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University of South Bohemia in České Budějovice
Faculty of Science

Functional analysis of newly described 45S SSU* complex in *Trypanosoma brucei*

Ph.D. Thesis

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Annotation

This thesis addresses several hypothetical roles of a newly described and very unusual 45S SSU* complex in procyclics *Trypanosoma brucei*. RNAi interference of several 45S SSU* subunits revealed essentiality of this complex for its mitochondrial translation machinery. The effect of ablation of selected subunits on mitochondrial-encoded mRNAs is complex and not fully understood.

Declaration

Prohlašuji, že svoji disertační práci jsem vypracovala samostatně pouze s použitím pramenů a literatury uvedených v seznamu citované literatury.

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Lucie Ridlon

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List of papers and author's contribution

The thesis is based on the following papers:

1. **Lucie Ridlon**, Ingrid Škodová, Songgin Pan, Julius Lukeš, Dmitri A Maslov (2013) The importance of the 45 S ribosomal small subunit-related complex for mitochondrial translation in *Trypanosoma brucei*. J Biol Chem. **288:** 32963-78
(published in The Journal of Biological Chemistry)

Lucie Ridlon prepared almost all RNAi constructs, did electroporations, growth curve analyses, Northern blot analysis, translation assay, sedimentation profile of 9S and 12S ribosomal RNA, in total about 70% of the data. Moreover, she analyzed the data and participated in writing the manuscript.

2. Zdeněk Verner, Somsuvro Basu, Corinna Benz, Sameer Dixit, Eva Dobáková, Drahomíra Faktorová, Hassan Hashimi, Eva Horáková, Zhenqiu Huang, Zdeněk Paris, Priscila Peña-Diaz, **Lucie Ridlon**, Jiří Týč, David Wildridge, Alena Zíková and Julius Lukeš (2014) The malleable mitochondrion of *Trypanosoma brucei*

(submitted in International Review of Cell and Molecular Biology)

Lucie Ridlon wrote the 'Translation and ribosomes' section, 5% contribution.

Table of Contents

1. Introduction

1.1. Trypanosomatids

Trypanosomatids is a very diverse group of parasitic protozoa which belong to the class Kinetoplastea. They are parasites of vertebrates, invertebrates and even plants. Many new trypanosomatid species have been described from insects, which suggest that they are the ancestral hosts of these parasites (Týč et al., 2013, Maslov 2013). The classical way to distinguish trypanosomatid species is ultra-structural characterization and their overall cell morphology (Hoare and Wallance, 1966). More recent studies show, however, that only molecular methods can be used for precise determination of individual species and mapping their evolutionary relationships (Simpson et al., 2006; Maslov et al., 2013).

Trypanosomatids are known for their characteristic features, many of which are unique for this order. However, some of the peculiar features originally found in trypanosomatids were later observed in other eukaryotes, albeit in modified versions. These biological phenomena include trans-splicing of nuclear RNAs, polycistronic transcription, and RNA editing (Schneider et al., 2008).

A common characteristic of all trypanosomatids is a single flagellum which is attached to the basal body and continues along the cell body (Vickerman 1976; Landfear and Ignatuschenko 2001). The flagellum facilitates the parasite's movement and it has also a role in host-parasite interaction and cell division (Ralston 2009). The other aspects are a single nucleus with 41 pairs of chromosomes (Weatherly 2009), an extremely varied cell surface and membrane transporters. However, it can be argued that the most attractive and distinct features and mechanisms are found in the single mitochondrion. This organelle is very well described and represents the best studied component of the trypanosomatid cell, because it is an essential for its energy production, synthesizes iron-sulfur clusters, and is essential for the cell in many other ways (Lukeš et al., 2005). While in the insect vector the mitochondrion is relatively big with a numerous cristae, in vertebrate blood it assumes as a simple tubular structure with reduced cristae. The excessive and highly complex mitochondrial DNA is called kinetoplast DNA, and its trascripts undergo mitochondrial RNA editing a very peculiar phenomenon which will be described in detail further in the text (Lukeš et al., 2002; Stuart and Panigrahi 2002).

Trypanosomatids are extensively studied not only because all members of this group are exclusively parasitic but also because of easy genetic manipulation and the aforementioned unique

molecular mechanisms. This thesis is focused only on members of the genera *Trypanosoma* and *Leishmania*, in particular a gecko parasite, *Leishmania tarentolae,* and an important pathogen of humans, *Trypanosoma brucei.*

1.1.1. *Leishmania* spp.

So far about 30 *Leishmania* species are known which cause a range of diseases collectively called leishmaniases. The parasite is transmitted by a phlebotomine (sand fly) vector. The infectious group includes *L. donovani, L. mexicana, L. tropica and L. major* species complexes. Most *Leishmania* species cannot be morphologically distinguished, but they can be identified by molecular methods, isoenzyme analysis or monoclonal antibodies. More than 350 million people are affected in 98 countries over the world except Australia and Antarctica.

Promastigotes living in the midgut of the sand fly vector can be transmitted to the mammal host when the fly feeds on host's blood. Non-dividing promastigotes phagocytozed by macrophages transform in aflagellar amastigotes, which multiply and infect various types of tissue, depending on a given *Leishmania* species. The cycle continues when a new fly feeds on this host and uptakes intracellular amastigotes that differentiate to promastigotes in the fly midgut (Fig.1.).

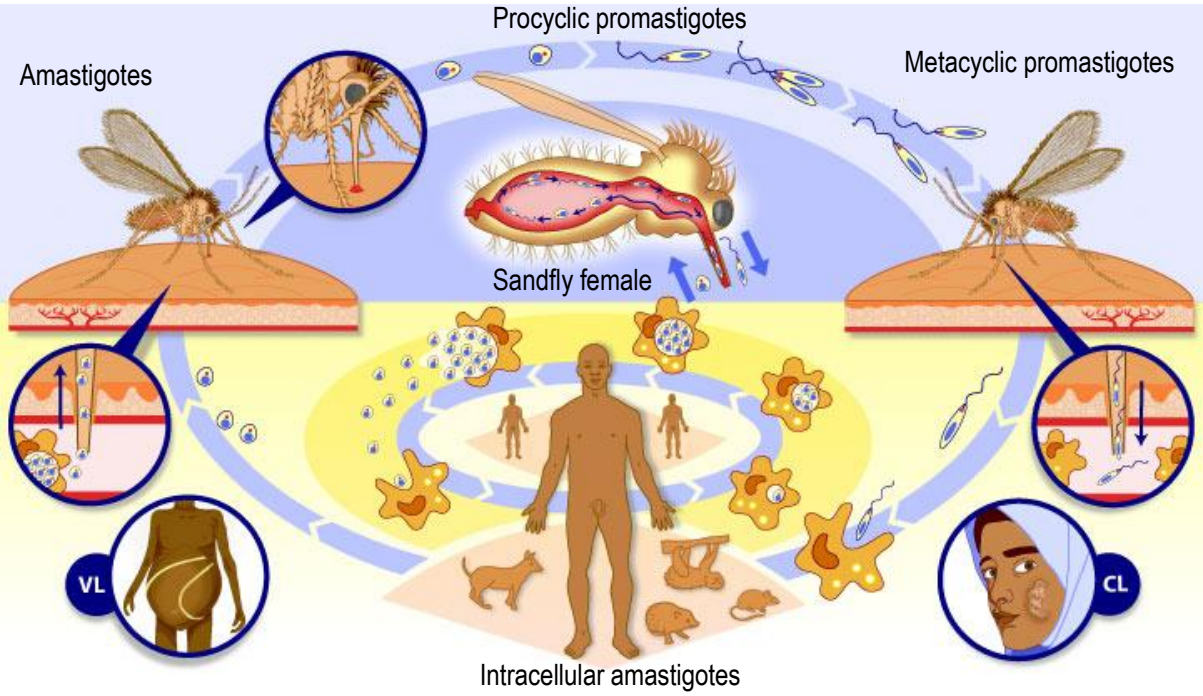

Figure 1: Life cycle of *Leishmania* in sand-fly vector and mammalian host. (Adapted from http://www.who.int/tdr/diseases/tryp/lifecycle.gif?ua=1).

1.1.2. *Trypanosoma brucei*

This trypanosome causes Human African trypanosomiasis (HAT) also known as 'sleeping sickness'. This disease affects 36 African`s countries and the risk increases especially in rural parts (Barret et al., 2003). Two different forms of the disease in humans are recognized: "West African sleeping sickness" and "East African sleeping sickness". The West African form covers 98% of all infections and is caused by *Trypanosoma brucei gambiense*. This type of HAT develops very slowly and usually without any major symptoms. Patients normally survive up to 3 years, sporadically even 6-7 years. However, once the disease evolves, it is already in a stage when the parasite already crossed the barrier to central nervous system, which leads to a permanent mental retardation and premature death. The second type of disease is less common but very rapid. It is caused by the subspecies *Trypanosoma brucei rhodesiense*. The symptoms can be observed within a few weeks or months after infection. The parasite also crosses the blood-brain barrier and invades the central nervous system. Both subspecies are morphologically indistinguishable and they are lethal if not treated. In the laboratory, the third subspecies, *T. brucei* is used, which is not pathogenic for humans but causes a deadly disease in cattle called nagana.

T. brucei has a very complex life cycle that includes four stages – a procyclic stage (in the mid-gut of the tsetse fly vector), which develops into a metacyclic form (in the tsetse fly salivary glands), and eventually into dividing long slender and non-dividing short stumpy bloodstream forms (in the mammalian host) (Fig. 2.). The procyclic (PF) and long slender bloodstream form (BF) can be cultivated in liquid media and are by far the most frequently studied life cycle stages.

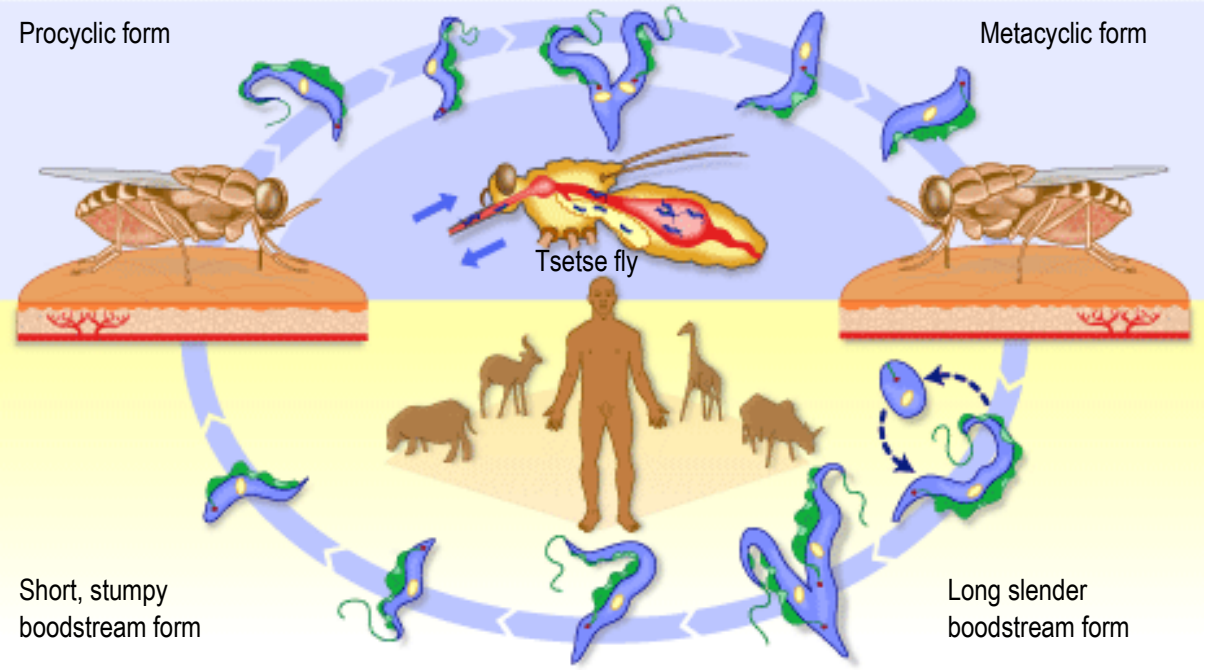

Figure 2: Life cycle of *Trypanosoma brucei* in tsetse fly vector and mammalian host (adapted from http://www.who.int/tdr/diseases/tryp/lifecycle.gif?ua=1).

1.2. Organization of kinetoplast DNA and processing of mitochondrial mRNA

This thesis is focused on the mitochondrial ribosomes and the process of mitochondrial translation in trypanosomatids. The goal of this study is to describe this so-far poorly known mechanism. For that, it is mandatory to describe what is known about the organization of the kinetoplast DNA (kDNA), as well as about the processing of mitochondrial mRNA known as RNA editing and polyadenylation/uridylation.

1.2.1. kDNA

kDNA is a dense network consisting of two types of circular DNA molecules – maxicircles and minicircles (Simpson, 1986; Chen et al., 1995; Lukeš et al., 2002) (Fig.3). Whereas identical maxicircles are present only in dozens of copies, minicircles that are heterogeneous in their sequence occur in thousands of copies. The approximately 22 kb-long maxicircles represent the equivalent to mitochondrial DNA of higher eukaryotes (Stuart and Panigrahi 2002, Lukeš at al., 2005). Maxicircles encode two ribosomal RNAs (rRNAs), 12S and 9S, as well as several proteins involved in the respiratory chain and one protein of the mitoribosome, namely subunits of NADH dehydrogenase (ND), cytochrome oxidase (Co), cytochrome b (CyB), and ribosomal protein subunit 12 (RPS12). In addition, they contain several genes of unknown function, such as MURF2 (maxicircle unidentified reading frame) and a handful of guide (g) RNAs (see below) (Fig.4). Maxicircle genes are transcribed polycistronically (Read et al., 1992) and require extensive processing to generate mature mRNAs, which includes RNA editing of some mRNAs, polyadenylation of mRNAs (Bhat et al., 1992), polyuridilation of gRNAs and rRNAs (Adler et al., 1991) and poly A/U extension of pre-existing poly-A tail on the mRNA's 3`end (Etheridge et al., 2008).

The size of minicircles varies between 0.5 and 10.0 kb depending on species. They encode 50 to 70 nucleotide-long gRNAs, which plays critical role in RNA editing (see below). One minicircle usually encodes 1 to 3 gRNA, which are probably transcribed polycistronically (Aphasizhev and Aphasizheva 2014).

