramatic shift was observed (Fig. 1B). This decrease was surprising, since it was expected that complex I shall be able to compensate for the loss of the alternative enzyme. The decrease of mitochondrial membrane potential thus has to be attributed to respiratory complexes III and IV. We suppose that the observed change reflects the decrease in number of electrons passing thru complexes III and IV, which consequently pump less protons per time unit. This data further support the hypothesis that complex I fails to outbalance the elimination of NDH2. Moreover, NDH2 seems to be a major NADH dehydrogenase of the respiratory chain.

Interestingly, a laboratory strain of *Crithidia fasciculata* was shown to feed respiratory chain mainly via complex II, rendering complex I to be likely dispensable for this flagellate. Studies performed on the *T. brucei* procyclics show that the situation is rather different in this species. While complex II is not essential for procyclics grown on glucose, it is complex I that is dispensable, yet still active in these cells (6). Even different situation evolved in *Phytomonas serpens*, which lost all mitochondrial- (16) and at least some nuclear-encoded subunits of these complexes (17). Therefore, this plant parasite relies on complex I, which is the only classical proton pump with complex V as a site of ATP synthesis.

Complexes I and III are reported to be places of high reactive oxygen species (ROS) production (16; 17). To test whether in the NDH2-depleted procyclics, complex I is active

without contributing to the mitochondrial membrane potential, the level of ROS was followed using dihydroethidium as described for complex I (6). We speculated that more electrons going thru complex I would mean their higher leak to oxygen and consequently higher ROS, although a decreased ROS production was actually measured. The most plausible explanation of this data is that the activity of complex I was not upregulated, hence no increase of ROS occurred. Instead, as there are fewer electrons going thru the whole system, less of them can leak onto oxygen, eventually leading to the decreased ROS production.

Respiratory chain enzymes are altered in NDH2 knock-down. Measurement of the overall NADH:Q2 oxidoreductase activity in the studied cell line, following a protocol described previously (6), showed that this activity was not significantly changed (data not shown).

To confirm that the respiratory chain was indeed altered in these cells, *in vitro* activities of mitochondrial glycerol-3-phosphate dehydrogenase (G3PDH), succinate dehydrogenase (complex II), cytochrome *c* reductase (complex III) and cytochrome *c* oxidase (complex IV) were measured following the protocols described elsewhere (Horváth et al., 2005). For the measurement of the G3PDH activity the following reaction mixture was prepared: 75 μg of mitochondrial proteins were added to 1 mL cuvette containing 50 mM Tris-buffer (pH 7.0), 0.15 mM 2,6-dichloroindophenol and 0.2 mM phenazine metosulfate. The G3PDH activity was measured by recording the decrease of absorbance at 600 nm triggered by the addition of 50 mM glycerol-3-phosphate (G3P). The obtained from the non-induced and RNAi-induced cells were compared, we uncovered a significant increase in the G3PDH activity (+40 %; t-test: n = 10, P = 0,01), while the remaining enzymatic activities were not influenced by RNAi against NDH2.

Since no change in the overall NADH:Q2 oxidoreductase activity occurred, we performed a glycerol gradient fractionation to see whether there is a shift in distribution of this activity. Mitochondria isolated from cells 4 days upon induction of RNAi were lyzed, protein content in each fraction was measured using Bradford and the NADH:Q2 activity and rotenon and DPI sensitivity were followed as described previously (6). Table 1 and Suppl. Table 1 show that comparison of fractions with specific activity ≥ 5U revealed a different distribution in the induced cells as compared to the non-induced ones. Moreover, the sum of NADH:Q2 oxidoreductase activity was higher in the RNAi-induced cells, suggesting a compensatory activity. We suspected complex I might be upregulated following the loss of

NDH2, yet we did not measure any difference in the sensitivity to either rotenone, a specific inhibitor of complex I, or to DPI, a general flavine-enzyme inhibitor. Distribution of both sensitivities in the glycerol gradient fractions followed the same general pattern. We therefore conclude that: i/ NDH2 does not seem to use Q2 as a prefered electron acceptor; ii/ NDH2 probably physically interacts with complex I, since the distribution of the NADH:Q2 activity was altered; iii/ NDH2 is the major electron source for respiratory chain since its change leads to an increase of the mitochondrial G3PDH activity in vitro.

The lack of effect on the NADH dehydrogenase activity led us to measure the overall respiration of the NDH2 knock-downs. Approximately 2 x 10^7 cells were spun, resuspended in fresh SDM-79 medium and their oxygen consumption was measured using Clark electrode as described elsewhere (8). After stabilization, KCN and SHAM, the inhibitors of complex IV and TAO, respectively, were added to final concentrations 0.1 mM and 0.03 mM. In an alternative experiment the drugs were added in reversed order. Since lower sensitivity to cyanide was observed (Fig 1D), although overall oxygen consumption was not statistically different between the non-induced and RNAi-induced cells (data not shown), we conclude that the remaining electrons were shuffled to TAO. This observation also suggests that up-regulation of G3PDH either does not occur *in vivo* and can be detected only *in vitro*, or this enzyme shuffles all electrons solely to TAO. This would not be surprising given the fact that in the absence of cytochromes, the G3PDH-TAO system is used in the bloodstream stage *T. brucei* (1).

Concluding remarks

In a previous study on complex I we speculated that the compensation by NDH2 maybe responsible for the lack of phenotype (6). However, a more complex picture emerges from the newly obtained results. . The only direct proof that we indeed hit complex I was a change in the distribution of NADH:Q2 activity and its sensitivity to rotenone and DPI in glycerol gradient fractions. In light of the results obtained with NDH2, one would predict the existence in the respiratory chain of another flavine-containing enzyme capable of NADH:Q2 oxidoreductase activity.

This study also shows an urgent need of functional approach in descriptive biochemistry. Fang and Beattie identified NDH2 and performed NADH dehydrogenase

activity assays with a limited set of ubiquinone derivatives, namely duroquinone, Q0 and Q1 on a recombinant protein (4). To our surprise, when NDH2 was knocked-down, we did not observed any decrease of the NADH:Q2 activity. Since all acceptors used are mere derivatives of the naturally occurring Q9/Q10, the possibility that i*n vivo* activities are substantially different from the *in vitro* ones cannot be excluded. For the same reasons one cannot rule out the possibility that NDH2 is localized in the intermembrane space like in yeasts or plants (18) (19). If this was true, not only ubiquinone could accept electrons but a soluble cytochrome c1 would be accessible as well. NDH2 being associated with complex I from outside of the mitochondrion and passing electrons to cytochrome would explain the observed phenomena, such as lower cyanide sensitivity, lower membrane potential and different distribution of NADH:Q2 activity.

Acknowledgements

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Figure legends

Table 1

Distribution of NADH:Q2 oxidoreductase activity and rotenone- and DPI-sensitivity in glycerol gradient fractions of the NDH2 knock-down. For activity lines, + denotes 5-10 U

activity, ++ 10-15 U and +++ >15 U. U was calculated as ΔOD (340 nm) per min per mg of protein × 1000. For inhibitory lines, + denotes inhibition 1-30 %, ++ 30-70 %, +++ >70 % as compared with the activity without inhibitor. Tet- represents the non-induced cells; Tet+ represents the RNA-induced cells.

Figure 1

Phenotypes of *T. brucei* procyclics interfered against the NDH2 protein.

(A) Growth curves of RNAi cell lines. Full and dashed lines correspond to the non-induced (tet-) and RNAi-induced (tet+) cells, respectively. *x* - hours post-induction; *y* - cell concentration/ml. Insets show effect of RNAi (left lane – parental cell line, middle lane – non-induced cells; right lane – RNAi-induced cells). As a control, the gel was stained with ethidium bromide to visualize rRNA bands. Mitochondrial membrane potential **(B)** and ROS production **(C)** were measured in the non-induced cells (full lines) and cells 4 days of RNAi-induction (dashed lines). *x* - log scale of fluorescence; *y* - number of events. **(D)** Respiration of parental (29-13), non-induced (tet-) and induced (tet+) cell lines. Respiration before adding of inhibitors was considered 100 %. *x* – inhibitors; *y* – per cent of untreated cells.

Supplementary Table 1

Biochemical analyses of glycerol gradient fractions. The distribution of total protein, NADH:Q2 oxidoreductase activity, rotenone and DPI sensitivity was followed in glycerol gradient fractions obtained from the non-induced (tet-) and RNA-induced (tet+) cells. **Protein** - protein content established by Bradford assay in mg/ml × 100; **OD** - NADH:Q2 oxidoreductase activity calculated as ΔOD (340 nm) per min per mg of protein × 1000; **Rot** - effect of rotenone on NADH:Q2 oxidoreductase activity calculated as % of activity with

rotenone divided by activity without the drug; **DPI** - effect of DPI on NADH:Q2 oxidoreductase activity calculated as % of activity with DPI divided by activity without the drug. ND - activity was not detected. Coloured areas correspond to cross-coded fractions in Table 1; protein content and activity higher than 5 were taken arbitrariry; orange – protein peak; light blue – the first NADH:Q2 oxidoreductase peak; dark blue – the second NADH:Q2 oxidoreductase peak; green – rotenone-sensitive parts of any NADH:Q2 oxidoreductase peak; red – DPI-sensitive parts of any NADH:Q2 oxidoreductase peak

Fraction nr.	1-9	10	12	13	14	15	16	17	18	19	20	21	22	23	24
Activity (Tet-)		+		+		+++	+	++	+				+	+++	
Activity (Tet+)			+				+	+++		+++	++	+	+++	+++	
Rotenone (Tet-)		++		++		++	+	+	+++					++	
Rotenone (Tet+)			+++					++		++	+++				
DPI (Tet-)		+++		++		+++	++	+++					+++	++	
DPI (Tet+)			++				++			++			++	+	