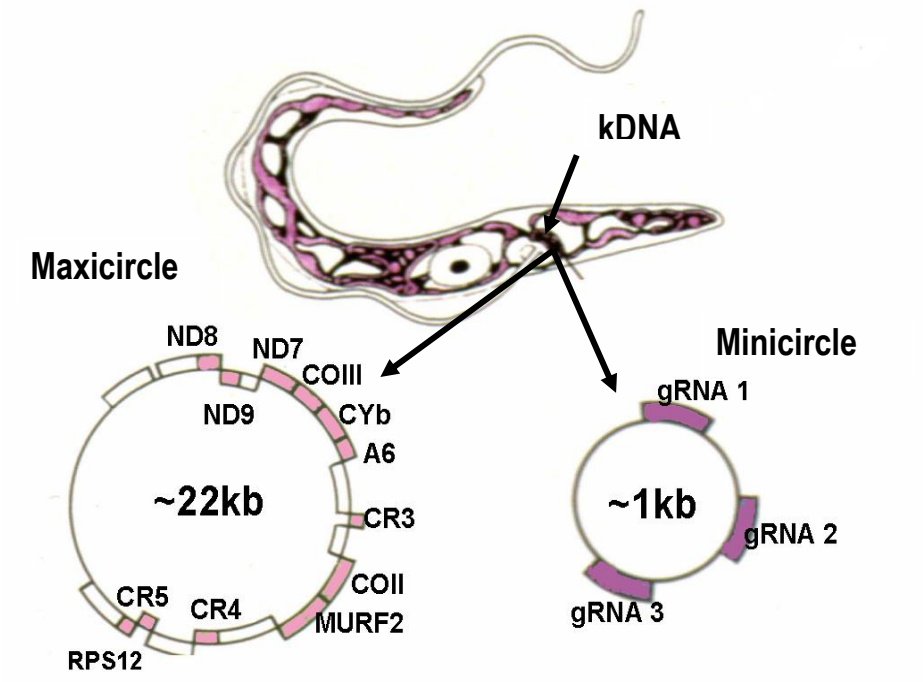

Figure 3: structure of mitochondrial kDNA.

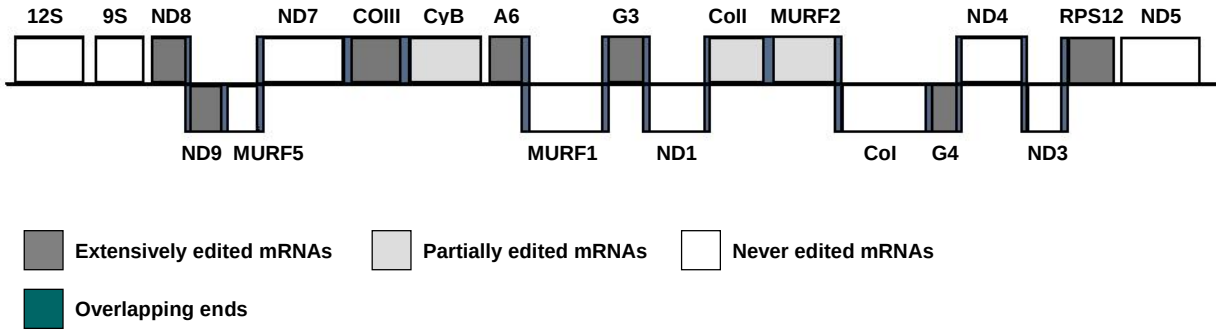

Figure 4: Maxicircle encoded genes. MURF1 = ND2 (Kannan and Burger, 2008).

1.2.2. RNA editing, polyadenylation and polyuridylation

The three processes listed in the chapter's title are essential for cells' viability and they are also critical for production of conventional mitochondrial proteins such as components of the mitochondrial respiratory chain and F_1F_0 ATP synthase (Fig. 4). RNA editing is a massive post-transcriptional maturation of mitochondrial mRNAs represented by deletions and/or insertions of uridine (U) residues that starts at 3'

end and continues to the 5' of mRNA (Maslov and Simpson 1992). This process is directed by gRNAs, which determine where and how many U will be inserted or deleted. The gRNA interacts with the pre-edited mRNA just downstream of the pre-edited regions and the following U insertion/deletion creates a new site that can be recognized by another gRNA (Fig. 5) (Aphasizhev and Aphasizheva, 2011). The whole mechanism requires multiple enzymatic activities, such as U-specific exonuclease, terminal uridylyl transferase and RNA ligase (Simpson et al., 1993, Benne 1994; Simpson et al., 2004; Stuart et al., 2005; Hashimi et al., 2013). RNA editing is a very complicated process that may create a start codon and correct an encoded frame shift, or can create an entire open reading frame (ORF).

Figure 5: Small gRNA molecules directing mitochondrial mRNA editing begin the editing by annealing to 3' end of the mRNA and continuing the editing in the 5' direction. This editing creates binding site for a subsequent gRNA which would in turn extend the editing further to the 5' end. Multiple cycles of editing are required for 'pan-edited' mRNAs such as ND8, RPS12 or A6. (Adapted from Aphasizhev and Aphasizheva, 2011).

Polyadenylation, in which a poly(A) tail is added to the 3´ end of the mRNA, is also a part of the mRNA maturation process in trypanosome mitochondria. Almost all eukaryotic mRNAs are polyadenylated. The poly(A) tail in nuclear mRNAs is important for nuclear transport and mRNA stabilization (Manley and Proudfoot, 1994). For example, human mitochondrial mRNAs with the poly(A) tail are relatively stable, whereas deadenylated mRNAs are not (Nagaike et al., 2005). *T. brucei* mitochondrial mRNAs contain two different poly(A) sizes (Apasizhev and Apasizheva, 2010, 2011). The short (20-25 nucleotides) poly(A) tail stabilizes edited mRNAs and protects them from degradation (Kao and Read, 2005) but the same tail can also destabilize pre-edited molecules (Aphasizhev and Aphasizheva, 2011). On the other hand, long poly(A/U) tail (200-300 nucleotides) is present on mRNA molecules that are intended for translation, such as never-edited (=those which do not require editing, e.g. COI) and fully edited mRNAs (Etheridge et al., 2008; Aphasizheva et al., 2011). mRNAs that are not extended by poly(A/U) tail are rapidly degraded (Ryan et al., 2003; Kao and Read, 2005) (Fig. 6). This 3' poly(A) tail is catalyzed by kinetoplast polyadenylation factors KPAF 1 and 2 and kinetoplast polyadenylation polymerase KPAP (Aphasizhev and Aphasizheva 2011). It was expected that also RET1 contributes to this

process, and in the experiment to synhesize a poly(A/U) tail *in vitro* by using recombinant KPAP, RET1 and KPAF 1 and 2, the poly(A/U) tail was created in the length usualy seen in fully and never-edited mRNAs (Aphasizhev and Aphasizheva 2014). The exact function of poly(A/U) tail is still unsolved, but because mRNAs with this long 3‘ overhang are preferentially found in association with the mitoribosomes (Aphasizheva et al., 2011; Ridlon et al., 2013), it is believed that this step of mRNA processing is necessary for identification of mature mRNAs and their recruitment to mitoribosomes.

Polyuridylation occures also on the 3` end of the maxicircle-endoded rRNAs and gRNAs, and the minicircle-encoded gRNAs (Adler et al., 1991). This process is performed by terminal uridylyl trasferase called RNA editing TUTase 1 (RET1) (Aphasizhev at el., 2002; Aphasizhev et al., 2010). rRNA and gRNA molecules receive 15 nt oligo(U) tail (Aphaszheva and Aphasizhev 2010). It was also believed that the U-tails stabilize both of these molecules, however experimental studies show that a loss of these tails has no significat impact on gRNA or rRNA stability (Aphasizhev and Aphasizheva 2010).

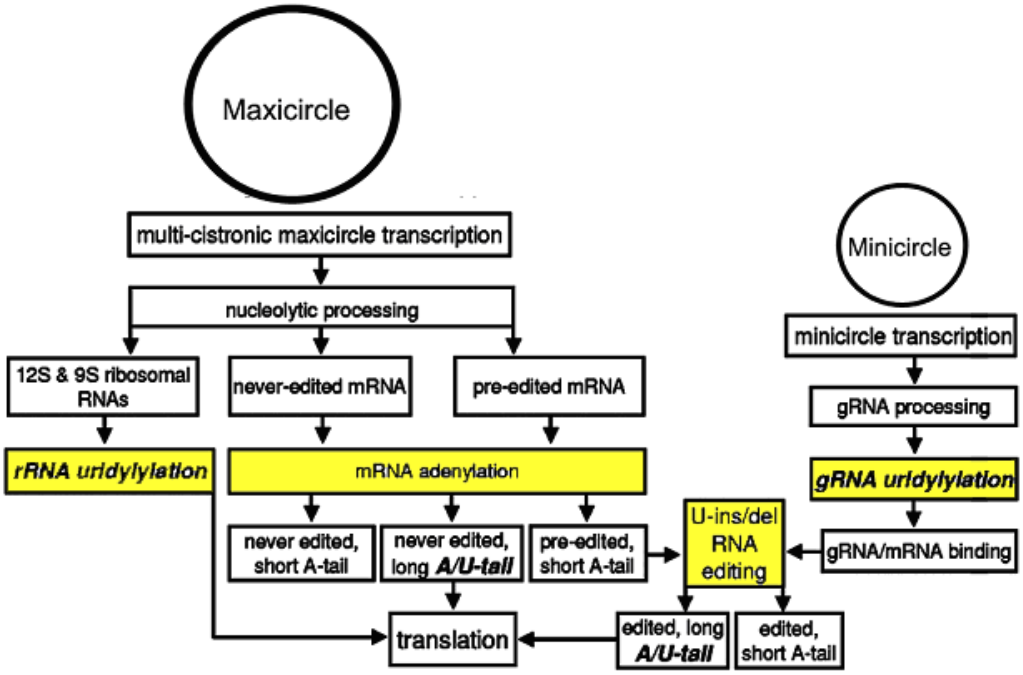

Figure 6: Mitochondrial RNA processing (Adapted for Aphasizhev and Aphasizheva, 2010).

1.3. Mitochondrial ribosome

Ribosomes of every living cell translate mature mRNAs to proteins. A ribosome is composed of two unequally sized subunits: a small ribosomal subunit (SSU) and a large ribosomal subunit (LSU). Each particle is made up of highly conserved ribosomal RNAs (rRNA) and numerous proteins, usually with the ratio of 65% rRNA and 35% protein. Translation of mitochondrial mRNAs was first observed in 1958 in human mitochondria (Mc Lean et al., 1958). This discovery drew a lot of attention to the mitochondrial ribosome, its composition and function. Mitochondrial ribosomes originated by acquisition of the archeal cell by aerobic bacterium or a pre-eukaryotic cell through a process called endosymbiosis (Sagan 1967; Gray et al., 2001; 2004). Therefore, mitoribosomes are expected to share many features in structure and composition with bacterial ribosomes. Numerous early studies pointed at similarities among mitochondrial ribosomes from various organisms and at a huge divergence between mitochondrial and cytoplasmic ribosomes (O`Brien 2002; O`Brien and Denslow, 1996; O`Brien and Kalf, 1967).

1.3.1. Bacterial ribosome

A procaryotic ribosome is 70S in size and its subunits are 30S (SSU) and 50S (LSU). The small 30S subunit contains 21 proteins and the 1.500 nt-long 16S rRNA, the structure of which is formed by shoulder, platform, base and head (Sharma et al., 2013). These functional domains of the SSU are defined by the folded 16S rRNA rather than proteins. Among the most important structures are the three tRNA binding sites: A - aminoacyl, P - peptidyl and E - exit sites. SSU is also responsible for keeping the mRNA in the correct open reading frame. The main functional structures of large 50S subunit are L1 protuberance, the central protuberance and the base stalk. The bacterial LSU contains two rRNAs types: 23S (2.900 nt) and 5S (120 nt) (Schmeig and Ramakrishnan, 2009). The 23S rRNA forms six structural domains and its essential function is the catalysis of the peptide bond formation, which occurs in the peptidyl transferase center in domain V. The second 5S rRNA is much smaller, and it looks like a seventh structural domain of the LSU. This part is predicted to have a function in maintaining structural stability of the LSU, and it is mainly found in association with the LSU domains II and V. Proteins from both ribosomal subunits are mostly situated on the ribosomal surface and/or filling gaps between rRNA structures. This means that proteins associated with ribosomes play roles in structural formation or stability of the ribosome rather than contribute to the translation process (Mears et al., 2006).

1.3.2. Trypanosomatid mitochondrial ribosome

As mentioned above, because of the endosymbiotic event, mitochondrial ribosome is derived from its bacterial ancestor. The same applies to the trypanosomatid mitoribosome, although its sizes differ substantially from those seen in prokaryotes. In *T. brucei* and *L. tarentolae* the mitoribosome is only 50S and is composed of the 30S small subunit (SSU) and 40S large subunit (LSU) (Maslov, 2006).

The SSU particle contains 9S rRNA (610 nt), while the LSU contains 12S rRNA (1.173 nt) both are encoded by the maxicircle kDNA (Eperon et al., 1983, de la Cruz et al., 1985). Interestingly, both rRNAs belong to the smallest known rRNA molecules, and are even smaller than 12S SSU and 16S LSU rRNAs of the mammalian mitochondria. By comparing the 9S rRNA of *T. brucei* with its bacterial 16S rRNA homolog, Eperon and colleagues showed some small similarities in the studied rRNA molecules but also noted a lof of gaps in the 9S rRNA sequence compared to the 16S which explains its relatively small size Eperon et al., 1983). One example of a highly diverged region is the 3` end which is highly conserved in prokaryotic 16S rRNA but with no sequence similarity remaining in *T. brucei* or *L. tarentolae* (Eperon et al., 1983; de la Cruz et al., 1985). However, there are three small regions of the 9S rRNA with detectable homology to the bacterial 16S, and the mitochondrial mammalian 12S and yeast 15S SSU rRNA. The first region is the binding site for S4 ribosomal protein, and this part is very well conserved in bacteria (Noller and Woese, 1981) and it is also found in mammalian (Anderson et al, 1982) and yeast mitochondrial ribosomes, as well as in cytoplasmic ribosomes (Salim and Maden, 1981). This sequence in trypanosomes does not perfectly match the *E. coli* sequence but the secondary structure of trypanosome S4 also has a few differences compared to its bacterial S4 orthologue (Nomura et al., 1980). The second homology regions is a 8 nt-long sequence that is relativelly poorly conserved as compared to the bacterial counterpart but is highly similar to the respective segment of the yeast 15S mitochondrial SSU rRNA. The last and longest matching region is well conserved in human and bovine rRNAs and its 8 nt-long motif segment is also similar to other mammals (Eperon et al., 1983). Comparison of the 9S and 16S secondary structures was published also for *L. tarentolae* (de la Cruz et al., 1985) (Fig.7). It was conluded that although the 9S is highly unusual in its size and the primary structure shares only a few similarity regions, the secondary folding is very close to the structure observed in the bacterial 16S rRNA. Moreover, it was also found that the aligned 9S sequences of *L. tarentolae* and *T. brucei* exhibit 84% similarity that is higher than in some other closely related organisms (de la Cruz et al., 1985).

An alignment of the *T. brucei* 12S rRNA with *E. coli* 23S LSU rRNA revealed some highly conserved regions, but a number of domains and stem-loops typical for other LSU rRNAs were reduced (Eperon et al., 1983). Several highly conserved regions of LSU rRNAs are preserved in mammalian and yeast mitochondrial ribosomes and even in the chloroplast ribosomes (Glotz et al., 1981, Branlant et al., 1981; Bear and Dubin, 1981; Edwards and Kossel, 1981). The first of them is highly conserved in mitochondrial rRNAs of mammals (Eperon et al., 1983; Bear and Dubin, 1981), yeast (Dujon, 1980), or chloroplast (Allet & Rochaix, 1979) and also in the cytoplasmic rRNAs (Nomiyma et al., 1981: Veldam et al., 1981). The high preservation level of this structure in such a wide range of organisms clearly points at the functional importance of this domain, which has a role in the formation of the ribosomal A site. Another important region, composed of two decamers separated by a stem-loop is implicated in chloramphenicol sensitivity. Both decamers are conserved in most of mitoribosomes, as well as ribosomes residing in chloroplasts and bacteria. Point mutations in this reagion lead to chloramphenicol resistance (Dujon, 1980; Kearsey and Craig, 1980; Bkan et al., 1981; Blanc et al., 1981). While rRNA in yeast lost only a fraction of this sequence, the 12S of *T. brucei* lacks this entire region and its mitochondrial translation became consequently resistant to chloramphenicol (Eperon et al., 1982, Veldman et al., 1981). The *E. coli* 23S rRNA contains binding sites for proteins L5, L8, L25 and also for the 5S rRNA (Branlant et al., 1976). This domain is regarded as the one with the most drastic size reduction in evolution - in mammals it has lost a part of the 5S binding, which dwindled to 31 nt (Glotz et al., 1981; Branlant et al., 1981), while a complete loss of this region has occurred in trypanosomes. To the contrary, the α-sarcin region that is involved in the formation of the A-site is well conserved (Eperon et al., 1983; Veldman et al., 1981).

In 1985 de la Cruz et al. published a comparison of the six bacterial LSU domains with the *L. tarentolae* and other mitochondrial LSU rRNAs (Fig. 7). Even though the sequence similary between the leishmania 12S and bacterial 23S rRNAs is low in domain I, its secondary structure remains retained. In contrast, only 17% of the sequence corresponding to domain II of bacterial LSU was retained in this flagellate, and it is regarded as one of the largest deletions in the 12S rRNA. Domain III is extremely divergent between *T. brucei* and *L. tarentolae* 12S, and the question remains whether this part remains functionally essential for the trypanosomatid mitoribosome. The next region, domain IV consists of two helices. It is well conserved in other rRNAs, but in trypanosomatids the sequence of the first helix is slightly different and only a small part of the second helix was identified. Domain V is considered as the most important functional region of LSU because it forms the peptidyl transferase center. The preservation of the

primary and secondary structure of this domain is confirmed in all rRNAs. Finally, the size of domain VI is reduced (in comparison to the bacterial structure) in mammalian mitoribosome and the loss of this structure is even more remarkable in *L. tarentolae*. The only conserved part of this domain is the loop that serves for the α-sarcin cleavage (Endo and Wool, 1982).

The reduction of the 12S and 9S rRNAs in kinetoplastids can be explained by two hypotheses. The first one is that the function of the missing parts are not crucial for mitoribosome of these early-branching eukaryotes and/or these functions can be substituted by proteins (Eperon et al., 1982; Cruz et al., 1985). The latter hypothesis is also supported by the finding that in spite of the small size of the 9S and 12S RNAs, the respective subunits have a relatively big mass, indicating that the trypanosomatid mitoribosome is protein rich (Maslov et al., 2006; Maslov et al., 2007; Zíková et al., 2008).

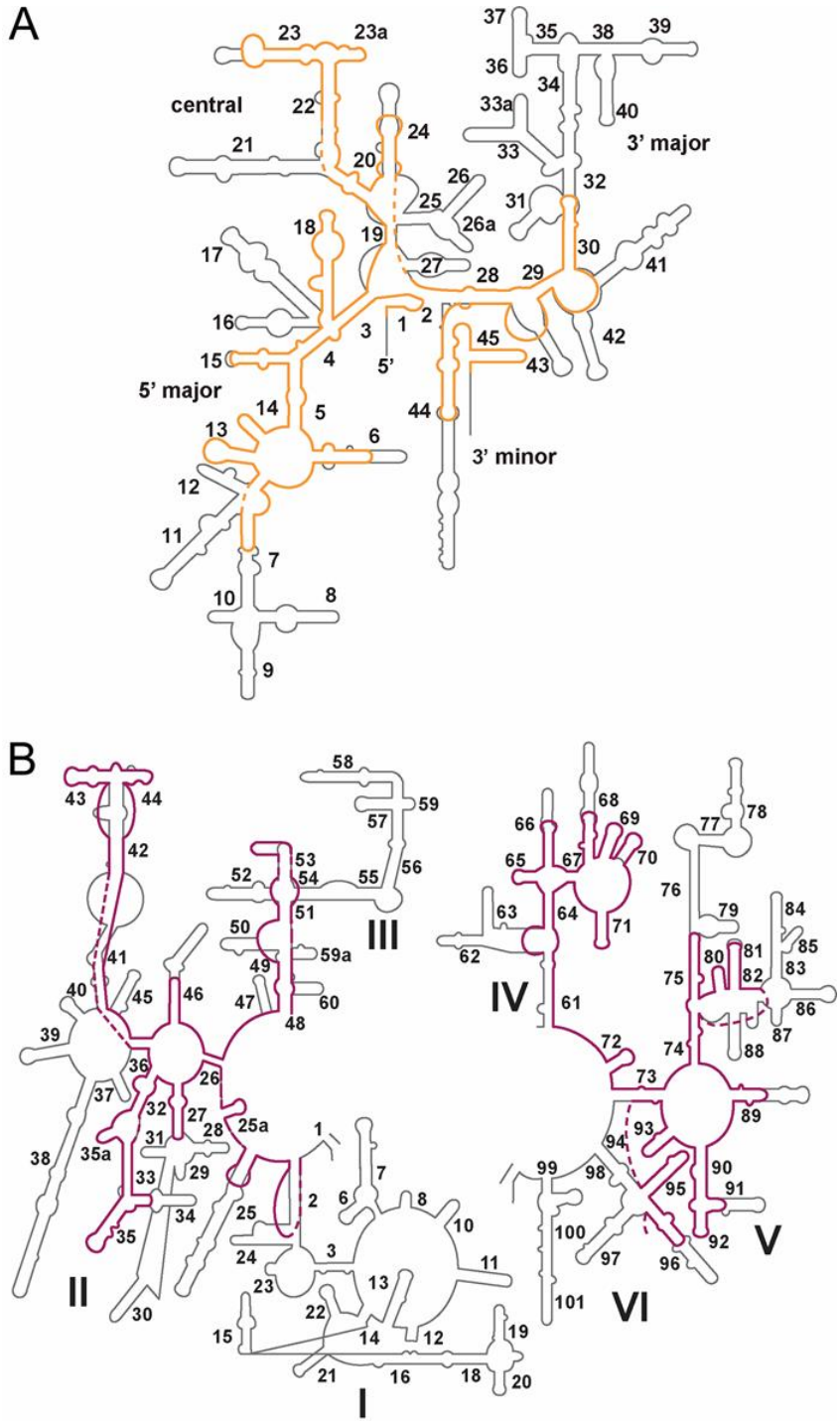

Figure 7: Secondary structures of *Leishmania* 9S SSU (A - orange) and 12S LSU (B - magenta) rRNAs in comparison to *E. coli* 16S SSU and 23S LSU (gray) rRNAs (adapted from Maslov and Agrawal, 2012).

Mitochondrial ribosomes in trypanosomatids were characterized by several studies showing the composition, organization and arrangement of the ribosomal subunits (Maslov et al., 2006; Sharma et al., 2003, 2009; Sheinman et al., 1993). The mitoribosomes appear in six different ribonucleoprotein complexes.

The simplest complexes are the 40S and 30S representing the LSU and SSU, respectively. A dimer of LSU is detectable in sucrose gradient as a 65S particle. The "real" mitoribosome, also called monosome, is represented by a 50S particle and contains the LSU and SSU rRNA and mitoribosomal proteins. This complex is also detected as a dimer sedimenting at 80S. Moreover, a very unusual 45S particle is also found containing only the 9S SSU rRNA, SSU protein homologues and other proteins which are not typical for ribosome. The last complex is the dimer of ther 45S complex - a 70S particle. The 45S complex from *L. tarentolae* was isolated and characterized by electron microscopy and mass spectrometry and termed 45S SSU* (Maslov et al., 2007). A few years later the 50S monosome was also isolated and studied by cryo-electron microscopy (Sharma et al., 2009). The results of the study confirmed the uniqueness of this mitoribosome, yet also identified numerous typical features seen in eubacterial ribosomes. For example, the size and morphology (head, body and platform for SSU or central proturbulance in LSU) are quite similar to other eubacterial counterparts, but positions of several functionally relevant regions are different. In any case, the trypanosome mitoribosome substantially differs from its bacterial ancestor – by the aforementioned chloramphenicol resistance, the reduced sizes of rRNAs due to the loss of functional domains, and by a higher number of ribosomal proteins. Electron microscopy analysis of *L. tarentolae* 50S monosomes pointed at additional differences from the *E. coli* ribosome. It showed not only huge gaps, pores and several tunnels in the mitoribosome, but also the fact that the SSU and LSU subunits are positioned relatively far apart within the monosome. Only nine bridges were detected between the subunits in *L. tarentolae* compared to eleven bridges in *E. coli*. Moreover, the RNA-RNA bridges seen in bacteria were substituded by RNA-protein or protein-protein interactions (Sharma et al., 2003, 2009). Figure 8 shows cryo-electron microscopy structure of the *L. tarentolae* 50S mitoribosome (Maslov and Agrawal, 2013).

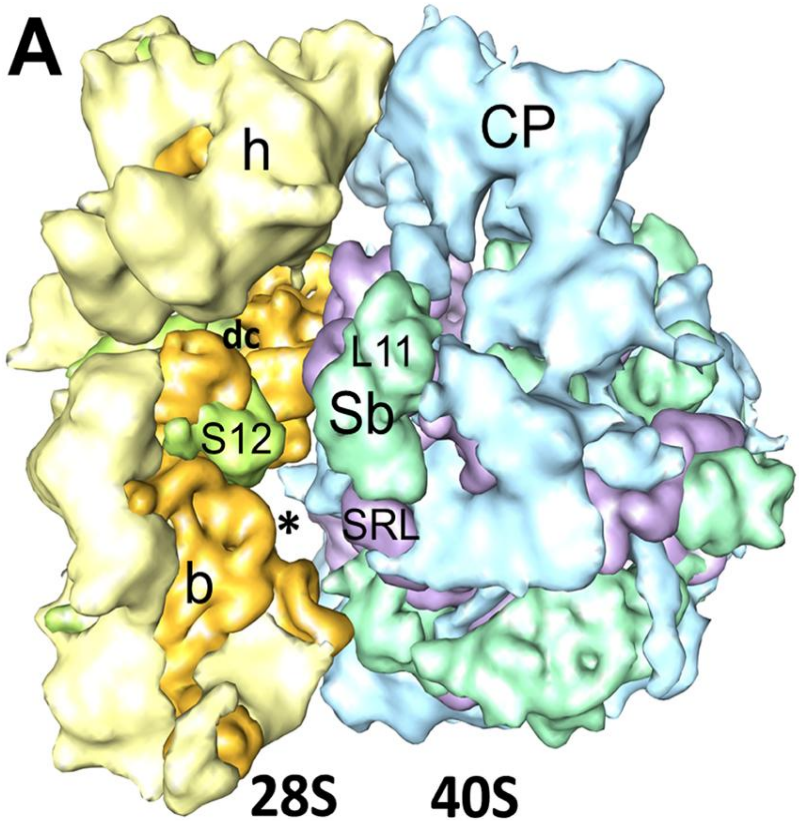

Figure 8: Cryo-EM structure of *L. tarentolae* 50S mitochondrial ribosome (From Maslov and Agrawal, 2013). Ribosomal proteins that are not universally conserved are colored yellow (SSU) and blue (LSU); conserved proteins are colored green (SSU) and aquamarine (LSU); and rRNAs are colored orange (SSU) and magenta (LSU). b, body; dc, mRNA decoding site; h, head; h44, SSU rRNA helix 44; pt, platform; S12, ribosomal protein S12 also called RPS12; CP, central protuberance; L1, protein L1 stalk; Sb, stalk base or L11 region; SRL, α-sarcin-ricin stem-loop.

1.3.3. Mitochondrial translation

A consequence of endosymbiosis was that most genes originally present in the plastid or mitochondrial genomes were subsequently transfered to the nuclear genome (Martin et al.,1998; Waller et al., 1998). In most organisms mitochondrial genome was reduced to 37 or less genes compare to its bacterial ancestor (Sharma et al., 2013). Those that remained are genes for subunits involved in oxidative phosphorylation (OXPHOS), rRNAs, few mitochondrial ribosomal proteins (MRPs) and tRNAs (the latter genes are absent from the trypanosomatid kDNA).

Trypanosomatid mitochondrial translation still remains to be characterized in detail. The mitoribosomes translate only 18 genes encoded by maxicircles kDNA, with most of them encoding subunits of the respiratory complexes with the function in the mitochondrial inner membrane. In addition to other maturation steps common to all transcripts, mRNAs for 12 of them undergo the above-described process of RNA editing (Arts et al., 1993; Benne et al., 1986; Feagin 1990). Due to the high hydrophobicity, the respective proteins are translated in the close vinicity of the mitochondrial inner membrane and they are likely cotranslationally incorporated into the membrane via the conserved Oxa1 pathway (Stuart 2002).