Table 1

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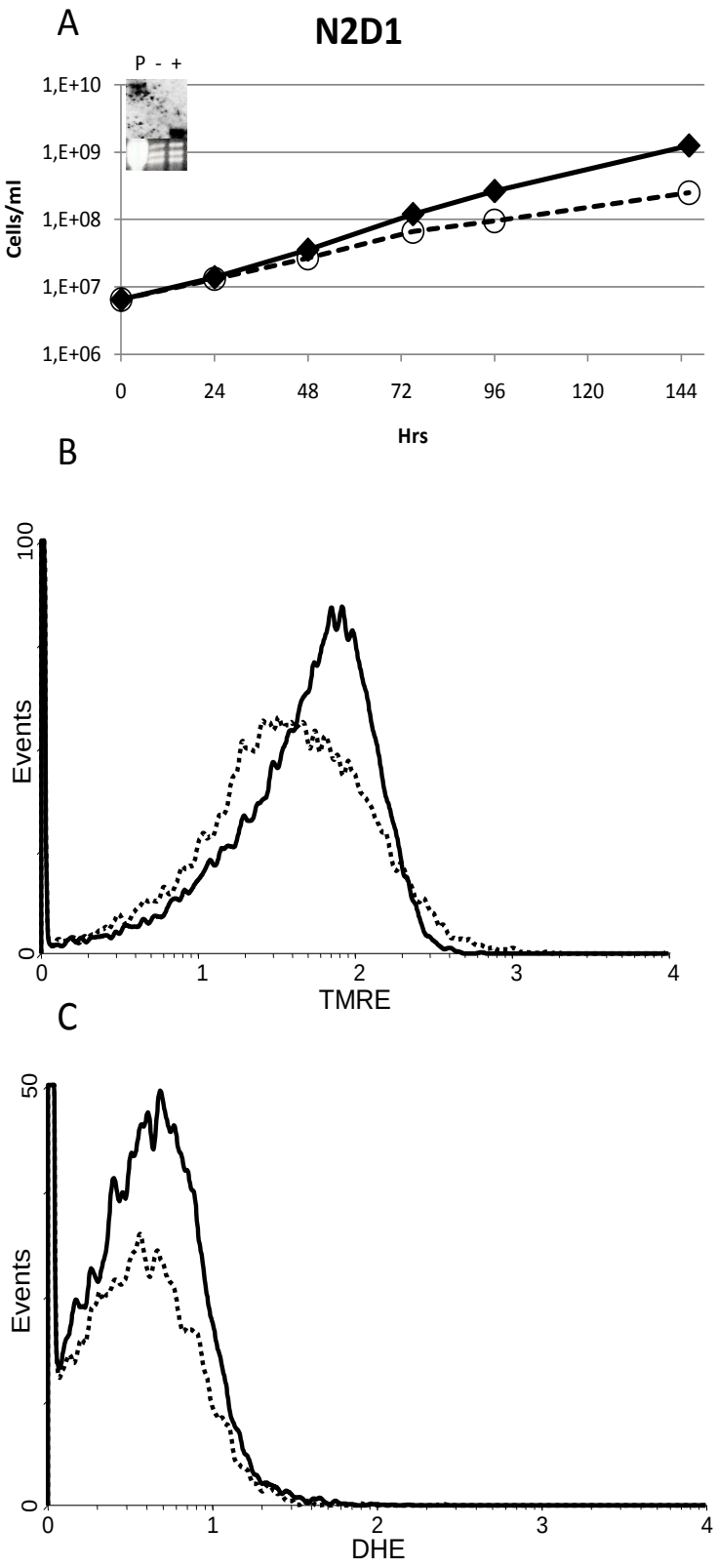
Fig. 1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P - +
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1,E+09
1,E+08
1,E+07
1,E+06
Cells/ml
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48
72
96
120
144
Hrs
B
100
Events
0
0
1
2
3
4
TMRE
C
50
Events
0
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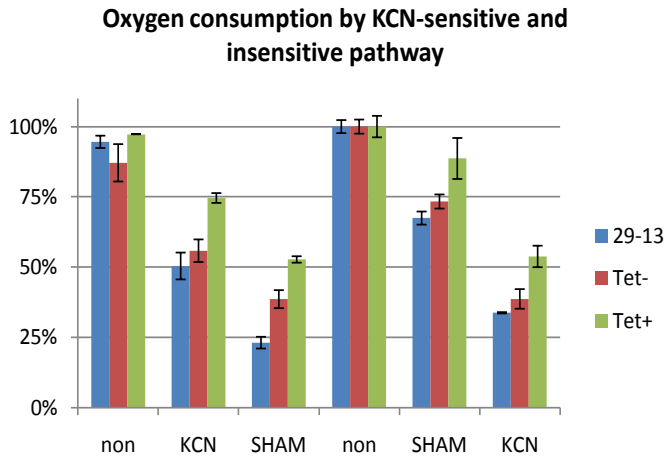
1
2
3
4
DHE

D


Oxygen consumption by KCN-sensitive and insensitive pathway
100%
75%
50%
25%
0%
non
KCN
SHAM
non
SHAM
KCN
29-13
Tet-
Tet+

Verner, Z. 2011. Mitochondrial energy metabolism in *Trypanosoma brucei:* Complex I and the others.

Supplementary Table 1								
	NDH2 (tet-)				**NDH2 (tet+)**			
Fraction nr.	**Protein**	**OD**	**Rot**	**DPI**	**Protein**	**OD**	**Rot**	**DPI**
1	1,47	6,53	0%	0%	1,30	0,72	800%	0%
2	1,55	3,09	78%	137%	1,35	5,91	164%	0%
3	2,37	3,04	52%	104%	1,17	12,27	100%	46%
4	5,78	1,46	19%	0%	4,27	1,12	0%	98%
5	6,57	1,53	111%	19%	5,92	0,81	0%	67%
6	7,11	0,45	205%	100%	6,79	3,04	0%	0%
7	6,97	1,26	107%	27%	6,73	2,50	105%	0%
8	6,16	1,65	138%	0%	5,63	2,16	0%	177%
9	4,88	3,29	0%	47%	4,92	2,28	0%	59%
10	4,02	7,15	45%	8%	3,60	3,12	133%	0%
11	3,21	1,00	300%	234%	2,80	3,99	186%	0%
12	2,33	1,72	320%	94%	2,04	6,89	23%	53%
13	1,81	7,77	67%	60%	1,63	4,42	200%	0%
14	1,60	1,50	200%	273%	1,65	3,40	67%	17%
15	1,36	20,05	62%	28%	1,29	1,24	800%	234%
16	1,28	5,64	89%	52%	1,28	8,09	192%	54%
17	1,50	12,25	87%	25%	1,13	25,64	55%	113%
18	1,62	7,15	28%	178%	0,79	2,03	650%	0%
19	1,46	1,09	750%	0%	0,97	20,25	53%	65%
20	1,25	1,93	67%	234%	0,83	11,34	10%	220%
21	1,43	4,59	110%	439%	0,80	9,37	192%	212%
22	1,25	7,69	167%	0%	0,82	15,63	132%	65%
23	1,00	20,23	66%	32%	0,23	37,48	300%	95%
24	2,34	4,79	33%	114%	0,70	2,67	811%	1000%

10.6 Biochemical characterization of FAD-dependent glycerol-3-phosphate dehydrogenases in trypanosomatids with emphasis on *Trypanosoma brucei* (Škodová I., Verner Z., Fabián P., Lukeš J., Horváth A.) (in preparation)

Biochemical characterization of FAD-dependent glycerol-3-phosphate dehydrogenases in trypanosomatids with emphasis on *Trypanosoma brucei*

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Introduction

Members of the order Kinetoplastida represent an interesting group of organisms important from both medical perspective and as a subject of basic research. Within this protist order, virtually all obligatory parasites of vertebrates and insects belong to the family Trypanosomatidae, for which a host of unique features is characteristic (Lukeš et al., 2010).

The variability of life strategies that evolved during the long evolutionary history of these widespread and significant parasites is represented by the following four species, each

representing a species-rich taxon: i/ the dixenous *Leishmania tarentolae* rotates between an insect vector (*Phlebotomus* spp.) and the macrophages of a gecko. Other members of the genus *Leishmania* are etiological agents of a range of serious human diseases termed leishmaniases; ii/ the monoxenous *Crithidia fasciculata* is confined to the intestinal tract of insect hosts, in this case a mosquito; iii/ *Phytomonas serpens* parasitizes the tissues of various plants, including coconut or palm trees. It uses various heteropteran insects as passive vectors and causes economically important diseases, such as hartrot and marchitez sorpresiva; iv/ last but certainly not least, *Trypanosoma brucei* circulates between the bloodstream of warm-blooded vertebrates including humans, and the midgut of its insect vector, the tse-tse fly (*Glossina* spp.). It is responsible for lethal African sleeping sickness of humans and nagana of livestock.

As a reflection of different life strategies, each parasite has adapted its energy metabolism to a different environment. Together, they represent excellent models for the comparison of the resulting adaptations (Tielens et al., 2009). *L. tarentolae* and *C. fasciculata* dispose with fully developed respiration except for a non-functional complex I (Santhamma, et al., 1995)(Speijer, et al., 1997). Although *P. serpens* lacks complexes III and IV (Maslov et al., 1999), it is the only trypanosomatid with proved active complex I (Čermáková et al., 2007). In all these trypanosomatids the presence of two alternative respiratory enzymes, namely trypanosomal alternative oxidase (TAO) and alternative NADH dehydrogenase (NDH2) has been predicted. Electron flux from ubiquinone can be shifted via TAO directly to the final acceptor, molecular oxygen, without protons being pumped out of the mitochondrial matrix. NDH2 bypasses complex I sending electrons to ubiquinone without generating proton gradient on the inner mitochondrial membrane (Verner et al., in preparation).

Not surprisingly, of these flagellates *T. brucei* is the most studied species. Its main proliferative stages are the long-slender bloodstream form (BF) in mammals and the procyclic form (PF) residing in the midgut of the tse-tse fly vector. Major differences in energy and metabolic transduction pathways are characteristic for these two forms. The PF consumes mainly the amino acids proline and threonine, which are abundantly present in the tse-tse fly (Bringaud et al., 2006). This stage has a single fully developed mitochondrion, which contains a full complement of cytochromes and most of the enzymes of the Krebs cycle (Schneider 2001; Bringaud et al., 2006). Unexpectedly however, the Krebs cycle is not spinning in a way common for other eukaryotes, since it uses various intermediates produced by the amino acid

metabolism as the sources of reduced equivalents (van Hellemond et al., 2005). Reducing equivalents generated by the amino acid metabolism are then oxidized via a classical respiratory chain (Bringaud et al., 2006).

The BF is adapted to an environment rich in glucose and consequently totally depends on it for energy production, as the ATP-generating functions of its mitochondrion are repressed (Opperdoes, 1987). Respiratory chain of the BF cells consists only of TAO and FAD-dependent glycerol-3-phosphate dehydrogenase (FAD-G3PDH) (Grant and Sargent, 1960). Respiratory complex II together with the other Krebs cycle enzymes is missing, while complex I and NDH2 seem to be present, although their contribution to the electron flow remains unknown (Fang and Beattie, 2002).

Mitochondrial respiration and glycolysis that is confined to the specialized organelle termed glycosome are connected by glycerol-3-phosphate/dihydroxyacetone phosphate shuttle (G3P:DHAP). This shuttle usually consists of two enzymes: FAD-dependent glycerol-3-phosphate dehydrogenase (FAD-G3PDH) located on the outer face of the inner mitochondrial membrane (Allemann and Schneider; 2000) and NAD-dependent glycerol-3-phophate dehydrogenase (NAD-G3PDH) located in the glycosomes (Opperdoes et al., 1976). The main distinction of this pathway from that in other eukaryotes is the invariably cytosolic location of NAD-G3PDH (Opperdoes et al., 1976). The sequestration of first seven steps of glycolysis into the glycosome (Opperdoes, 1987) makes the whole pathway independent of the cytosolic NADH/NAD^+ ratio. The glycolytic NAD^+ requirement is saturated by the G3P:DHAP shuttle. Electrons from G3P are shuffled by FAD-G3PDH to ubiquinone in the respiratory chain. Further fate of electrons is known in BF, where the TAO is their only possible electron acceptor (Clarkson, et al., 1989). In PF, alternative pathways operate, namely one from ubiquinone to TAO, and the other from ubiquinone to complex III. The importance of NAD^+ regeneration through the G3P:DHAP shuttle is evident in the BF cells, where glycolysis is the main source of energy. These electron transports do not seem to play a crucial role in ATP generation in the PF cells. With glucose being scarce in the tse-tse fly, amino acids constitute the main source of energy (Opperdoes, 1987).

Trypanosomal NAD-G3PDH is encoded by a single gene, in contrast to yeasts and plants, which carry more isoforms with different functions in their genomes (Gee et al., 1988; Wei et. al, 2001). On the other hand, *T. brucei* and *L. major* code for two genes for FAD-dependent glycerol-3-phosphate dehydrogenases (FAD-G3PDH). In the genome of *T. brucei*,,

they are annotated as mitochondrial FAD-G3PDH (Tb11.02.5280) (mtG3PDH) and putative FAD-G3PDH (Tb927.1.1130) (putG3PDH), with the latter dehydrogenase containing an unknown signal sequence. Mitochondrial localization has been predicted by MitoCarta (http://www.broadinstitute.org/pubs/MitoCarta/index.html) (Pagliarini et al., 2008), the signal peptide can possibly target this protein to the endoplasmic reticulum (see www.genedb.org entry for Tb927.1.1130). Mitochondrial localization of the former dehydrogenase was experimentally confirmed by ectopic expression in the yeast (Guerra et al.; 2008) and this result was subsequently confirmed by a high-throughput TAP-tag-based proteomic analysis (Panigrahi, et al., 2009) (compare results for both proteins at www.trypsproteome.org). However, functional analysis of trypanosomal G3PDH enzymes has been lacking.

Trypanosoma brucei possesses functional RNA interference (RNAi) machinery, and an inducible system for its employment in reverse genetics has been established (Wirtz et al., 1999; Wang et al., 2000), qualifying this pathogen as a principal model kinetoplastid flagellate. The aim of this study was to characterize more deeply the G3PDH activity and to determine localization of protein products of both FAD dependent G3PDHs in *T. brucei*. Moreover, we have compared the mitochondrial G3PDH activity in four different trypanosomatid species, shedding some light on substantial differences in their respiratory chains.

Experimental Procedures

Trypanosome cultures

The PF of *T. brucei* (strain 29-13) was cultivated at 27˚C in SDM-79 medium containing 10 % (v/v) heat-inactivated fetal bovine serum, 7.5 µg/mL hemin and the following antibiotics when relevant: 50 µg/mL hygromycin, 15 µg/mL neomycin and 205 µg/mL phleomycin. The BF of *T. brucei* was cultured at 37˚C with 5% CO_2 in air in HMI-9 medium supplemented with bathocuproinedisulphonic acid, l-cysteine, hypoxanthine, 2-mercaptoethanol, pyruvate, thymidine and 10 % fetal bovine serum. Cells were diluted 10 times as soon as they reached the concentration of 10^7 and 10^6 cells per ml for PF and BF, respectively.

C. fasciculata and *P. serpens* were cultured at 27 ˚C in Brain Heart Infusion medium (BHI) (37 g/l) *L. tarentolae* was grown under the same condition with BHI medium supplemented with haemin (10 μg/ml).

Inhibition of gene expression by RNAi

The PF and BF *T. brucei* were transfected with the p2T7-177 vector (Wickstead et al., 2002) allowing RNAi induction against the mitochondrial FAD-G3PDH. This construct was prepared by cloning PCR-amplified fragment of the region spanning nucleotides 493-1303 of the FAD-G3PDH gene (Tb11.02.5280), with BamHI and HindIII restriction sites added into the primers. The resulting 810 bp fragment was cloned into the pCR-TOPO vector (Invitrogen), cut using the above restriction sites and re-cloned into the p2T7-177 vector. Finally, p2T7-177-G3PDH construct was linearized using NotI and 10 μg of DNA was electroporated into the PF and BF cells. For the selection of transformants, phleomycin was added 24 hours after electroporation, and clones were obtained as described elsewhere (Vondrušková et al., 2005). Sythesis of double-stranded (ds) RNA was induced by the addition of 1 μg/mL tetracycline (tet) to the medium.

Preparation of the fused protein putG3PDH with V5tag

The PF *T. brucei* was transfected with the pLEW79 vector containing whole putG3PDH gene with the V5 tag attached to its 3' end. The construct was prepared by cloning PCR-amplified gene with added BamHI and Hind III restriction sites into pLEW79, which was prior electroporation in the PF T. *brucei* cells linearized with NotI. Transformants were grown in medium with neomycin(15μg/ml) and hygromycin (50μg /ml) and were selected with puromycin (15 μg/ml). Expression was induced by the addition of 1 μg/ml tet to the medium one day after transfection.

Spliced leader RNA

cDNA from total cellular RNA, prepared using the High Pure Isolation kit (Roche) following the manufacturer's instructions, was used as a template for nested PCR with the following design. In the first reaction, a primer specific for the gene of interest was used together with a primer derived from the splice leader (SL) RNA gene. In the second step, the

gene-specific and SL primer located closer to each other than the previous pair were used. Products of both reactions were resolved on an agarose gel, the amplicons were cut-out, gel-purified, cloned in the pCR-TOPO vector and sequenced.

FAD-dependent glycerol-3-phosphate dehydrogenase activity

75 μg of mitochondrial protein lyzed with 0,5 mol/L aminocaproic acid and 2% dodecylmaltoside was added to 1 mL cuvette containing 50 mM Tris-buffer (pH 7.0), 0.15 mM 2,6-dichloroindophenol and 0.2 mM phenazine metosulfate. The FAD-G3PDH activity was measured by recording the decrease of absorbance at 600 nm that was triggered by the addition of 50 mM glycerol-3-phosphate (G3P).

Digitonin permeabilization

For this experiment a modification of a protocol described elsewhere (Riviére et al., 2009) was used. The pellet of PF *T. brucei* was resuspended to the final concentration of 2 mg of cell protein/ml in 50 mM NaCl, 50 mM imidazole/HCl (pH 7.0), 2 mM aminocaproic acid and 1 mM EDTA. To 25 μl of cell suspension containing 50 μg of protein, 5 μl of different concentrations of digitonin were added (for final concentrations see Fig. 2), incubated for 5 min on ice and spun for 30 min at 21,000 *g* at 4 °C. The resulting pellet was resuspended in SDS-PAGE loading buffer (62.5 mM Tris-HCl, pH 6.8; 10% glycerol; 2% SDS; 2.5% 2-mercaptoethanol; 0.0025% bromophenol blue), boiled for 3 min and loaded on a SDS-PAGE gel.

Western blot analyses

Digitonin-extracted fractions were separated onto a 12% SDS-PAGE gels, blotted and probed with polyclonal antibodies against enolase (kindly provided by P. Michels), trypanosome cytochrome *c* oxidase subunit 4 (trcoIV) (Maslov et al., 2002), glycosomal NAD-G3PDH (kindly provided by P. Michels) and a monoclonal antibody against the V5 tag (Sigma-Aldrich) were used at 1:150,000, 1:1,000, 1:1,000, and 1:1,000 dilutions, respectively. Appropriate secondary antibodies (1:2,000) (Sevapharma) coupled to horseradish peroxidase were visualized using the ECL kit according to the manufacturer's protocol (Pierce).

Isolation of mitochondria, cell and mitochondrial lysates

Mitochondria were isolated from 5 x 10^5 cells by hypotonic lysis as described previously (Horváth et al., 2005). Mitochondrial pellets were stored in -80˚C for further spectrophotometry measurements of the G3PDH activity. To obtain cleared lysates, mitochondria isolated from 5 x 10^8 cells or 5 x 10^8 cells were incubated with 2% (w/v) dodecylmaltosid in 0,5 M aminocapronic acid in the final volume 50 µL on ice for 1 hr, then spun 30 min at 21,000 g at 4˚C. The supernatants were used for in gel-staining or spectrophotometric measurement.

Results

Expression of mtG3PDH and putG3PDH

Expression of both genes coding for FAD-G3PDH was evaluated using a SL RNA-based strategy. cDNA prepared form total RNA was used as a template for nested PCR with two pairs of SL RNA specific primers. Attachment of the SL RNA via *trans*-splicing to the 5' end of the FAD-dependent G3PDH mRNA provides evidence that the gene is transcribed into a translatable mRNA.

Fig. 1 shows that both FAD-mtG3PDH (Tb11.02.5280) and putG3PDH (Tb927.1.1130) are expressed in the PF and BF stages of *T. brucei*. To exclude possible DNA contamination of the RNA samples, control PCR reactions were performed with RNA in the absence of reverse transcriptase (Fig. 1). Amplicons of ~ 350 bp were obtained for both genes. Since this method is not quantitative, we checked the expression profile on http://splicer.unibe.ch/, where data from SL trapping can be accessed (Nilsson et al., 2010). The information mined from this database clearly shows differential expression of the two genes.

Subcellular localization of putG3PDH

Gene database entry at www.genedb.org for putG3PDH contains two possible organellar targets: mitochondrion, predicted by MitoCarta (Pagliarini et al., 2008), and endoplasmic reticulum (F. Opperdoes, unpubl. data). To clarify this conundrum, the PF cells

were transfected with an inducible construct expressing putG3PDH fused with the V5 tag (putG3PDH-V5). Subcellular localization of putG3PDH-V5 was determined in subcellular fractions obtained by digitonin titration. The increasing concentration of the drug sequentially permeabilizes cell membranes on the basis of their different sterol content.

Western blot analysis revealed that the markers for various *T. brucei* cell compartments were indeed released at different concentrations of digitonin (Fig. 2). While cytosolic enolase appeared at concentration lower than 0.04 mg digitonin/mg protein, glycosomal NAD-G3PDH was released only at a higher concentration (0.25 mg digitonin/mg protein). Finally, 0.5 mg digitonin/mg protein was required to rupture the mitochondrial inner-membrane, as followed by the subunit IV of cytochrome *c* oxidase (trcoIV) antibody (Fig. 2).

The putG3PDH-V5 protein was released from the pellet along with NAD-G3PDH, suggesting that this enzyme is not localized within the mitochondrion (Fig. 2). As digitonin titration depends on different amount of sterol in cellular membranes, co-localization with a glycosomal enzyme does not necessarily mean its glycosomal localization, as peroxisomes, glycosomes, Golgi apparatus and the endoplasmic reticulum all posses the same type of membranes in this respect. Thus, we can exclude inner mitochondrial membrane, mitochondrial matrix as well as cytosol as a place of putG3PDH residence.

G3PDH activity in subcellular fractions

Pellets of the parental 29-13 cell line after digitonin treatment were used for a spectrophotometric assay of the G3PDH activity. In the absence of digitonin, the pellet contains intact cells. With increasing digitonin concentration, the pellet is sequentially stripped of cytosol, single-membrane organelles and finally double-membrane ones. Thus, a drop in the overall G3PDH activity was expected. A decrease of activity was recorded in the fraction treated with 0.25 mg digitonin/mg proteins, corresponding to the lower putG3PDH-V5 signal in Western blot (Fig. 2). Another drop occurred in the fraction treated with 0.6 mg digitonin/mg proteins, a concentration sufficient to open the inner mitochondrial membrane. These findings further support the notion that FAD-G3PDH has indeed dual localization. While mitochondrial G3PDH was described previously (Guerra et al., 2006), product of the *putG3PDH* gene seems to be located outside of the organelle. An expression of the tagged version of putG3PDH might due to an unnatural level of expression,cause artificial

localization. However, an activity measured in the parental cell line dropped at two different digitonin concentrations, further confirming that two trypanosomal FAD-G3PDHs are localized in two different cell compartments.