Transfer RNA molecules (tRNAs) play one of the most important functions in translation (Adams and Palmer, 2003). Their role is to bring amino acid (AA) to the growing polypeptide chain. Sometimes, several tRNAs involved in mitochondrial translation are encoded in the nuclear genome and they have to be imported into the organelle; this takes place in yeast (Martin et al., 1979), plants (Marechal-Drouard et al., 1988) and even mammals (Rubio et al., 2008). An extreme situation is observed in trypanosomes, which completely lack mitochondrially-encoded tRNAs and all tRNAs needed for mitochondrial translation have to be imported from the cytosol (Hanckock and Hajduk, 1990). The same set of tRNAs is therefore involved in the cytoplasmic and mitochondrial translation, necessitating certain editing and other maturation processing events required for acommodating these molecules for mitochondrial import and functioning inside the mitochondrion (Alfonzo 1999, (Alfonzo & Soll, 2009). The list of imported tRNAs includes the cytoplasmic methionyl-tRNA. Upon import, a fraction of these molecules gets formylated to form the initiation fMet-tRNA, while the non-formylated fraction serves for elongation.

The exact mechanism of translation initiation in trypanosomatid mitochondria is not yet completely understood. In bacterial systems initiation factors 1-3 (IF1-3) are involved in this process (Schmeing and Ramakrishnan, 2009). It was proposed that IF3 binds to the SSU and mRNA and that IF1, IF2 and initiator tRNAs create 30S initiation complex. IF2 also creates a complex with GTPase and this brings the SSU and LSU together to create 70S mitoribosome. However, some recent cryo-electron studies are inconsistent with this theory (Simonetti et al., 2008). A homologue of IF2, known to bring fMet-tRNA to the ribosome, was found in the trypanosomatids genomes. , while the other two IFs are lacking (Maslov and Agrawal, 2012), which points at the peculiarity of translation initiation in this system.

Usually the initiation of translation occurs at an AUG start codon. Some of the maxicircle genes contain non-canonical translation initiation codon or the start codon is created during the RNA editing process, as exemplified by ribosomal protein 12 (RPS12) that has a Auu start codon instead of AUG (Maslov 2010). In bacteria the recognition of start codon (translation initiation) is facilitated by a specific

sequence (5`-AGGAGGU-3`) called the Shine-Dalgarno sequence located a few nucleotides upstream of the AUG start codon. A "cap“ on the 5` end of the mRNA (Campbell et al., 2003) helps to recruit a capped mRNA to the ribosome. However, in mRNAs of *T. brucei*, the 5` end is only mono-phospohorylated and does not contain a cap. So, it seems that none of these mechanisms is applicable for trypanosomatid translation initiation, a situation that may be similar to yeast. In these organisms, mitochondrial mRNAs possess a long untranslated 5` region (UTR) that often contains out-of frame AUGs (Dunstan et al., 1997; Fox 1996; Green-Wilms et al., 1998). Even though the UTRs have received a lot of attention in yeast, the precise mechanism is still unknown. Recognition of the correct start codon is facilitated by several different protein called "translation activators“ (TAs). They are all nuclear-encoded (Cabral and Schalz, 1978) and it is speculated that they play more than only one role in processes needed for translation initiation (Poutre and Fox, 1987). Some studies show the specificity of a given TA to a certain mRNA (Krause et al., 2004). However, TAs are not evolutionary conserved, and we can therefore predict that if there are similar recognition factors for trypanosomatids, they will be trypanosome-specific (Maslov and Agrawal, 2012).

Elongation is next step in the translation process. It is a cyclic reaction when the mRNA sequence is translated to a peptide. In comparison to initiation, elongation is a relatively well-conserved process (Spremulli et al., 2004; Koc and Koc 2013), which also requires dedicated proteins called elongation factors (EF). These factors are responsible for the correct tRNA binding and the mRNA and tRNA translocation on the ribosome. tRNAs with proper amino acids are delivered to the growing polypeptide via elongation factors in complex with GTP (Agrawal et al. 2003; Schmeing and Ramakrishnan, 2009). Elongation turns to termination (end of the protein synthesis) as soon as the stop codon on mRNA is recognized. In trypanosomes, one of the stop codons is UGA which in the standard genetic code represents tryptophan (Alfonzo et al., 1999). The remaining stop codons are standard (UAG and UAA)

The last stage of mitochondrial translation is "recycling“ (Schmeing and Ramakrisnan, 2009). In this step the newly created protein is released from the ribosome and because of its highly hydrobhobic nature probably immediatelly gets incorporated into the inner membrane.

Numerous questions related to various aspects of the translational processes in trypanosomatids remain unaswered. A lot of work will be necessary to investigate and describe this machinery in detail. To this end, the functional analysis of a recently described 45S SSU* complex (defined bellow) will bring some new information, which would fill the gap in our knowledge of initiation and other aspects of mitochondrial translation in trypanosomatids.

1.3.4. 45S SSU* complex

Trypanosomatid mitochondrial translation machinery is unique due to the presence of the 45S SSU-related complex that forms a characteristic bilobed structure observable under electron microscopy (Maslov et al, 2007; Sharma et al., 2009). The main goal of my PhD project was to investigate the function of this unusual particle called the 45S SSU* complex. The lobes of this complex are created by 9S rRNA and at least 39 proteins (Maslov et al., 2007). One of the two lobes is identical with the functional mitoribosomal SSU. However, the second lobe might be more interesting because its approximate size is the same as the SSU but its shape and composition are different. Figure 9 illustrates the bilobed structure of the 45S complex. Red dashed lines indicate boundaries between the two lobes, with the right-side part being identified as the SSU. The left lobe represents the putative non-SSU moiety, which is expected to contain a score of unique proteins not usually found in mitoribosomes. Some of these unusual SSU* proteins have well characterized motives such as the coiled coil, rhodanese, pentatricopeptide repeat (PPR) or tetratricopeptide repeat (TPR) domain.

PPR and TPR domains are mainly known from plants but are also numerous in trypanosomatids (Pusnik et al., 2007; Pusnik and Schneider, 2012). The PPR proteins are characterized by 35 amino acid-long domain repeated 2 to 26 times in tandem (Small and Peeters, 2000). This family of proteins is phylogeneticaly extremely divergent. While hundreds of them are found in plants (Delannoy et al., 2007), only few were detected in yeasts and mammals (Small and Peeters, 2000). The PPR motifs can specificaly bind to any type of RNA molecules, at property which defines their function in RNA processing including RNA splicing and editing. Unexpectedly, in *T. brucei* there are at least 28 PPR and 6 TPR proteins (Aphasizhev & Aphasizheva, 2011a; Aphasizhev & Aphasizheva, 2011b) (Zíková et al., 2008, Pusnik et al., 2007), but the function of most of them has not been specified yet. Most were found in association with mitoribosomes, RNA editing complexes or the polyadenylation machinery (Aphasizheva et al., 2011). It is frequently hypothesized that the PPRs, because of their specificity to various RNAs, play a role in recognition of mature mRNAs intended for translation or are involved in other aspects of translation.

The function of the non-SSU lobe of 45S SSU* complex was not characterized yet but there are two working hypotheses. Both are based on the fact that it is connected with the SSU, so it can be also somehow involved in the mitochondrial translation machinery. Following the first hypothesis, the non-SSU moiety can specifically recognize and bind mature mRNAs and recruit them to the inner membrane for

translation. The second hypothetical function is prevention of free SSU from uncontrolled reassociation with free LSU at the mitochondrial inner membrane. It is also possible that both scenarios are correct. However, more studies are needed to shed light on this question.

At least 30 out of 39 orthologs of the proteins found in the 45S SSU* complex originally described in *L. tarentolae* were also found in *T. brucei* (Zíková et al., 2008). We decided to undertake a functional analysis of this complex in *T. brucei* because this parasite has advantage of applying RNA interference to shut down gene activity, which would allow us to observe direct effects of silencing the functions of the 45S SSU* subunit on trypanosomes. For the RNA ablation were chose 3 subunits that are unique for the non-SSU part of 45S SSU*. Each of the selected protein has a well characterized domain. Tb.927.5200 ("200 kDa") contains a coiled coil motif. Coiled coil domain is formed by 2 to 7 alpha helices, which are involved in protein-protein interactions. Because of the large size of this protein, we anticipated that it can play a role in structural stability of the complex. Tb927.4930 ("rhodanese“) protein is characterized by the rhodanese domain and is also expected to play a structural role. Thus, it was assumed that RNAi silencing of these components will destabilize the entire complex. Finally, Tb927.1790 is a protein with a PPR motif.

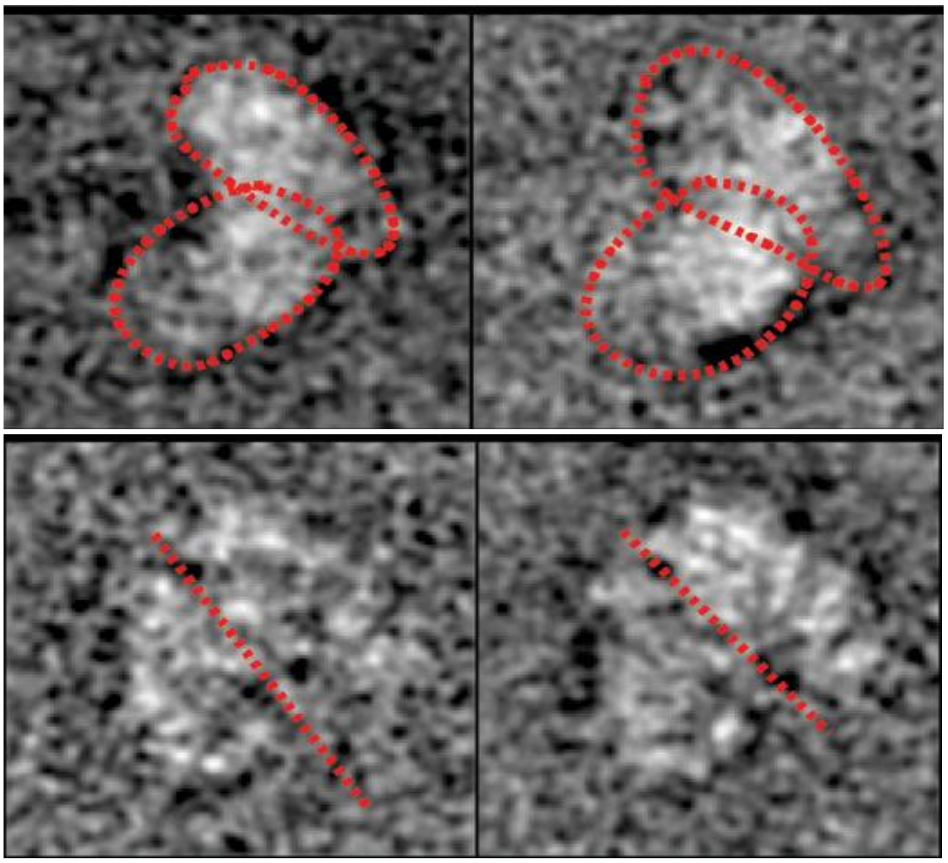

Figure 9: Cryo-EM of the 45S SSU* complexes (From Maslov et al., 2007)

2. Publications

2.1. The Importance of the 45 S Ribosomal Small Subunit-related Complex for Mitochondrial Translation in *Trypanosoma brucei*

2.2. The malleable mitochondrion of *Trypanosoma brucei*

This thesis contains only a chapter written by Lucie Ridlon, contributions of other authors to this extensive review are not included.

Running title: Mitochondrion of *T. brucei*

The malleable mitochondrion of *Trypanosoma brucei*

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ABSTRACT

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

KEY WORDS: Heme; kDNA; kinetoplast; import; metabolism; mitochondrion; transport; *T. brucei*

Translation and ribosomes

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

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

Trypanosome mt ribosomes display several unique properties in comparison to their bacterial counterparts; the fully assembled ribosome (monosome) is much smaller, sedimenting at 50S (Maslov et al., 2006). The 30S SSU particle contains 9S rRNA (610 nt), while the 40S LSU carries 12S rRNA (1,173 nt) with both rRNAs encoded by the kDNA maxicircle (de la Cruz et al., 1985a, 1985b; Eperon et al., 1983). Interestingly, these rRNAs belong to the smallest known rRNAs molecules. A comparison of *T. brucei* 9S rRNA to its 16S rRNA bacterial homologue revealed similarities but also identified large gaps in the former molecule (de la Cruz et al., 1985a). Similar comparison of the *T. brucei* 12S rRNA and bacterial 23S rRNA depicted both the highly conserved regions, as well as the loss of some typical domains and stem loops regions (Eperon et al., 1983). Importantly, a stem-loop region responsible for binding chloramphenicol, a potent inhibitor of prokaryotic translation, is extensively altered and thus *T. brucei* mt translation is chloramphenicol-insensitive. The extremely reduced 12S and 9S rRNAs indicate essential regions for the function of the mt ribosomes. Moreover, existence of these protein-rich mt ribosomes supports a hypothesis that some of the rRNAs structures can be replaced by proteins (Maslov et al., 2007, 2006; Zíková et al., 2008b).

Mitochondrial ribosomes were characterized by several studies demonstrating the ribosomal composition, organization and arrangement of their SSU and LSU (Maslov et al., 2006; Sharma et al., 2003; Scheinman et al., 1993). Electron microscopy of the *L. tarentolae* 50S monosomes suggested several important structural features: i/ the overall structure is very porous; ii/ the SSU and LSU are hold together only by 9 intersubunit bridges (consisting of protein-protein, RNA-RNA and protein-RNA interactions) in contrast to 13 bridges in eubacterial ribosomes and 15 in mammalian mt ribosomes; iii/ an intersubunit space, which is involved in interaction with translation factors and tRNA, has a unique topology. Furthermore, trypanosomatid-specific proteins form major portions of the mRNA channel, the tRNA passage and the polypeptide exit tunnel, implying significantly different mechanisms to recruit mRNA, bind tRNA, and

release the nascent polypeptide. Despite the specific structural differences and strikingly small rRNAs, the overall morphology of the trypanosomatid mt ribosome is remarkably similar to its bacterial counterpart, suggesting the existence of strong functional and structural constraint on this early diverged mt ribosome (Sharma et al., 2009, 2003).

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

2. Unpublished data

My PhD work is mostly summarized in the attached publication (Ridlon et al., 2013), however, this chapter contains the complete data-set (all investigated time points) of published experiments and some not-shown data. Additionally it shows initial results obtained in the study of the 45S SSU* complex and proposes future plans to shed a light on its function. Furthermore, as an extension of the above-mentioned studies of the 45S SSU* complex, I show initial results of functional analysis of proteins "Tb927.8.2650 and Tb11.02.2710" that are associated with mitochondrial ribosomal complexes, yet their function remain unchanged.