G3PDH activity in *T. brucei*

We prepared RNAi cell lines with downregulated mtG3PDH (Tb11.02.5280) and employed the previously prepared putG3PDH-V5 cell line to follow the G3PDH activity. The RNAi cell line was induced for 4 days, while putG3PDH-V5 was overexpressed for 12 hours. Then the G3PDH activity was measured in either total cell or mitochondrial lysates, and compared with the same activity in the parental 29-13 strain. In total cell lysate of the PF cells, the specific activity per mg of protein was lower than in the mitochondrial lysates (Fig. 3). Surprisingly, high increase of the G3PDH activity was observed in lysates from the cell line with overexpressed putG3PDH in comparison with the 29-13 sample (+65%). Increased activity was observed in the mitochondrial lysate of putG3PDH-V5 (+15%) when compared with the 29-13 mitochondrial lysate. However, the ratio of cellular to mitochondrial activity clearly shows an increase of the former activity (0,74 for 29-13 and 1,08 for putG3PDH-V5) (Fig. 3).

RNAi drastically reduced the mitochondrial G3PDH activity, which dropped from 25 mU/mg in the non-induced cells to 1.6 mU/mg in the RNAi-induced cells (Table 2.). The same experiment was done with the BF mitochondrion with a similar outcome. The 105 mU/mg activity in the parental strain dropped in the RNAi-induced samples to 35 mU/mg.

Comparison of G3PDH activity in four trypanosomatids

For comparative purposes, the FAD-G3PDH activity was measured in the mitochondrial lysates of trypanosomatids *P. serpens*, *L. tarentolae*, *C. fasciculata* and two life stages of *T. brucei*. To reduce potential influence of any interfering activity from the cytosol, lysates of enriched mitochondrial fraction were used for activity measurement. Specific G3PDH activity significantly differs between the tested strains and clearly reflects differences in their environment and various adaptations of metabolic pathways (Table 3). The highest activity was detected in flagellates lacking classical respiratory chain, in particular the *T. brucei* bloodstreams. *P. serpens* possessing functional complex I has an intermediate activity

that is significantly lower than that in BF yet is still almost three times higher than in the PF *T. brucei*. *L. tarentolae* and *C. fasciculata* contained the lowest activity of G3PDH.

Discussion

Glycerol phosphate - dihydroxyacetone phosphate shuttle links the glycosomal and mitochondrial metabolisms. While in the glycosome the NADH dependent G3PDH is active, its FAD-dependent isoform is typical for the mitochondrion. Along with TAO, mitochondrial FAD-G3PDH is the only part of the respiratory chain of *T. brucei* transferring electrons to oxygen that is active in both the BF and PF cells. This cyanide-insensitive and SHAM-sensitive system formerly called α-glycerophosphate oxidase (GPO) is known for more than 30 years (Njogu et al., 1981; Opperdoes, 1987). However, while enzymes between ubiquinone and SHAM-sensitive TAO (Nakamura et al. 2010), as well as the cyanide-sensitive branch of the respiratory chain were studied quite extensively, much less attention was given to the physiological importance of electron pathways preceding ubiquinone.

The actual role of G3PDH in the metabolism of *T. brucei* became even more enigmatic after two genes encoding the FAD-dependent isoforms of this enzyme were found (http://www.genedb.org/gene/Tb927.1.1130). Since both genes were expressed in both studied life stages with the localization of only one of them experimentally established (Guerra et al., 2006), we decided to determine *in vivo* localization of the product of the second FAD-G3PDH gene. For that we have measured specific FAD-G3PDH activity in cell fractions prepared by increasing concentrations of digitonin. Drop of this activity in two different concentrations of the detergent strongly indicated that the enzyme is present in two different cellular compartments.

In the absence of specific antibodies against any of the FAD-G3PDH isoforms, to further address the intracellular localization of this protein, we prepared fusion of putG3PDH with the V5 tag attached to its C-terminus. Digitonin fractionation showed that the tagged protein disappears at concentrations higher than those that release cytosolic enolase, but lower than concentrations influencing the glycosomal NADH-G3PDH. There was certainly no common release with the mitochondrial marker, here the trCOIV subunit of complex IV. Another independent confirmation of the extra-mitochondrial localization of putG3PDH came from the comparison of the G3PDH activity in the whole cell and mitochondrial lysates of

parental cells and those over-expressing the V5-tagged putG3PDH. While in the parental strain relative activity per mg proteins was higher in the mitochondrial than in the total cell lysates, the situation was reversed in the, over-expressing strain, in which higher specific activity was detected in the whole lysate than in the organelle. The most convincing evidence came from RNAi knock-downs of mtG3PDH, in which a drop of mitochondrial G3PDH activity to virtually zero values occurred, while in the total cell lysates about 20% of the activity remained refractive to RNAi. This value can be interpreted as a reflection of the 1 to 4 ratio between the putG3PDH to mtG3PDH activities in the parental strain. At this point, however, differences in the activity of both enzymes under *in vitro* and *in vivo* conditions cannot be excluded. In any case, four independent approaches showed that the product of the putG3PDH gene has glycerol-3-phosphate dehydrogenase activity localized outside of the mitochondrion. The approach based on digitonin fractionation does not allow more exact localization though, because several organelles possess membranes with very similar sensitivity to this detergent. Presence of G3PDH in the endoplasmic reticulum involved in the lipid biosynthesis (Athenstaedt and Daum, 1997) along with the predicted targeting signal lead us to propose the localization of putG3PDH in this cell compartment. Results available so far do not allow to address possible association between put- and mtG3PDH.

Comparison of the mtG3PDH activities in five types of trypanosomatid cells shows that there is no functional connection between this enzyme and complex I. Indeed, in *C. fasciculata* and the BF of *T. brucei*, both lacking complex I, the lowest and highest G3PDH activities, respectively, were measured. The lack of correlation is further reflected by the rather high activity of G3PDH in *P. serpens* and three times lower one in the PF of *T. brucei*, with fully and partially functional complex I, respectively (Čermáková et al., 2007; Verner et al., 2011). However, the level of G3PDH activity seems to reflect energy dependence on glycolysis, since all three trypanosomatids with lowest activities have functional complexes III and IV, which produce membrane potential that is a source for ATP production by mitochondrial ATP synthase. On the contrary, *P. serpens* with complex I being the only proton pump, likely unable to fully compensate for the absence of other respiratory complexes, has the G3PDH activity several times higher. The *T. brucei* BF without any oxidative source of membrane potential has the highest G3PDH activity.

Presented finding clearly show that putG3PDH is active and has an extra-mitochondrial localization implicating all G3PDH activity present in the isolated

mitochondria exlusively with its mtG3PDH isoform. Relationship between mtG3PDH and substrate phosphorylation is obvious, yet further study is needed to show whether this connection is mainly due to the regeneration of dihydroxyacetone phosphate for glycosomal metabolism, or it has some significance for mitochondrial energetics as well. G3PDH activity was detected in all studied cell lines and physiological importance of this enzyme is subject of an ongoing study to better characterize the roles played by mtG3PDH in the BF and PF stages of *T. brucei*. Moreover, additional information is needed to address possible interactions between both isoforms and/or between mtG3PDH and other systems transfering electrons to ubiquinone.

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Figure legends

Fig 1. Spliced leader PCR of mtG3PDH and putG3PDH

RT PCR using primers for putG3PDH (lanes 1,2,3 and 4) and mtG3PDH (lanes 5,6,7 and 8) with mRNA isolated from the procyclic (lanes 1,2,5 and 6) and the bloodstream forms of *T. brucei* (lanes 3,4,7 and 8). Samples in lines 2, 4, 6, and 8 without reverse transcriptase served as negative controls.

Fig. 2. Determination of FAD-G3PDH localization by digitonin fractionation

Equal amounts of the cells were treated with different amounts of digitonin (concentration in mg of digitonin per mg of cell is indicated above the appropriate lane). Following centrifugation, the pellet was resuspended in SDS PAGE sample buffer and loaded on the gel (enolase – cytosolic marker; NAD-G3PDH – glycosomal marker; trcoIV –mitochondrial marker**.**

Fig. 3. Relative activities of G3PDH

Whole cell (cell) and mitochondrial (mito) lysates of *T. brucei.* Dodecylmaltoside of respective lysates were used for activity measurement. WT = parental strain, putG3PDH strain overexpressing putG3PDH-V5.

Fig. 4. Relative activities of G3PDH in *T. brucei*

Activity was measured in whole cells (cell) and mitochondrial (mito) lysates before (-) and after (+) RNAi-induction targeting mitochondrial G3PDH. Dodecylmaltoside respective lysates were used for activity measurement.

Table 1. G3PDH activity in digitonin fractions.

Equal amounts of cells were treated with different amount of digitonin and pelleted by centrifugation. Dodecylmaltoside lysates of the pellets were used for activity measurements (for details see Experimental Procedures). mg digitonin/mg protein - concentration of digitonin used; *1 U is an enzymatic activity that converts 1 μmol of DPIP per min. Presented data are from one representative experiment.

Table 2. G3PDH activity in mitochondria of *T. brucei* RNAi cell lines.

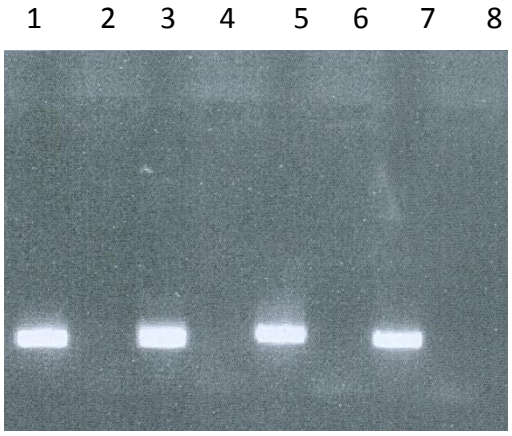
* For definition of enzymatic activity see Table 1; For measurements, dodecylmaltoside lysates of isolated mitochondria were used. PF = procyclic form; BF = bloodstream form. Presented data are average of three experiments.

Table 3. G3PDH activity in mitochondrial lysates of **different trypanosomatids**

* For definition of enzymatic activity see Table 1. Dodecylmaltoside lysates of isolated mitochondria were used for activity measurements. Data represent average of more than three different measurements.

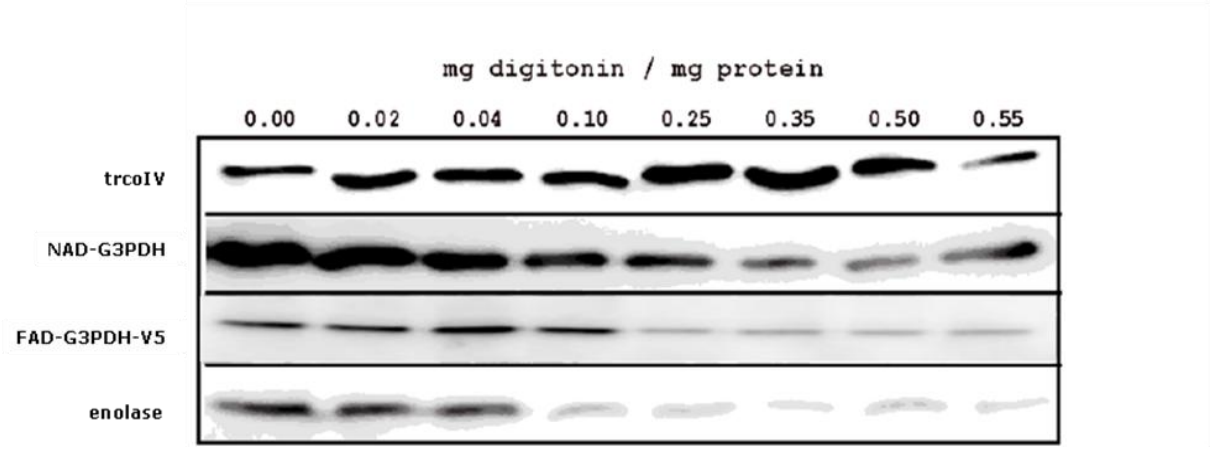
Verner, Z. 2011. Mitochondrial energy metabolism in *Trypanosoma brucei:* Complex I and the others.

Figure 1



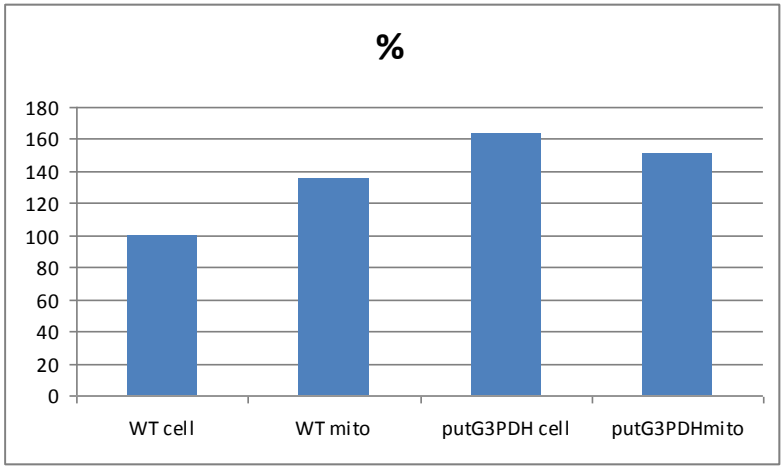
Verner, Z. 2011. Mitochondrial energy metabolism in *Trypanosoma brucei:* Complex I and the others.

Figure 2



Verner, Z. 2011. Mitochondrial energy metabolism in *Trypanosoma brucei:* Complex I and the others.

Figure 3



Verner, Z. 2011. Mitochondrial energy metabolism in *Trypanosoma brucei:* Complex I and the others.

Figure 4

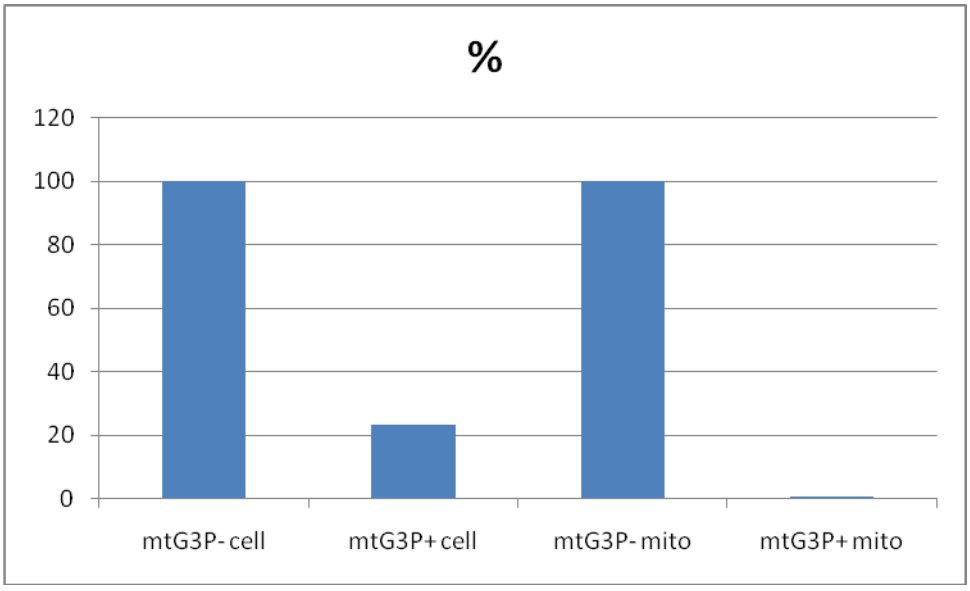


Table 1

mg digitonin/mg protein	Specific activity [mU*/mg]
0.0	6.3
0.04	6.4
0.25	4.5
0.3	4.2
0.4	4.2
0.5	4.0
0.6	2.0
4.0	1.0

Table 2

T. brucei **mtG3PDH**	**Specific activity [mU*/mg]**
PF noninduced	25 ± 4.5
PF induced	1.6 ± 1.5
BF noninduced	105 ± 27.3
BF induced	35 ± 13.3

Table 3

organism	**Specific activity [mU*/mg]**
L. tarentolae	13 ± 3.8
T. brucei PF	29 ± 13.5
T. brucei BF	122 ± 23
P. serpens	88 ± 36.3
C. fasciculata	11 ± 1.6

10.7 Insight into the functional organization of cytochrome c oxidase in Trypanosoma brucei (Gnipová A., Paris Z., Verner Z., Horváth A., Lukeš J., Zíková A.) (in preparation)

Insight into the functional organization of cytochrome *c* oxidase in *Trypanosoma brucei*

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Introduction

Kinetoplastid flagellates *Trypanosoma brucei* and *Leishmania* spp. are unicellular parasites of major medical and veterinary significance, since they causes diseases in humans (sleeping sickness and leishmaniasis) and animals (nagana) [1]. At present, there are no vaccines against these diseases and only a few drugs are available. In addition, drug resistance is demanding an urgent need for replacement and supplementary drugs. Currently, some anti-

trypanosome and anti-leishmania drugs target the mitochondrion, signifying the therapeutic potential of disrupting mitochondrial (mt) function [2,3,4].

The *T. brucei* mitochondria undergoes dramatic metabolic and structural changes during the life cycle of the parasite, as it alternates between a mammalian host and the blood-feeding insect vector, a tsetse fly [5]. In the mammalian host, the mitochondrion of the trypanosomes lacks a functional cytochrome-mediated respiratory chain [6], while in the tsetse fly host, the organelle becomes fully developed with many cristae and an active respiratory chain [7]. In addition to the conventional respiratory chain, the *T. brucei* mitochondrion contains alternative respiratory pathway composed of alternative NADH dehydrogenase and plant-like alternative oxidase [8,9]. *Leishmania* spp. also alternate between a mammalian host and the blood-feeding insect vector, in this case, a sand fly. However, in contrast to t*rypanosom*es, both stages of Leishmania are strongly dependent on the cytochrome-mediated respiratory chain for their energy generation [10,11].

The mt respiratory pathway, carried out by protein complexes I thru IV, generates mt membrane potential across the inner mt membrabe which is then used by F_oF_1-ATP synthase (respiratory complex V) to produce ATP by oxidative phosphorylation.

In *T. brucei*, it has been shown that the respiratory complex IV, cytochrome (cyt) *c* oxidase, is essential for the insect procyclic (PF) stage but not for the mammalian bloodform (BF) stage of *T. brucei* [12,13]. This observation is in agreement with metabolic changes that occur during parasite's life cycle, as no cytochrome-mediated respiratory chain is present in the BF stage. In contrast, dependence on the cytochrome-mediated respiratory chain makes cyt *c* oxidase essential for both *Leishmania* life stages [14]. Furthermore, it has also been shown that cells of *Leishmania donovani* and *L. major* with a decreased cyt *c* oxidase activity are less virulent or non-virulent at all, suggesting that parasites devoid of this activity could be considered as a live attenuated vaccine candidate against leishmaniasis [15,16].

Trypanosomatid complex IV, like all eukaryotic cyt *c* oxidases, is a multi-component complex composed of three large mitochondrial-encoded subunits and at least 15 nuclear-encoded subunits [17,18,19]. Moreover, complex IV of *T. brucei* appears to be highly diverged, as only the two nuclear-encoded subunits COVI and COVIII possess recognizable homology to the human subunits coxVIb and coxIV, respectively. In addition to its core subunits, the *T. brucei* complex IV is transiently associated with additional 24 proteins, most of them of unknown function [19]. Associations with these proteins raise the possibility that cyt *c* oxidase is part of the larger (super)complex within the inner mt membrane possessing

more than one function or that some of the protein components have dual functions and may constitute different complexes [19].

In this study we characterized three proteins that have been shown to associate with the trypanosomatid cyt *c* oxidase to various degree: i/ Tb11.01.4702 – Tbcox4702 (coxX) was purified as a core subunit in both *Crithidia fasciculata* and *T. brucei* complexes; ii/ Tb927.3.1410 – coxTb1410 (coxVII) is consider a core subunit in *C. fasciculata*, but is only transiently associated with the *T. brucei* complex; iii/ Tb927.8.6080 – coxTb6080 (hypothetical protein) has not been detected in the *C. fasciculata* complex, however, it binds transiently with the *T. brucei* complex [17,19]. In order to explore putative structural and functional associations of these subunits with the cyt *c* oxidase complex in *T. brucei*, we silenced all three genes in the PF stage and examined the ensuing phenotype. Surprisingly, all three subunits are important for structural integrity of the cyt *c* complex and their silencing caused severe phenotypes related to mitochondrial functions. These results suggest that the *T. brucei* cyt *c* oxidase not only highly diverged from its homologues in other eukaryotes, but may be a very complex enzyme, which might have acquired novel subunits and/or functions throughout the evolution.

Results

Inhibition of coxTb4702, coxTb6080 and coxTb1410 gene expression leads to growth defects and to a decrease abundance of trCOIV

To evaluate functional associations of all three analyzed putative subunits of cyt *c* oxidase we constructed cell lines in which expression of Tb11.01.4702, Tb927.3.1410 and Tb927.8.6080 can be inducibly silenced using RNA interference (RNAi). In all cases, RNAi was mediated by p2T7-177 construct that allows tetracycline (tet)-dependent and thus regulatable expression of dsRNA which results in RNAi-mediated degradation of the target mRNA. After the addition of tet into the culture medium the growth of coxTb1410 cell line was strongly inhibited and the reduced growth rate was apparent by day 3 (Fig. 1A). The growth inhibition effect of induced coxTb4702 and coxTb6080 cells was less apparent though the RNAi- induced cells grew slower comparing to the uninduced cells (Fig. 1A). The efficiency of RNAi was confirmed by Northern blot analysis using specific probes, showing that mRNA for Tb4702, Tb6080 and Tb1410 is almost eliminated by day 2 after RNAi induction (Fig. 1B). Based on the growth curves, coxTb1410 cells were collected at days 3 and 5 following RNAi induction, while coxTb4702 and coxTb6080 cells were harvested at

days 5 and 7 after tet addition for all subsequent experiments. Depletion of target proteins could not be verified since the specific antibodies against coxTb1410, Tb4702 and Tb6080 proteins are not available. However, using specific antibody against cyt *c* oxidase subunit IV (trCOIV) we tested if the depletion of the studied proteins caused a decrease in abundance of this previously studied subunit. Indeed, Western blot analysis of cleared cell lysate prepared at days 3, 5 and 7 after RNAi induction revealed a substantial loss of subunit trCOIV, as compared to the uninduced cells (Fig. 1C). The trCOIV protein was almost undetectable in cells ablated for coxTb1410, whereas a milder reduction of the trCOIV abundance was observed in coxTb6080 and coxTb4702 RNAi-induced cells.

Absence of coxTb4702, coxTb6080 and coxTb1410 affects cyt c oxidase activity, structural integrity of the complex and steady-state abundance of two cyt c oxidase subunits.