"PPR29, "Rhod" and "200 kDa" proteins described in the introduction represent a unique complex termed 45S SSU*. These proteins were chosen for further analysis to investigate a function of the 45S SSU* complex. We prepared a RNAi-mediated knockdown cell line (KD) for each gene and monitored the effect of the gene down-regulation on mitochondrial metabolism.

3.1. Quantitative Real Time PCR

The first step was to verify the prepared KD cells by quantitative PCR. This method was used also for monitoring levels of other mitochondrial encoded mRNAs, such as CoI, CyB, edited (E) and pre-edited (P) RPS12, and 9S and 12S rRNAs.

3.1.1. Methods

- Use Filter barrier tips for every step to avoid contamination with genomic DNA, RNases, PCR products etc. in a separate room (not the same room where PCR products were analyzed)
- Use highly distilled ("nano-pure") water, which is only used with filter tips
- Use dedicated reagents for qPCR
- Use a dedicated set of pipettes (after the step "DNase digestion")

- Pipette the qPCR reactions in a dedicated environment where no genomic DNA (gDNA) is isolated, no PCR tubes are opened, etc., to avoid contamination
- Handle reagents only with gloves
- Grow cells, isolate RNA and make cDNA at the same time for all samples
- Harvest 10^8 cells at a concentration of 1-2x10^7 cells/ml
- Perform DNase digestion using TURBO DNA-free™ kit (Ambion)
- Dissolve 20-25 μg RNA in 50 μl of "nano-pure" H_2O following the Ambion manual
- After DNA digestion, precipitate the sample with ethanol and sodium acetate
- precipitation :
  - 250 μl ethanol (96%)
  - 10 μl 3 M sodium acetate, pH 5.2
  - 1 μl glycogen (5 mg/ml stock, Ambion)
  - Mix well, keep at -20 °C for 1 h or overnight
  - Spin for 30 min at 4 °C
  - Wash with 70% ethanol
  - Spin again for 15 min at 4 °C
  - Decant supernatant, spin briefly, dry on air then resuspend in 20 μl water

- Measure RNA concentration by Nanodrop spectrophotometer (Thermo Scientific)
- Verify RNA integrity by electrophoresis in 1% agarose gel
- Transcribe 4-5 μg of DNase-treated total RNA to cDNA using random hexamer primers /Reverse Transcription (SuperScript® III) (Invitrogen)
- Prepare two reactions each with Reverse Transcriptase (RT), one without; called the RT+ reactions A and B and the RT- reaction (without reverse transcriptase)
- Follow the SuperScript® III Reverse Transcriptase manual (Invitrogen)
- PCR program
  Step 1: Heat to 65 °C for 5 min
  Step 2: keep on ice 2 min and add remaining components
  Step 3: 25 °C for 5 min
  Step 4: 50 °C for 60 min

Step 5: 70 °C for 15 min

Step 6: Keep at 4 °C

- Take the volume of the cDNA to 200 µl with 180 µl H_2O
- Prepare 1:50 dilutions of the RT+ samples (= A and B)
- primers for real time PCR in 5'-3' polarity (Tab.3.1.1. and 3.1.2.)

Gene	**Forward**	**Reverse**
PPR29	TGCGTGTCATTCGCTGCCG	CGACGCACTGTGGCTGAGCA
Rhod	TCGTACTTGCGTCTGCAGGCA	TGATGCCTTGTGCGCCTGTCT
200 kDa	GGCGGCAGGAGGAGGTGGTA	TCAACCAAGGCTGCTCCGCG

Table 3.1.1. Primers for Real Time PCR analysis designed using http://www.ncbi.nlm.nih.gov/tools/primer-blast/index.cgi?LINK_LOC=BlastHome

Gene	**Forward**	**Reverse**
CoI	TGCCTATAACTATGGGTGGGTTTACAAAC	ACTAAGCAACCAAATCCTCCAATAAACATTC
CyB E	AAATATGTTTCGTTGTAGATTTTTATTATTT	CCCATATATTCTATATAAACAACCTGACA
RPS12 P	GATTTTAAGATTGGCTTTGATTGA	AATATAAAATCTAGATCAAACCCATCACA
RPS12 E	CGTATGTGATTTTTGTATGGTTGTTG	ACACGTCGGTTACCGGAACT
9S	ATTAGATTGTTTTGTTAATGCTATTAGATG	ACGGCTGGCATCCATTTC
12S	GGGCAAGTCCTACTCTCCTTTACAAAG	TGAACAATCAATCATGGTAATAAGTAGACGATG

Table 3.1.2. Oligonucleotides for Real-Time PCR analysis of investigated maxicircle mRNAs (Carnes et al., 2005) and ribosomal rRNAs.

- Use 96-well plate (Eppendorf), add 2 µl of cDNA RT+ (A) to the three qPCR tubes and do the same for the second cDNA RT+ (B) to prepare triplicate of each reaction
- Add 2 µl of the cDNA RT- to the one tube on the 96-well plate (Eppendorf) (as a control)

- Add 18 μl of the master mix (Tab. 3.1.3.) with 18 S rRNA-specific primers to the each tube
- Choose cDNA RT+ A or B for further analysis
- To the three different qPCR tubes add 2 μl of chosen cDNA and add 18 μl of the master mix. Do the same for all primers, see Tab.3.1.2.
- Do the negative control with cDNA RT-

Master mix	Reaction	
	1	17
2X Sybr Green MM (Applied Biosystem)	10	170
cDNA	0	0
1.5 μM primers	8	136

Table 3.1.3. calculation of master mix for qPCR reaction

- PCR reaction program using Eppendorf Mastercycler® ep realplex S

Step 1	95 °C 10 min	
Step 2	95 °C 15 sec	45X
Step 3	60 °C 60 sec	
Step 4	95 °C 15 sec	
Step 5	60 °C 15sec	

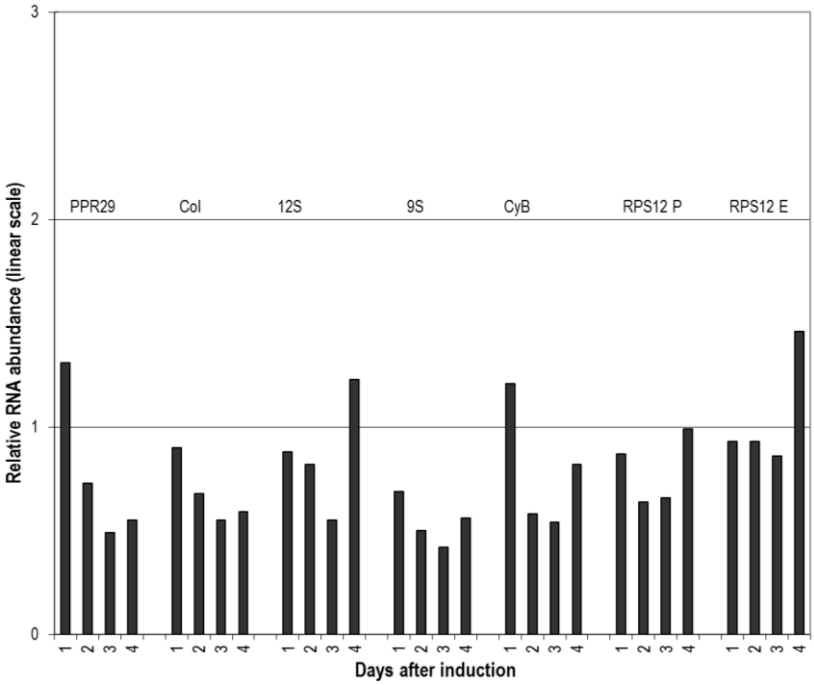
Data output generated by the Eppendorf Mastercycler Realplex Software were analyzed by the Pfaffl method (Pfaffl, 2001), the principle of which is a relative quantification of transcripts based on the relative expression of a target gene versus an unaffected reference gene.

3.1.2. Results

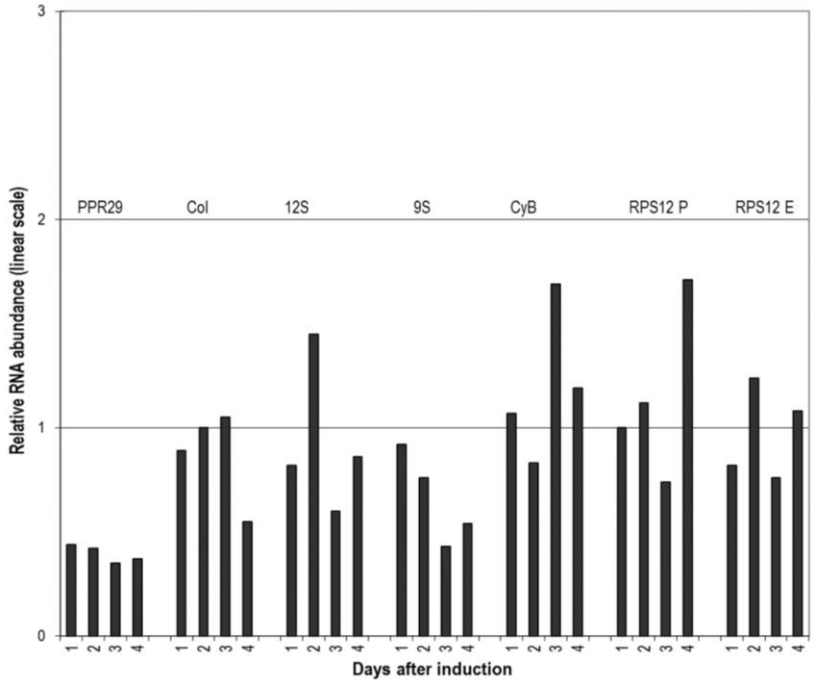
The template for qPCR was the cDNA generated from the non-induced and induced cells of procyclics (PS) *T. brucei* harvested on 1st, 2nd, 3rd, and 4th day after RNAi induction. This assay verified the RNAi silencing of each monitored gene. The maxicircle-encoded CoI, CyB, RPS12 (P and E) and ribosomal 9S and 12S RNAs were screened also with this method (Fig 3.1.1, panels A, B and C). The data were

normalized to the measured level of cytoplasmic 18S rRNA, because it was not affected by RNAi, and processed using the Pfaffl method (Pfaffl et al., 2001). We show that already on the 1st or 2nd day after RNAi induction, the levels "PPR29", "Rhod" and "200 kDa" mRNA were down-regulated. However, other results from this analysis were not fully consistent and in some cases they are difficult to interpret. But we can conclude that after silencing of "PPR29" the levels of 9S rRNA, COI, CyB and P RPS12 mRNAs were decreased as soon as on 2nd day after initiation of silencing. Even the level of 12S rRNA dropped, although this happened only on 3rd day after "PPR29" silencing (Fig. 3.1.1. A). Ablation of "Rhod" caused down-regulation of CoI mRNA starting on 4th day after RNAi silencing and the 9S rRNA was affected on 2nd day; this down-regulation was maintained till day 4 post-induction (Fig 3.1.1. B). The results for "200 kDa" KD cell line were the most consistent and they corresponded to other data that were obtained by Northern blot analysis. The level of CoI mRNA was declining linearly with each day since initiation of 200 kDa ablation, the CyB mRNA was also down-regulated, as well the 9S rRNA. The 12S rRNA and RPS12 remained unchanged (Fig 3.1.1. C). A possible explanation of why the qPCR data did not show a gradual decrease (or increase) of the mRNAs or rRNAs levels along with progression of RNAi silencing is that qPCR is extremely sensitive method easily affected even by minor sampling errors. The second possible explanation is the fact that mitochondrial mRNAs exist in two different forms: short tail mRNA (ST) and long tail (LT) mRNAs. The mRNAs containing LT are intended for translation and, as mentioned above, one of the hypothesized function of 45S SSU* is a specific recognition of long tail mature mRNAs and their recruitment to mitochondrial ribosome. By qPCR analysis, we were not able to distinguish between LT and ST forms of mRNA. For example, if the LT mRNA is down-regulated but ST mRNA accumulates in the cell, the qPCR would not show any significant differences in the mRNA level. This makes qPCR an inappropriate method for monitoring RNAi effects that selectively impact either LT or ST mRNA levels.

A


Relative RNA abundance (linear scale)
PPR29
Col
12S
9S
CyB
RPS12 P
RPS12 E
Days after induction

B


Relative RNA abundance (linear scale)
PPR29
Col
12S
9S
CyB
RPS12 P
RPS12 E
Days after induction

C

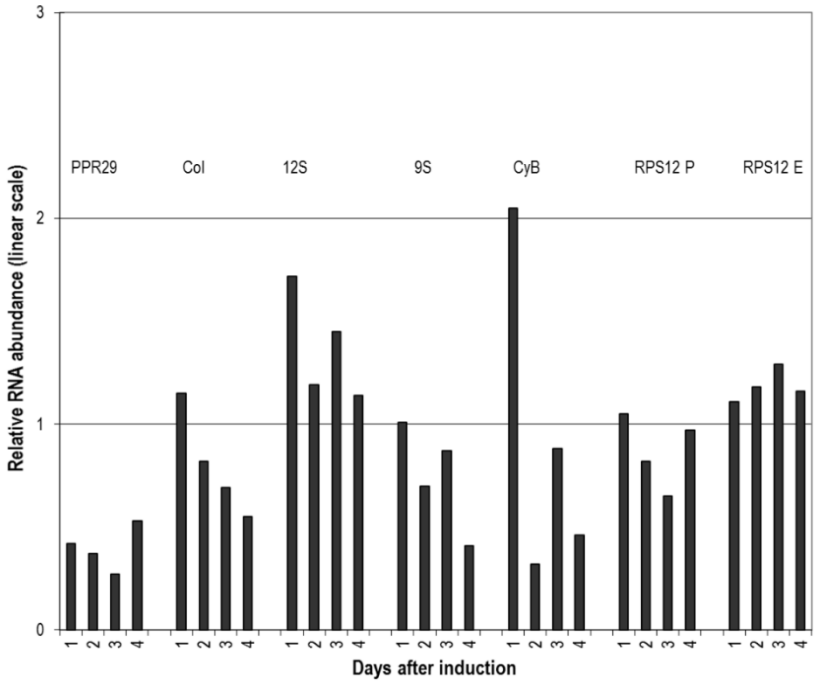


Figure 3.1.1. Quantitative real-time PCR analysis of the "PPR29" - Tb927.5.1790 (A), "Rhod" - Tb927.6.4930 (B) and "200 kDa" - T.927.8.5200 (C) mRNAs performed on knockdown of each gene. The RNA levels averaged from three replicates were normalized to 18S rRNA. The relative RNA abundance calculated by PfaffI method is in linear scale and it shows the increase or decrease of the measured mRNAs or rRNAs after RNAi silencing of each gene. The cells were harvested 1st, 2nd, 3rd and 4th days after protein ablation.