We employed several approaches to investigate whether the activity and/or stability of the cyt *c* oxidase complex are impaired in the RNAi-induced cells. First, we used Blue-native (BN) gel-based assay for the detection of multisubunit cyt *c* oxidase [12]. The mt lysates from uninduced and RNAi-induced cells were fractionated on a BN gel and cyt *c* oxidase activity was detected by histochemical staining. We observed a very strong decrease of the followed activity in all RNAi-induced cells (Fig. 2A). Next, we separated solubilized mt lysate by 2D BN/Tricine SDS PAGE and migration of cyt *c* oxidase subunits were identified on the basis of previous work [20]. While mitochondria from uninduced cells contains several observable cyt *c* oxidase subunits, these proteins were lost from the lysates of the RNAi-induced cells (Fig. 2B).

To further confirm that the depletion of targeted subunits also affects activity of the complex, specific *in vitro* activity of the cyt *c* oxidase complex was measured. As shown in Fig. 2C, in a representative experiment the specific cyt *c* oxidase activity was decreased by 67, 54 and 40% in coxTb1410, coxTb4702 and coxTb6080 induced cells, respectively, as compared to the uninduced cells. These results suggest that both structural integrity and activity of the cyt *c* oxidase complex are impaired after RNAi silencing of all three proteins. Since specific antibodies are available against trCOIV and COVI of the trypanosomatid cyt *c* oxidase complex, abundance of these two subunits was followed in total mitochondrial lysates from uninduced and RNAi induced cells. As a consequence of failed assembly of the cyt *c* oxidase complex, subunits trCOIV and COVI were almost eliminated in the induced coxTb1410 cells and significantly decreased in the cells depleted for coxTb6080 and

coxTb4702 (Fig. 2D). It thus appears that the lack of *de novo* assembled cyt *c* oxidase complexes is responsible for the degradation of the unincorporated trCOIV and COVI subunits.

To examine whether the activity of cyt *c* oxidase complex is affected specifically, activities of other respiratory complexes were measured in the studied knock-down cell lines. Activities of respiratory complexes II and V were detected using BN and high resolution clear native gel electrophoresis followed by specific staining. No significant differences in the activity and size of these complexes were observed (data not shown). Since no gel-based activity staining is available for the cyt *c* reductase complex, this activity was measured directly in the mt lysates *in vitro*. Unexpectedly, activity of this complex was only slightly increased following the depletion of coxTb1410, yet it was considerably decreased in cells lacking the other two cyt *c* oxidase subunits, coxTb4702 and coxTb6080 (Fig. 2C). This result may suggest differential role of these subunits in the mitochondrion of *T. brucei*. While in RNAi-induced coxTb1410 cells, there seems to be a lack of cross-talk between both the cyt *c* oxidase and reductase complexes, results obtained with the other two analyzed cell lines reflect functional dependence between these large protein complexes.

ATP production by oxidative phosphorylation and mitochondrial membrane potential are reduced following coxTb4702, coxTb6080 and coxTb1410 silencing

Cyt *c* oxidase is one of the component involved in ATP production by oxidative phosphorylation. Since this pathway depends on proper function of all respiratory complexes, we investigate whether the generation of ATP via oxidative phosphorylation is affected in the RNAi-induced cells. Indeed, we observed a significant decrease of ATP production by 85, 48 and 30% in RNAi-induced coxTb1410, coxTb4702 and coxTb6080 cells, respectively (Fig. 3). The ATP synthesis was sensitive to malonate, an inhibitor of succinate dehydrogenase (complex II) and atractyloside, an inhibitor of ATP/ADP translocator. In PF *T. brucei* cells, mt ATP is known to be produced via three different pathways [21]. In addition to the oxidative phosphorylation pathway, triggered *in vitro* by succinate, there are two substrate phosphorylation pathways, which are part of the incomplete citric acid cycle and of the acetate-succinate CoA transferase/succinyl-CoA synthetase cycle. Importantly, both pathways were not significantly affected in the RNAi interfered cells, with only a slight increase of ATP production by substrate phosphorylation pathways observed (data not shown). Taken together,

these results show that coxTb1410, coxTb4702 and coxTb6080 are important for ATP synthesis via oxidative phosphorylation.

An essential function of cyt *c* reductase and cyt *c* oxidase during the electron transport in the respiratory chain, is proton pumping across the inner mt membrane. This mt membrane potential depends on the intact function of respiratory complexes and is indispensable for the import of mt proteins. Analysis of TMRE-stained uninduced and RNA-induced cells revealed a decrease in fluorescence intensity, which is indicative of reduced mt membrane potential (Fig. 4). As expected, the strongest phenotype was observed in cells with downregulated coxTb1410, in which the mt membrane potential was decreased by ~60% of the uninduced cells. A milder decrease in membrane potential (by ~26 and ~50% as compared to the uninduced coxTb4702 and coxTb6080 cells, respectively) was observed in their RNAi-induced counterparts (Fig. 4).

Depletion of coxTb4702, coxTb6080 and coxTb1410 causes a shift from cytochrome-mediated respiration to alternative oxidase

The mitochondrion of PF *T. brucei* cells is equipped with two oxygen-dependent terminal oxidases, namely the cyt *c* oxidase and the plant-like alternative oxidase (TAO). Importantly the electron flow from the cytochrome-mediated chain can be redirected to TAO when the former is disrupted. The treatment with drugs that selectively inhibit each pathway enables to distinguish between cyt *c* oxidase- and TAO-mediated oxygen consumption. After the addition of KCN, which selectively inhibits the activity of cyt *c* oxidase, oxygen uptake decreased by about 70% in the uninduced cells. In contrast, in the RNAi-induced cells less dramatic decrease of KCN-sensitive oxygen consumption was observed (Fig. 5). In particular in cell ablated for coxTb1410 cells, the addition of KCN decreased oxygen consumption only by 15%, validating the anticipated activation of the KCN-insensitive TAO in these cells. Similar, although not as strong phenotype was observed in cells with decreased coxTb4702 and coxTb6080. Relative contribution of the SHAM- and KCN-sensitive pathways to the overall respiration is shown in Fig. 5.

Discussion

In this study we have characterized three mt proteins previously shown to be associated with the cyt *c* oxidase complex. We strengthened the hypothesis that these proteins

are *bona fide* subunits of the cyt *c* oxidase complex since its activity was affected after repression of the target mRNAs. The presented results are fully consistent with coxTb1410, coxTb4702 and coxTb6080 being structurally and functionally important components of this respiratory complex in *T. brucei*.

A variety of biochemical methods has been used to purified cyt *c* oxidase from *L. tarentolae*, *C. fasciculata* and *T. brucei* [17,18,19,22]. These purifications yielded to some extent overlapping sets of proteins suggesting that in the trypanosomatid flagellates, the cyt *c* oxidase complex has at least 15 core nuclear-encoded subunits. In addition to those, up to 18 proteins were identified only in the purified *T. brucei* cyt *c* oxidase, implying either transient or weak interactions of these proteins with the complex or unusually different, species-specific composition of the cyt *c* oxidase complexes. Furthermore, association of cyt *c* oxidase with the MIX protein, which was shown to play a role in mitochondrial segregation and virulence in *L. major* [15], adds further complexity and opens a possibility that cyt *c* oxidase constitutes just a part of the larger membrane-bound multifunctional (super)complex.

So far only two core subunits of the trypanosomatid cyt *c* oxidase complex were functionally characterized. Importantly, COVI (Tb10.100.0160) was shown to play an essential role in structural integrity of the complex in *T. brucei* [12], while the *L. donovani* homolog of Tb11.0400 (Ldp27) apparently enhances the cyt *c* oxidase activity and plays a role in the virulence of these parasites [16]. The highly diverged composition of the cyt *c* oxidase complex and the fact that *Leishmania* parasites with lower activity of the complex have been investigated as possible candidates for genetically attenuated vaccine [16] underline the importance of further dissecting this intriguing complex in the trypanosomatid parasites.

In order to gain an insight into the function of the three novel candidate subunits we prepared RNAi knock-downs, each with one of these subunits downregulated. CoxTb1410 and coxTb4702 were identified in the *L. tarentolae*, *C. fasciculata* and *T.brucei* complexes, while coxTb6080 was detected only in the *T. brucei* complex. Notably, all three proteins were essential for structural integrity and activity of the cyt *c* oxidase complex. Moreover, their repression resulted in a severe decrease of membrane potential, had a major negative effect on ATP production via oxidative phosphorylation and induced a switch to respiration via alternative oxidase.

Silencing of coxTb1410 had a major impact on cell growth, cyt *c* oxidase activity and mt membrane potential and respiration, while coxTb4702 and coxTb6080 exhibited generally

much milder growth phenotypes accompanied by weaker phenotypic changes. This discrepancy in observed phenotypes might be explained by an ineffective RNAi against Tb4702 and Tb6080, a claim difficult to test in the absence of specific antibodies, although Northern blot analysis confirmed effective ablation of both target transcripts. Therefore, it is likely that the function of coxTb4702 and coxTb6080 can to limited extent be complemented by other subunits.

Cyt *c* oxidase passes electrons from cytochrome *c* to the final acceptor, a molecule of oxygen. This process is coupled with the translocation of protons through the inner mt membrane, upkeeping mt membrane potential. The essentiality for *T. brucei* PF cells of the cytochrome-mediated respiratory chain coupled with ATP production has been controversial, since the dependence on the oxidative phosphorylation seems to be strain specific [23,24]. In any case, the mt membrane potential is absolutely required for proper mt function and has to be kept at levels sufficient for protein import [25]. Since the complex I is non-essential for PF cells and does not play a role in maintaining mt membrane potential [26], this proton gradient has to be kept by proper function of cyt *c* reductase and cyt *c* oxidase. Thus, it is plausible to hypothesize that the ablation of coxTb1410 leads to the drop of membrane potential under the threshold level causing a strong growth phenotype, whereas decrease of membrane potential by only 25 to 50% following the downregulation of coxTb4702 or coxTb6080 resulted in just a rather slight growth phenotype. This is with agreement with the previous study when two subunits of cyt *c* reductase and COVI were RNAi-silenced and displayed only mild growth phenotypes with the membrane potential decreased to 60% of the wild-type level [12].

In many organisms the complexes I, III and IV constitute specific supercomplexes, and association of these complexes depends on the presence of cardiolipin within the inner mt membrane. Besides functional reasons, supercomplex formation is necessary for assembly and stability of its individual components (reviewed in [27]. In contrast to yeast, bovine and plants mitochondria, the *T. brucei* organelle apparently lacks such supercomplexes, since in the absence of cyt *c* reductase, cyt *c* oxidase is still properly assembled and remains functional and the same applies vice versa [12]. These findings are in agreement with the observed phenotype in the RNAi-induced coxTb1410, in which the *in vitro* activity of cyt *c* oxidase is reduced down to 33% of the uninduced level, while the activity of the preceding respiratory complex is slightly increased. However, certain mutual dependence of both complexes is reflected by the parallel reduction of both cyt *c* oxidase and reductase activities in cells ablated for coxTb4702 or coxTb6080. An equally plausible alternative is that the lower

activity of cyt *c* reductase is a secondary phenotype, an attempt of the affected cells to balance electron flow through the respiratory chain.

While in the COVI knock-down, the trCOVI subunit was not degraded [18], the repression of all three subunits led to the decrease in abundance of both trCOIV and COVI subunits, which implies that failure to incorporate these subunits into the *de novo* assembled cyt *c* oxidase complex causes their quick degradation. On the other hand, similar phenotype of mutual dependency between core subunits was observed when the Rieske and apoc1 subunits were down-regulated in *T. brucei* [12]. In summary, these results imply that a repression of a subunit usually affect the stability of another subunit within the same complex.

The Tbcox1410 and Tbcox4702 subunits, annotated as cyt *c* oxidase subunits VII and X, have no homology outside the Kinetoplastida and their assignments were based on the migration in SDS-PAGE gel of subunits of the purified *C. fasciculata* cyt *c* oxidase complex. Furthermore, only two trypanosomatid cyt *c* oxidase subunits, namely COXVIII and COXVI have some degree of similarity to human cyt *c* oxidase complex subunits IV and VIb (that correspond to COX5 and COX12 in yeast) [19,28]. With additional data obtained from the homologous *L. tarentolae* and *T. brucei* complexes we decided not to follow this designation and named the *T. brucei* cyt *c* oxidase-associated proteins using their geneDB identification number (e.g.Tb11.01.4702 = Tbcox4702).

Interestingly, Tbcox6080 is annotated as a hypothetical protein in geneDB database with the homology to glycerophosphoryl diester phosphodiesterases (GDPD). In eukaryotes, membrane proteins that contain the GDPD motif and GDPD activity form a large family of proteins involved in phospholipid metabolism and cytosketetal modification [29,30,31]. The role of these enzymes is to hydrolyze deacylated phospholipid to alcohol and glycerol-3-phosphate, thus participating in many biochemical pathways. The association of this protein with the *T. brucei* cyt *c* oxidase complex is intriguing and with the available data we cannot exclude or confirm the possibility that Tbcox6080 plays a role in phospholipid catabolic processes. Direct investigation of coxTb6080 RNAi-induced cells regarding its putative function in lipid metabolism would shed light on this interesting issue.

Experimental Procedures

Construction of plasmids

To create the construct for RNAi of Tb11.01.4702, Tb927.3.1410 and Tb927.8.6080 transcripts, fragments of 486bp, 326 and 731bp, respectively, were PCR amplified using oligonucleotides listed below and cloned into p2T7-177 plasmid [32] via XhoI and BamHI restriction sites (underlined).

CoxTb1410 Fw - 5' CTCGAGCCCTTTGGTGTGTGG

Rev – 5' GGATCCGGCAGGAATATAGAA

CoxTb4702 Fw -5' CTCGAGGTTGCGTGTGCTTGC

Rev – 5'GGATCCTACCAGCCGCGATGG

CoxTb6080 Fw – 5' CTCGAGCATCTAGTATGGCTG

Rev – 5' GGATCCATATGGGCATACCAT

Cell growth, transfection and RNAi induction

T. brucei PF cells of strain 29.13, transgenic for T7 RNA polymerase and the tetracycline (tet) repressor, were grown in vitro at 27°C in SDM-79 medium containing hemin (7.5 mg/ml) and 10% fetal bovine serum. The RNAi plasmids were linearized with NotI and transfected into the cell line as described previously [33]. The synthesis of ds RNA was induced by the addition of 1 μg/ml tet to the medium. The cells were counted using the Z2 cell counter (Beckman Coulter Inc.), and growth curves were generated for clonal cell lines over a period of 7 days.

Norther blot analysis

Total RNA was isolated from $1x10^8$ exponentially growing uninduced and RNAi-induced cells by extraction with TriReagent (MRC) according to instructions provided by the manufacturer. The RNAi-fragments were labeled using DecaLabel DNA labeling kit (Fermentas) with [α-^{32}P] dATP and used as a probe. Hybridization was carried out using standard procedures. Signal was visualized using Molecular Dynamics PhosphoImager screens and autoradiograms were analyzed by densitometry.

SDS-PAGE and Western blot analysis

The cleared whole or mt lysates fractionated by sodium dodecyl sulfate (SDS-) polyacrylamide gele electrophoresis (PAGE) were blotted onto a polyvinylidene difluoride

membrane (PVDF) and probed with polyclonal rabbit antibodies against *Leishmania tarentolae* cyt *c* oxidase subunit IV (trCOIV) [20], *T. brucei* cyt *c* oxidase subunit VI (COVI) [12], and mitochondrial RNA binding protein 1 (MRP1) [34], all used at 1: 500 dilution, and proteins were visualized using ECL system (Roche).

Cyt c oxidase assays

The mt vesicles from $5x10^8$ cells were isolated by hypotonic cell lysis as described previously [12] and stored as pellets at -70°C. Cyt *c* oxidase activity was measured in dodecyl-maltoside lysed mitochondria. In parallel, the same activity was detected by an in-gel assay following the electrophoresis of mt lysate (100 μg of protein per lane) on a 3-12% blue native (BN) PAGE gel. Furthermore, 100 μg of mt lysate was fractionated on 6% BN PAGE followed by resolution in a 10% Tricine-SDS-PAGE gels. After electrophoresis, the gel was stained with Coomassie Brilliant Blue to visualize mt respiratory complexes as described elsewhere [35].

Measurement of oxygen consumption and membrane potential

Logarithmically growing cells were harvested, washed and resuspended in 1 ml of SDM-79 medium at a concentration of $2x10^7$ cells ml^{-1}. Oxygen consumption at 27°C was determined with a Clark-type polarographic electrode (1302 Microcathode Oxygen Electrode, Strathkelvin Instruments). Potassium cyanide (KCN) and salicylhydroxamic acid (SHAM) were added in 2 min intervals to final concentrations of 0.1 mM and 0.03 mM, respectively. A 1 ml portion of mid-log phase cells was incubated in the presence of 125 nM tetramethylrhodamine ethyl ester (TMRE) (Molecular Probes) for 30 min at 27°C, harvested and washed with isotonic solution suitable for flow cytometry and analyzed for red fluorescence by a flow cytometer (BD FACSCanto II). Twenty thousand events were measured in each experiment. The data were analysed using WinMDI software.

ATP production assay

ATP production was measured as described previously [36]. Briefly, a crude mt preparation from the RNAi knockdown cell lines were obtained by digitonin extraction. ATP production was induced by 5 mM indicated substrates (succinate, pyruvate, and α-ketoglutarate), and 67 μM ADP was added. Inhibitors were preincubated with mitochondria

on ice for 10 min and used at the following concentrations: 6.7 mM malonate and 33 μg/ml atractyloside. The concentration of ATP was determined by a luminometer using the ATP Bioluminescence Assay Kit HS II (Roche).

Acknowledgements

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Figure legends

Fig. 1. Subunits coxTb1410, coxTb4702 and coxTb6080 are important for the *in vitro* growth of procyclic *T. brucei*.

(A) Growth curves of the uninduced and RNAi-induced coxTb1410 (left), coxTb4702 (middle) and coxTb6080 (right) cell lines. Cells were maintained in the exponential growth phase (between 10^6 – 10^7 cells/ml) and cumulative cell number represents the normalization of cell density by multiplication with the dilution factor.

(B) Northern blot analysis of the corresponding mRNA for coxTb1410, coxTb4702 and coxTb6080 in the parental 29-13 cells, uninduced cells (N) and cells 2 days upon induction of RNAi (I2).

(C) The steady state level of the trCOIV subunit was followed by Western blot analysis of whole cell lysates (20 μg of proteins per well) prepared from RNAi-induced cells after 3, 5 and 7 days and from the uninduced cells. The blots were probed with the α-trCOIV

polyclonal antibody [20]. The specific band recognizing the trCOVI protein is designated by an arrow. The lower unspecific band designated by an asterisk serves as an internal loading control.

Fig. 2. Activity and structural integrity of the cyt *c* oxidase complex is affected upon depletion of coxTb1410, coxTb4702 and coxTb6080.

(**A**) In-gel *T. brucei* cyt *c* oxidase activity after the individual ablation of subunits coxTb1410 (3 days upon RNAi), coxTb4702 and coxTb6080 (both 5 days upon RNAi). Mitochondrial preparations were solubilized using dodecyl maltoside and separated by 3-12% BN PAGE. Arrow points to bands visualized by the specific activity of cyt *c* oxidase.

(B) Two-dimensional (2D) gel analysis of the dodecyl maltosed-solubilized mitochondria of uninduced (N) and RNAi-induced (day 3 [I3] or 5 [I5]) coxTb1410, coxTb4702 and coxTb6080 cells. The 1st and the 2nd dimension was performed in 6% BN gel and 10% Tricine-SDS PAGE gel, respectively, and the gel was stained with Coomassie Brilliant blue. Position of the cyt *c* oxidase complex is indicated in the gel.

(**C**) Functional assay for cyt *c* oxidase and cyt *c* reductase activities. All activities were measured in mitochondrial lysates prepared from at least three independent induction experiments. Activities in the RNAi-induced cells related to the uninduced cells are expressed in percentages. coxTb1410 RNAi cells were induced for 5 days, while Tbcox4702 and Tbcox6080 were induced for 7 days.

(**D**) Effect of coxTb1410, coxTb4702 and coxTb6080 RNAi on the steady-state abundance of the cyt *c* oxidase subunits trCOIV and COVI. The steady-state abundance of the examined proteins was analyzed by Western blotting in mitochondrial extracts obtained from the uninduced cells (N) and those 3 (I3) or for 5 (I5) days of RNAi. Each lane was loaded with 20 μg of protein and blots were immunodecorated with polyclonal antibody against trCOIV and COVI. Mitochondrial RNA binding protein (MRP1) served as a loading control.

Fig. 3. ATP production by oxidative phosphorylation is severely affected in mitochondria depleted for coxTb1410, coxTb4702 and coxTb6080.

The generation of ATP was measured in digitonin-extracted mitochondria. The ATP-producing pathway was triggered by the addition of ADP and succinate. ATP production in mitochondria isolated from the uninduced cells and tested with succinate but without the

addition of malonate and atractyloside was set to 100%. All other values in each panel are means expressed as percentages of the sample. Malonate was used to inhibit ATP production and atractyloside was added to inhibit the import of ADP into the organelle. "No ADP" serves as a control for a background production of ATP from the endogenous source of ADP.

Fig. 4. Mitochondrial membrane potential is decreased following the depletion of coxTb1410, coxTb4702 and coxTb6080.

(**A**) Mitochondrial membrane potential was measured in the uninduced cells (black line) and RNAi-induced cells (grey line) by flow cytometry after staining with TMRE. The graphs are shown for coxTb1410 at day 3 and for coxTb4702 and coxTb6080 at day 5 after RNAi induction. The fluorescence distribution was plotted as a frequency histogram.

(**B**) Mitochondrial membrane potential measured in the uninduced cells was set to 100%. Values obtained from RNAi-induced cells are expressed as percentages of the uninduced sample and were obtained from at least three independent RNAi experiments.

Fig. 5. Shift from cytochrome-mediated respiration to alternative oxidase respiration follows the repression of coxTb1410, coxTb4702 and coxTb6080.

Uninduced and RNAi-induced cells were incubated in SDM-79 medium at 27°C and oxygen consumption was monitored with an oxygen electrode. Respiration of the uninduced and RNAi-induced cells in the absence of KCN was set at 100%. In each sample, oxygen consumption in the presence of KCN (0.1 mM) was subtracted from oxygen consumption of the same untreated cells and the difference was taken as a reference value for percentage calculations. The means and SD values of four experiments are shown. In this experiment the coxTb1410 cell line was induced for 5 days, while coxTb4702 and coxTb6080 cell lines were induced for 7 days.