3.2. Translation assay

Because the SSU* complex is associated with the SSU ribosomal subunit, in the next step we have focused on investigating the changes in the amounts of mitochondrial-translated proteins following the course of RNAi induction.

3.2.1. Methods

3.2.1.1. Labeling of PS *T.brucei* mitohondrial translational product by [^{35}S]-EasyTag Express Protein Labeling Mix (PerkinElmer Life Sciences)

- Use exponentially growing culture containing at least 10^7 PS *T. brucei* cells
- Harvest the cells and wash them twice with SoTE buffer (by pelleting at 3500 rpm, 7 min at 15 °C)

- Carefully aspirate the supernatant and place the cells resuspended in 90 μl SoTE into a Falcon 14 ml tube (with a round bottom)
- Add 1 μl 100 mM DTT
- Add 10 mM cycloheximide to final concentration 100 μg /ml
- Incubate 10 min at 27 °C on the shaker (110 rpm) to stop cytosolic translation
- Add 15 μl of ^{35}S-labeling mix and agitate at 27 °C for 1 hour
- Pellet cells and resuspend in 100 μl SoTE
- Resuspended cells can be used immeduately or can be frozen at -80 °C before use
- Perform this procedure for each of the 5 days after RNAi induction

3.2.1.2. Two-dimensional (2D) electrophoresis to analyze translation products

- count the cells before use and spin them for 5 min at 4 °C; use an equal number of cells (labeled at each day post-induction)
- Resuspend cells in 100 μl Sample buffer (SB) (see below)
- Incubate 30 min at 37 °C; pellet the debris from the lysate by centrifuhation for 20 min at 14,000 rpm
- Load the cleared cell lysate on the 9% Tris-Glycine SDS gel and run at 20 mA per gel until the front moves approximately 5 cm into the resolution gel (first dimension)
- Slice out the lanes of the gel and incubate the slices for 30 min at 37 °C in 0.125 M Tris-HCl, pH 6.8, 1% β-mercaptoethanol and 1% SDS
- Place the slices horizontally on the top of 14% Tris-Glycine SDS gel and run at 5 mA antil the front migrates for ~7 cm (second dimension)
- Fix the gel 15 min in “Fixing solution“ (see below)
- Stain the gel with commassie blue R250
- Destain overnight is “Destain buffer“ (see below)
- Wash the gel in water 3 X 20 min
- Incubate in 1 M sodium salicylate for 30 min
- Place the gel on the filter paper and dry using the vacuum gel dryer
- Place the dried gel in the cassette and expose to the X-ray film (Kodak)

Buffers:

Sample buffer
100 mM Tris-HCl, pH 6.8
200 mM dithiothreitol DTT
20% glycerol
4% sodium dodecyl sulfate SDS
0.01% Bromphenol blue

SoTE
0.6 M Sorbitol
20 mM Tris-HCl, pH 7.3
2 mM EDTA, pH 7.5

Fixing solution
10% Acetic Acid
50% Methanol

Destain buffer
10% Acetic Acid
10% Methanol

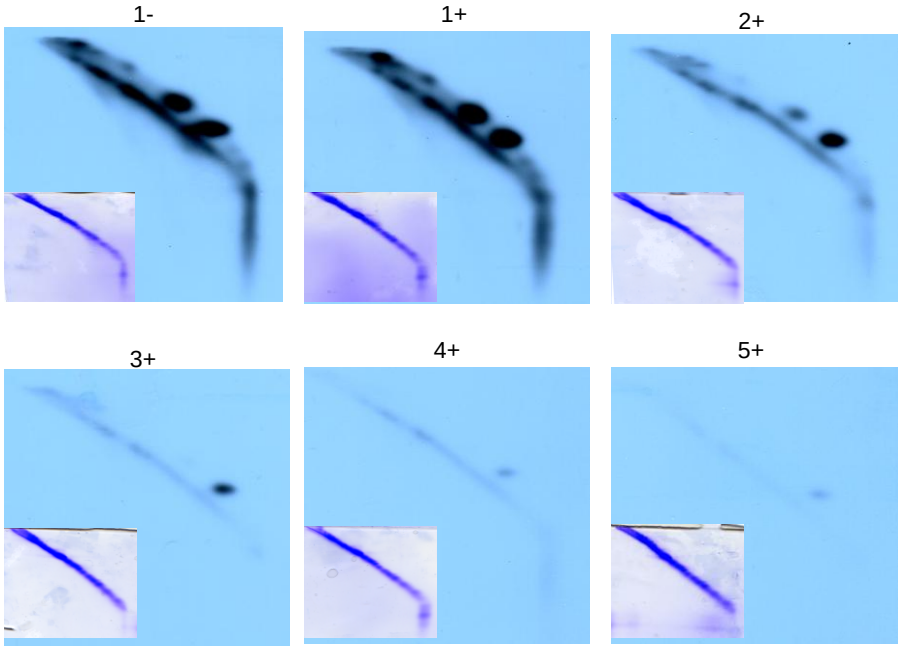
3.2.2. Results

The mitochondrial translation products of the non-induced and RNAi-induced cell lines were radioactively labeled on 1st, 2nd, 3rd, 4th and 5th day with ^{35}S-methionine and ^{35}S-cysteine mixture (EasyTag Express Protein Labeling Mix) after inhibition of cytosolic translation by cycloheximide. Approximately 10^7 cells collected at each post-induction day were labeled, lysed and then fractionated on the 2-dimensional gel.

The data show that the mitochondrial translation was drastically inhibited already a second day after the silencing of each targeted mRNA (PPR29, Rhod and 200 kDa) and this inhibition continued till day 5 at which time the experiment was stopped (Fig.3.2.1.).

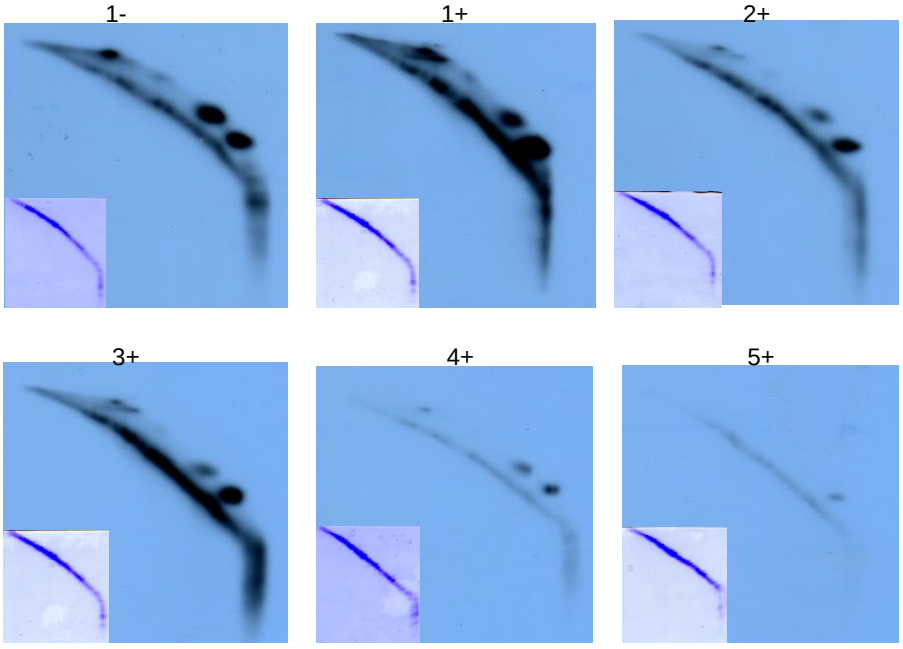
A

Tb927.5.1790



B

Tb927.6.4930



C

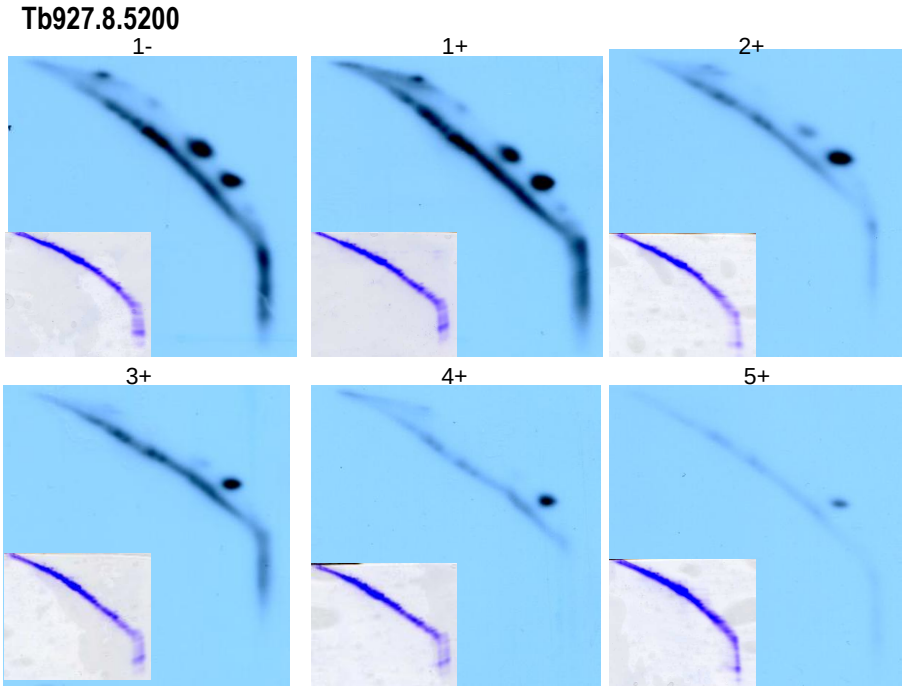
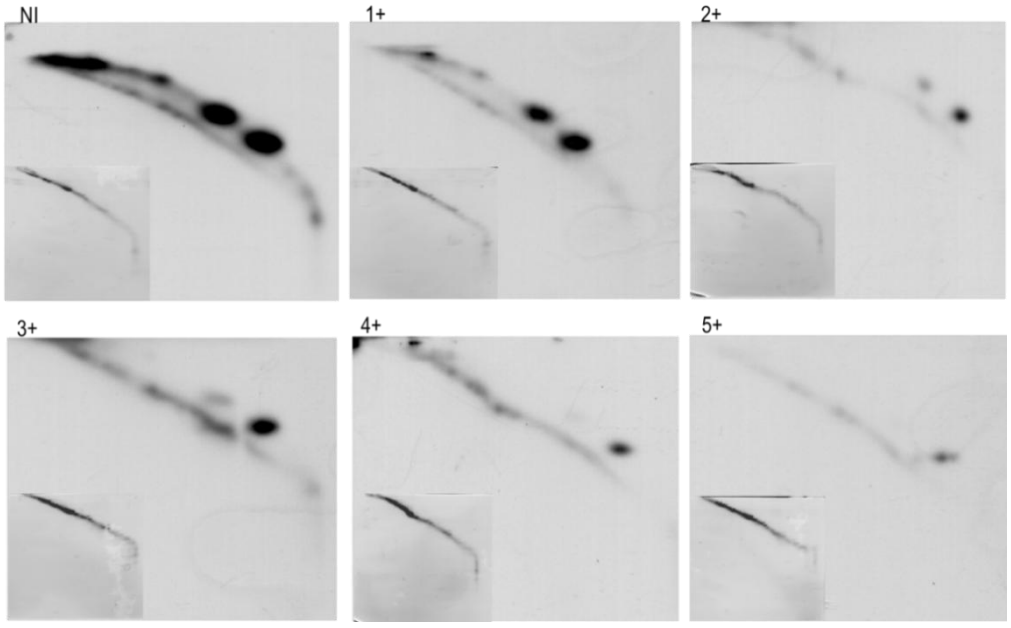


Figure 3.2.1. Analysis of translation products in the Tb927.5.1790 (A), Tb927.6.4930 (B) and T.927.8.5200 (C) RNAi induced cells. Radioactively (^{35}S) labeled mitochondrial translated products from cells harvested at 0 (non-induced, 1-), 1st, 2nd, 3rd, 4th and 5th (1+ to 5+) day after the RNAi induction was separated by 2D SDS electrophoresis. Coomassie-stained stained gels (insets in the left bottom corner) were dried and exposed to the X-ray film.

S17 and L3 proteins represent ribosomal proteins specific for mito-SSU or LSU, respectively. Ablation of these components was used as a control. Ablation of each protein caused a down-regulation of mitochondrial translation due to the damage of ribosome integrity (Fig.3.2.2.). However, unlike the SSU* components, ablation of L3 and S17 showed an effect on the mitochondrial translation as soon as day 2 after the RNA-induced silencing (Ridlon et al., 2013). This difference testified to the specificity of the observed effects of the SSU* ablation.

Tb09.211.2580

A



Tb927.3.5610

B

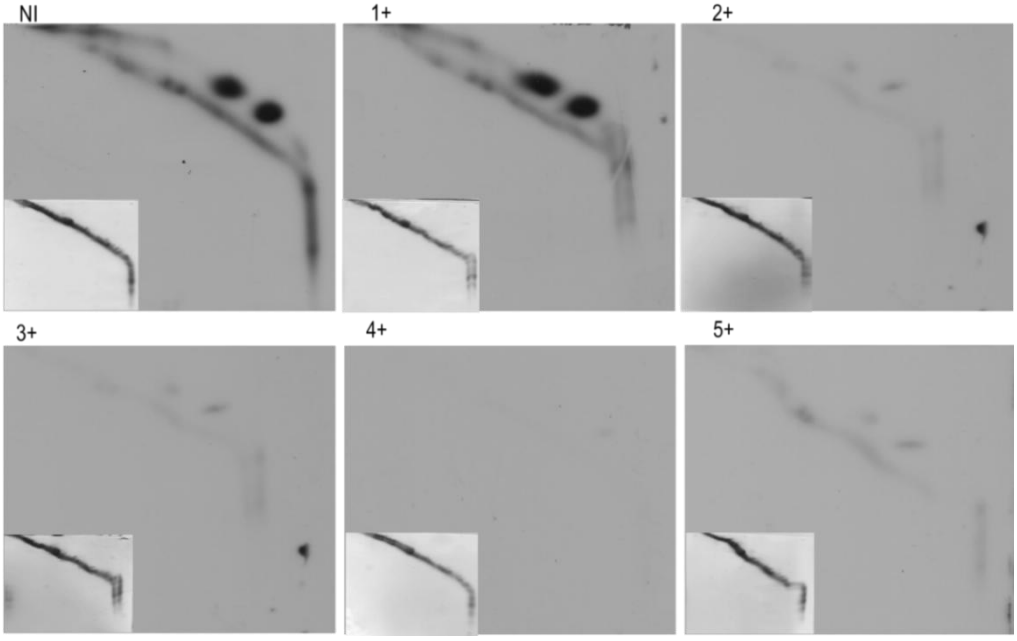


Figure 3.2.2. Effect of ablation of two ribosomal proteins Tb927.2580 S17 (A) and Tb927.5610 L3 (B) on mitochondrial translation. Radioactively labeled mitochondrial translation products of the cells harvested 0 (non-induced, NI), 1st, 2nd, 3rd, 4th and 5th (1+ to 5+) day after the RNAi induction were separated by 2D SDS electrophoresis.; Coomassie-stained gels (left bottom corner) were dried and exposed to the X-ray film.