Figure 1

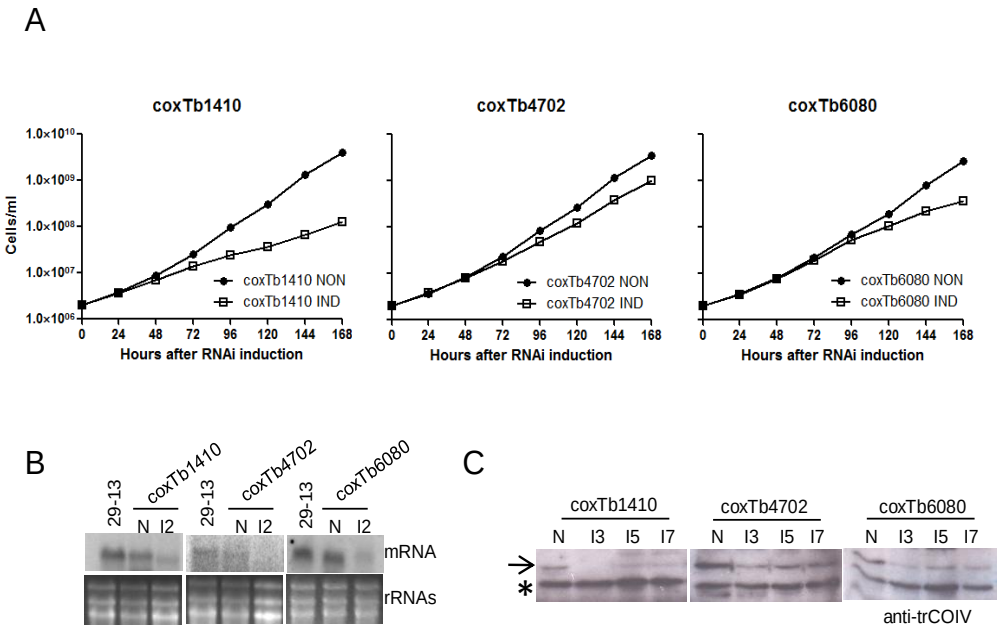
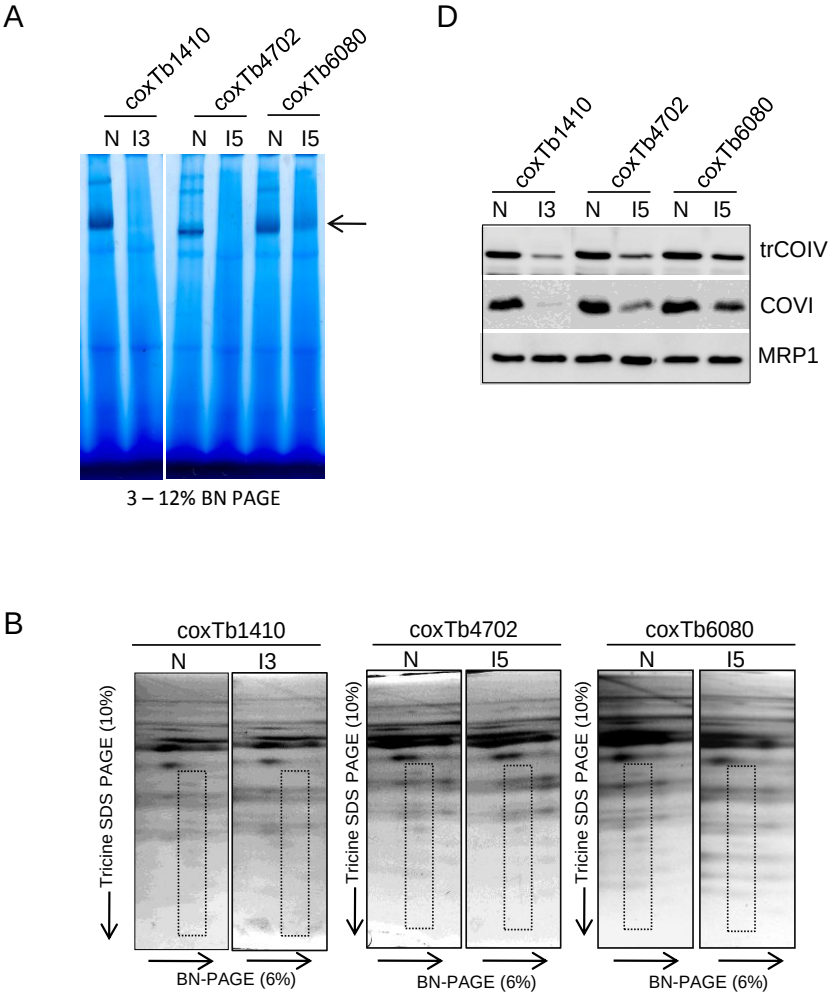

A
coxTb1410
coxTb4702
coxTb6080
Cells/ml
1.0×10¹⁰
1.0×10⁰⁹
1.0×10⁰⁸
1.0×10⁰⁷
1.0×10⁰⁶
0 24 48 72 96 120 144 168
Hours after RNAi induction
coxTb1410 NON
coxTb1410 IND
coxTb4702 NON
coxTb4702 IND
coxTb6080 NON
coxTb6080 IND
B
29-13
coxTb1410
coxTb4702
coxTb6080
N I2
mRNA
rRNAs
C
coxTb1410
coxTb4702
coxTb6080
N I3 I5 I7
anti-trCOIV

Figure 2



C

RNAi	Activity of cyt c oxidase	Activity of cyt c reductase
coxTb1410	32.95 ±5.15	131±7.2
coxTb4702	46.8±12.5	31.1±3.3
coxTb6080	59.55±4.05	53.6±3.7

Verner, Z. 2011. Mitochondrial energy metabolism in *Trypanosoma brucei:* Complex I and the others.

Figure 3

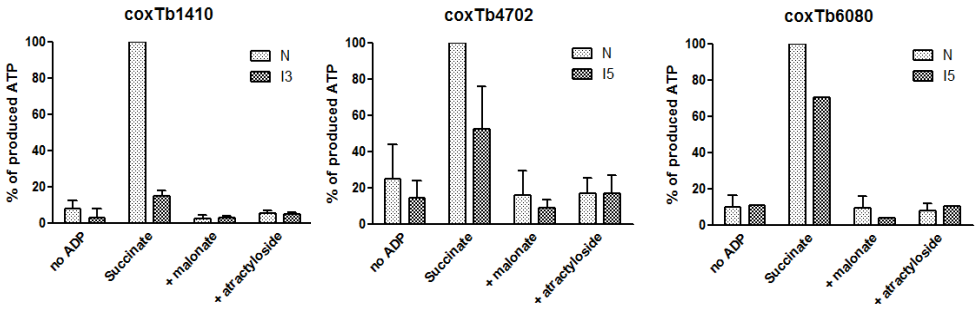
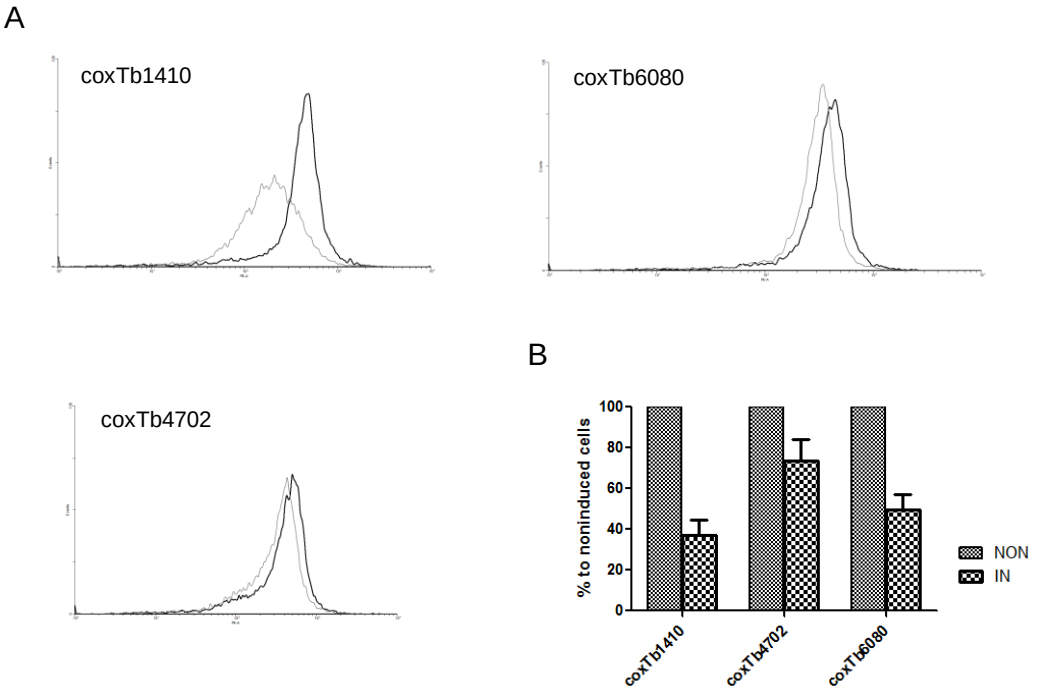
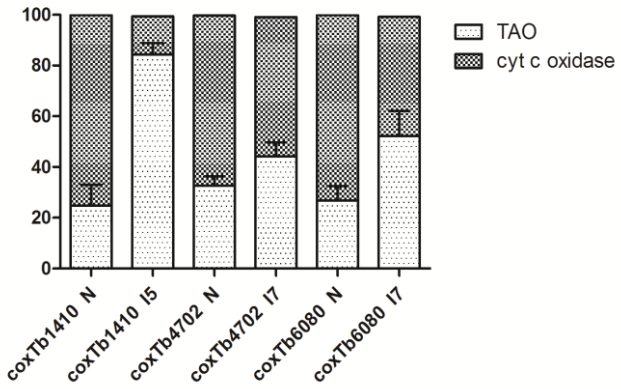


Figure 4


A
coxTb1410
coxTb6080
coxTb4702
B
% to noninduced cells
100
80
60
40
20
0
NON
IN
coxTb1410
coxTb4702
coxTb6080

Verner, Z. 2011. Mitochondrial energy metabolism in *Trypanosoma brucei:* Complex I and the others.

Figure 5



10.8 Other publications

Apart from enclosed papers, I have also been given a co-authorship on two other papers that are not connected to an energy metabolism of *T. brucei* and hence they are not included here.

The first one is an article addressing the way evolution is probably taking when an organism acquire new gene: Szabová, J.; Růžička, P.; Verner, Z.; Hampl, V.; Lukeš, J.: Experimental examination of EFL and MATX eukaryotic horizontal gene transfers: co-existence of mutually exclusive transcripts predates functional rescue. Mol. Biol. Evol. 2011. (epub ahead of print). My contribution to this article lies in teaching the basic molecular biology methodology and electroporating two control cell lines.

The second one is an article about FeS cluster assembly pathway: Long, S.; Changmai, P.; Tsaousis, A.D.; Skalický, T.; Verner, Z.; Wen, Y.-Z.; Roger, A.J.; Lukeš, J.: Stage-specific requirement for Isa1 and Isa2 proteins in the mitochondrion of Trypanosoma brucei and heterologous rescue by human and Blastocystis orthologues. Mol. Microb. (resubmitted) My contribution to this article was performing enzymatic measurements of the activities of complexes II and III and prepration of growth curves pictures for publication.