3.3. Isolation and sedimentation analysis of *E. coli* ribosomes

In order to monitor the impacts of RNAi induction experiments on the mitochondrial ribosome of *T. brucei*, we had to calibrate sucrose gradients used for these analyses. For this purpose, we isolated ribosomes from *E. coli* and separated the 70S monosomes, 50S LSU and 30S SSU in sucrose gradient under the same conditions that were used for separation of *T. brucei* ribosomes. This part was crucial for validation of the accuracy and reproducibility of our gradient maker and also for determination of the exact sedimentation coefficients (expressed in Svedberg units or S-values) of the *T. brucei* mito-ribosomal complexes.

3.3.1. Methods

3.3.1.1. Isolation of *E. coli* ribosomes

- Grow the *E. coli* DH5α of the 100 ml LB medium withouth antibiotics
- Harvest the cells (2000 rpm 10 min at 4 °C)
- Resusped the pellet in "French-press buffer" (see below) at the ratio of 2 ml of buffer per 1 g of bacterial pellet (wet weight)
- Add 1 μg of Rnase-free Dnase and incubate on ice for 5-10 min
- Break the cells with French-press (14,000 psi) or by sonication
- Centrifuge the suspension to remove the cell debris and the unbroken cells (12,000 rpm, 30 min, 4 °C)
- Layer the supernatant on the "sucrose cushion buffer" (see below) (Kurland, 1966)
- Centrifuge for 16 h, 28,000 rpm at 4 °C
- The pellet contains 70S ribosomes
- Resuspend the pellet in "Tight couples buffer"; measure A_{260} optical density (OD)
- Freeze the sample at -80 °C if necessary

Buffers:

French-press buffer	20 mM Tris-HCl, pH 7.5

50 mM $MgCl_2$
100 mM NH_4Cl
1 mM DTT
0.5 mM EDTA

20 mM Tris-HCl, pH 7.5
50 mM $MgCl_2$
500 mM NH_4Cl
1 mM DTT
0.5 mM EDTA
1.1 M sucrose

sucrose cushion buffer

3.3.1.2. Fractionation of ribosomes and subunits on a sucrose gradient

- Prepare 7-30% sucrose gradient by using Bio-Comp gradient station (model 153) following the manufacturer's manual
- Start by adding 5 ml of the 7% sucrose buffer to a centrifuge tube for a SW41 rotor
- Underlay 6 ml of the 30% sucrose buffer on the bottom of the tube
- Add more 7% sucrose buffer on the top of the gradient to the very edge of the tube
- Close the tube with the cap (provided as a part of the gradient station)
- Place the tubes to the magnetic stand and assemble to the Bio-Comp gradient station
- Run the gradient maker program: time 2.27 min; angle 81.5 degrees; speed 17 rpm
- Carefully remove the cap
- Aspirate ~ 200 μl of the solution from the top of the gradient and balance the tubes

Buffers:

7% sucrose buffer
40 mM Hepes
100 mM KCl
20 mM $MgCl_2$
7% sucrose

30% sucrose buffer
40 mM Hepes
100 mM KCl
20 mM $MgCl_2$
30% sucrose

- Load ~ 250 μl of the 70S ribosome sample (pellet ressuspended in "Tight couples buffer") on the top of the gradient
- Centrifuge in a Beckman SW41 rotor for 16 h, 17,000 rpm at 4 °C
- Separate the gradient on 35 fractions by using Bio-Comp gradient station

- Measure RNA concentration OD A_{260} of each fraction to determine which fractions contain the bacterail ribosomes
- Recover the ribosomes by spining for 16 h at 30,000 rpm at 4 °C in a Beckman 50.2 Ti rotor
- Resuspend the ribosomal pellet in "Tight couples buffer" and freeze (if necessary)
- In order to separate ribosonal subunits, the purified ribosomes have to be dialyzed against "Subunit gradient buffer" for 0.5-1 h at 4 °C
- Load the dialyzed material on the top of the 7-30% sucrose gradient (described above)
- Centrifuge in a SW41 rotor for 16 h, 17,000 rpm at 4 °C
- Fractionate the gradient and measure RNA concentration of each fraction using a Nanodrop spectrophotometer

Buffers :

Tight couples buffer
10 mM Tris-HCl, pH 7.5
6 mM $MgCl_2$
50 mM NH_4Cl
1 mM DTT
0.5 mM EDTA

Subunit gradient buffer
10 mM Tris-HCl, pH 7.5
1 mM $MgCl_2$
100 mM NH_4Cl
1 mM DTT
0.5 mM EDTA

3.3.2. Results

As mentioned earlier, we needed to perform a calibration of the sucrose gradients and determine the exact S values for mitochondrial ribosomal particles of *T. brucei*. For this purpose, the well characterized *E. coli* ribosomes were used. Isolated bacterial ribosomes were resuspended in the "Tight couples buffer" – designed to keep subunits of 70S ribosomes together; and in the "Subunit gradient buffer" – to dissociate the ribosomes onto 30S SSU and 50S LSU, and then separate them on 7-30% sucrose gradient under two different conditions (20,000 rpm 16 h at 4 °C) (Fig.3.3.1) or (17,000 rpm for 16 h at 4 °C) (Fig.3.3.2.). The gradients were separated into 35 fractions and RNA concentration of each obtained sample was measured by Nanodrop spectrophotometer (Thermo Scientific). Fraction's number and mesured concentrations were plotted to the graphs (Fig.3.3.1. and 3.3.2.).

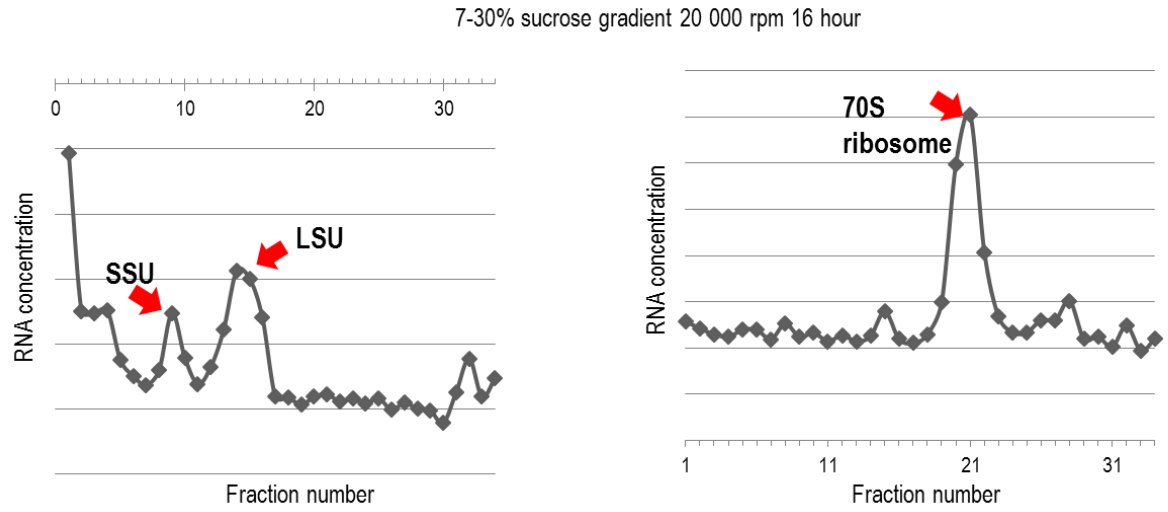


Figure 3.3.1.: 7-30% sucrose gradient for *E. coli* ribosome. Run in Sucrose buffer for 16 h, 20,000 rpm. Axis „x" shows measured RNA concentratation and axis „y" represents fractions of the gradient.

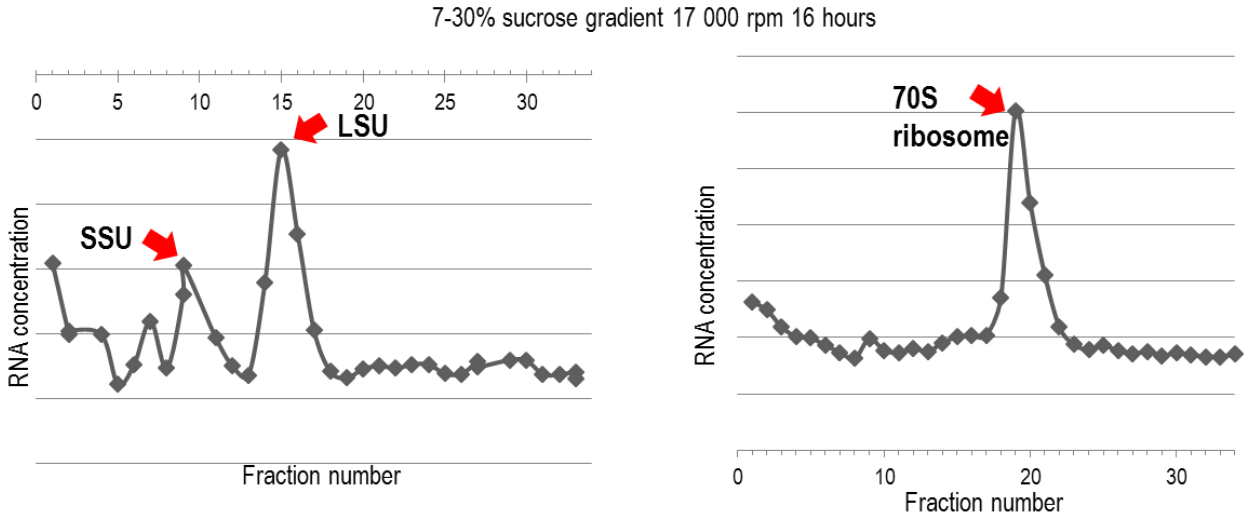


Figure 3.3.2.: 7-30% sucrose gradient of *E. coli* ribosome. Run in Sucrose buffer for 16 h, 17,000 rpm. Axis „x" shows measured RNA concentratation and axis „y" represents fractions of the gradient.

Figure 3.3.2., where the *E. coli* ribosome was sedimented under the conditions which were used also for *T. brucei* ribosome separation in further analysis, gave us the numbers for the fractions of the top of the peaks for 30S SSU – fraction 10; 50S LSU – fraction 15 and 70S ribosome - fraction 19. Because we know the S values for the *E. coli* ribosome, we created a graph with fractions numbers and corresponding S values and placed the numbers for the fractions of the top of the peaks for T. brucei (40S LSU – fraction 12; 45S

SSU*- fraction 13; 50S monosome – fraction 15 and 85S complexes – fraction 23). The aim was to confirm, if the *T. brucei* ribosomal subunits obtained with the same technique will fit to the trendline and correspond with published data about sizes of particular complexes (Fig.3.3.3.). By this method, we validated acuracy of our gradients.

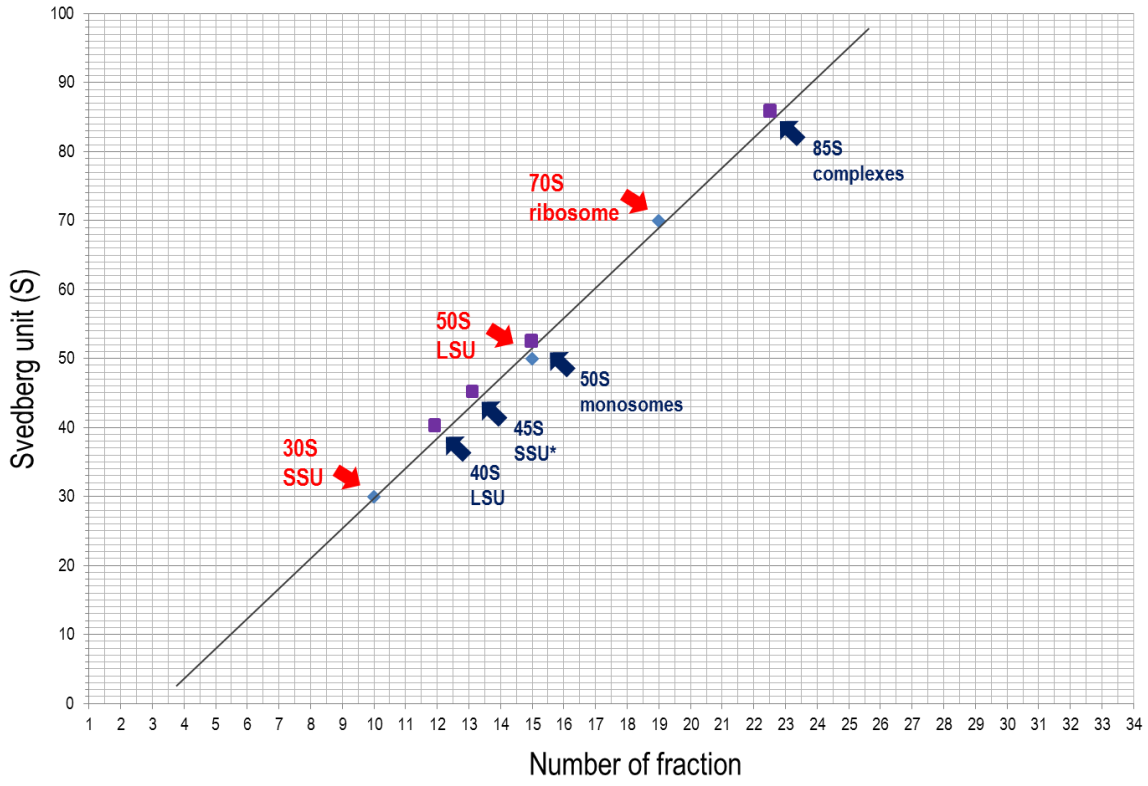


Figure 3.3.3.: Sedimentation of *E. coli* 30S SSU, 50S SSU and 70S ribosome (labeled red) and *T. brucei* 40S LSU, 45S SSU*, 50S monosome and 85S complexes (labeled dark blue) after separation in 7-30% sucrose gradient centrifuged in a Beckaman SW41 rotor at 17,000 rpm for 16 h.

3.4. Sedimentation Profile of 9S and 12S ribosomal RNA

Another important aspect in our research was to monitor the changes in sedimentation of the *T. brucei* mitochondrial ribosomal particles after the SSU* ablation.

3.4.1. Methods

- Harvest 1 x 10^7 – 2 x 10^7 of PS cells before induction of RNAi and 1-5 days after the induction
- Wash cells with 1 x SSC; use the same about of cells for each gradient
- Spin the cells and resuspend the pellet in 250 μl of ice cold SGB buffer (see the composition below)

- Perform cell lysis by additing 10% dodecyl maltoside to 1% final concentration
- Incubate lysate on ice for 30 min, clear the lysate by centrifugation at 14,000 rpm for 15 min in a benchtop Eppendorf microcentrifugeseparation of the gradient were also the same (16 h, 17,000 rpm at 4 °C)
- Fractionate the gradient on 34 fractions
- Isolate RNA from half (125 μl) of each fraction by a standard SDS/phenol-chloroform extraction, precipitate RNA with isopropanol using glycogen (Ambion) as carrier
- Load isolated RNA onto the BrightStar-Plus hybridization membrane (Ambion) using a Hoefer SlotBlot manifold device
- Immobilize RNA on the membrane by UV cross linking
- Prehybridize the membrane at 42 °C at least 30 min (see bellow)
- Hybridize the membrane in "Hybridization solution" mixed with radioactively labeled oligonucleotides specific for *T. brucei* mitochondrial ribosome rRNAs (9S and 12S) (Tab.3.4.1.) at 42 °C overnight

9S Rev	ACGGCTGGCATCCATTTC
12S Rev	TGAACAATCAATCATGGTAATAAGTAGACGATG

Table 3.4.1.: Primers specific for 9S and 12S rRNAs of mitochondrial ribosome of *T. brucei*.

Buffer:

SGB (Sucrose gradient buffer)
50 mM Tris-HCl, pH 7.5
100 mM KCl
10 mM $MgCl_2$
3 mM DTT
0.1 mM EDTA
0.05% dodecyl maltoside

- Radioactive labeling:
  - Label 10 pmole of a oligonucleotide with [γ-^{32}P]ATP (6000 Ci/mmol, Perkin Elmer) using T4 kinase (Invitrogen) using the manufacturer's protocol
  - Purify the radioactive probes using G25 spin columns (GE Healthcare) using the manufacturer's protocol

- Wash the membrane 3 X in “Washing buffer” (see bellow) at 42 °C
- Quantify results of the hybridization using phosphorimaging (Typhoon Phosphoimager (Amersham))

Buffers:

Prehybridization solution
6X SSPE
5x Denhardt’s solution
0.5 % SDS
20 mg/ml salmon sperm DNA

Hybridization solution
6X SSPE
5x Denhardt’s solution
0.1 % SDS
20 mg/ml yeast tRNA

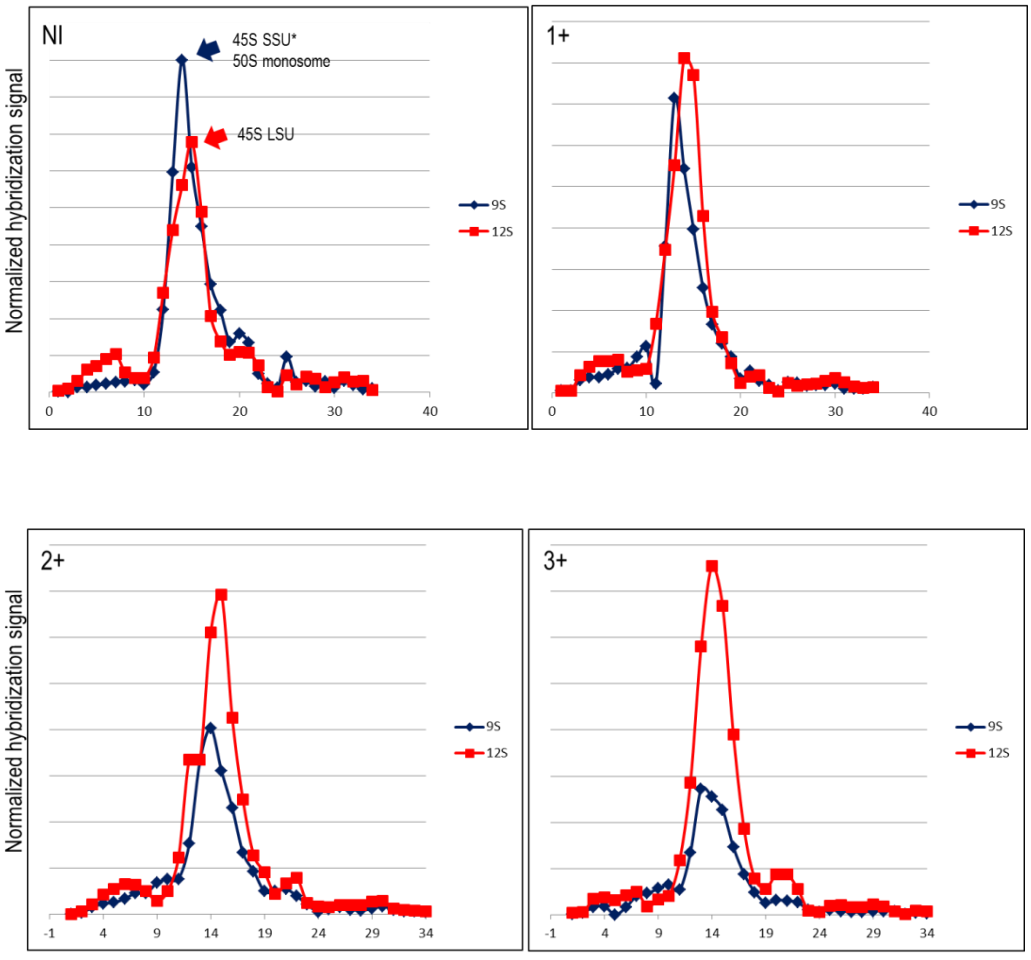
Washing buffer
2X SSPE
0.5 % SDS

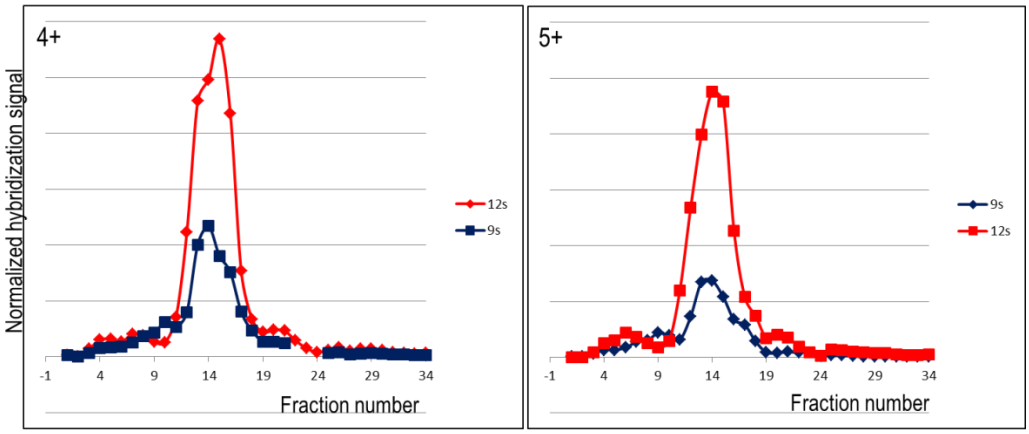
3.4.2. Results

Effects of RNAi-mediated ablation of the 45S SSU* complexes, as well as the ribosomal SSU and LSU complexes, were studied using total cell lysates obtained from $1x10^7$ to $2x10^7$ of the non-induced and induced cells (harvested on 1st - 5th day after the RNAi induction), fractionated in 7-30% sucrose gradients, as described in the Methods. RNA isolated from each fraction was blotted onto the membrane and hybridized with radioactively labeled oligonucleotide probes specific for the 9S or 12S rRNAs. Three different concentrations of the 12S-9S "tandem" transcript (prepared by an *in vitro* run-off T7 RNA polymerase transcription of the cloned maxicircle fragment encompassing the 12S and 9S rRNA genes from *T. brucei*; a courtesy of I. Škodová) were used to compare and normalize the amounts of the 9S and 12S rRNAs on the blots. Our data for “PPR29“, “Rhod“ and “200 kDa“ ablation experiments showed a drop of the signal for 9S rRNA already on 2nd or 3rd day after RNAi induction. “S17” and “L3” targets were used as controls. Their ablation is supposed to damage the ribosome's integrity. Silencing of “S17” affected the level of 9S rRNA of SSU ribosomal subunit as soon as on 2nd day after RNAi induction, but had no impact on 45S LSU complex. The ablation of “L3” subunit affected the integrity of 45S LSU with an obvious effect

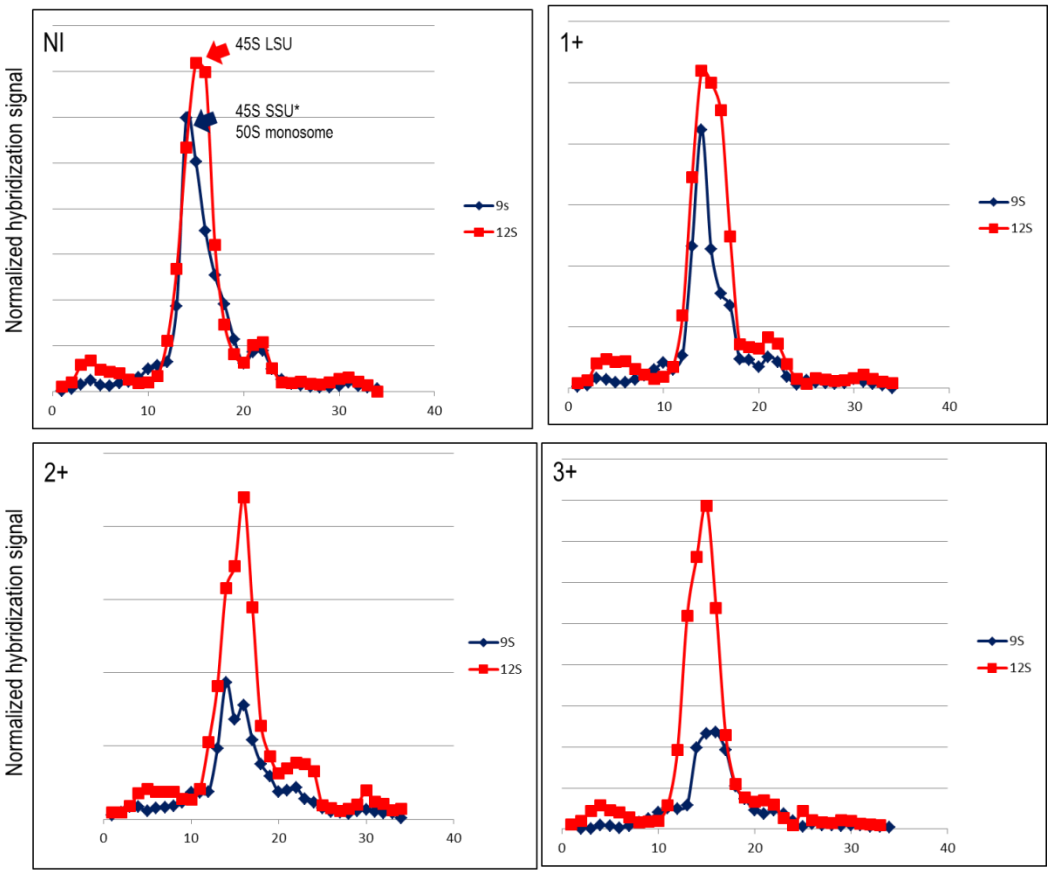
on 2nd day after the induction. The difference of the sedimentation profiles of 9S and 12S rRNA for silenced "S17' and "L3" compared to those seen during ablation of the 45S SSU* complexes showed that by targeting the specific components of the 45S SSU* complex (PPR29, Rhod, 200 kDa), we can influence only the targeted ribosomal complex without any noticeable impact on other ribosomal complexes (monosomes or LSU) and *vice versa* (Ridlon et al., 2013).

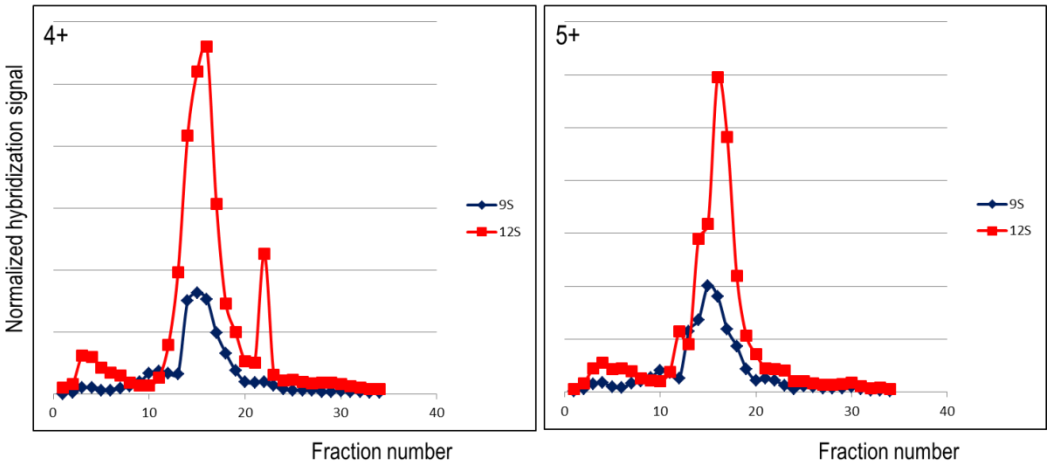
"PPR29"



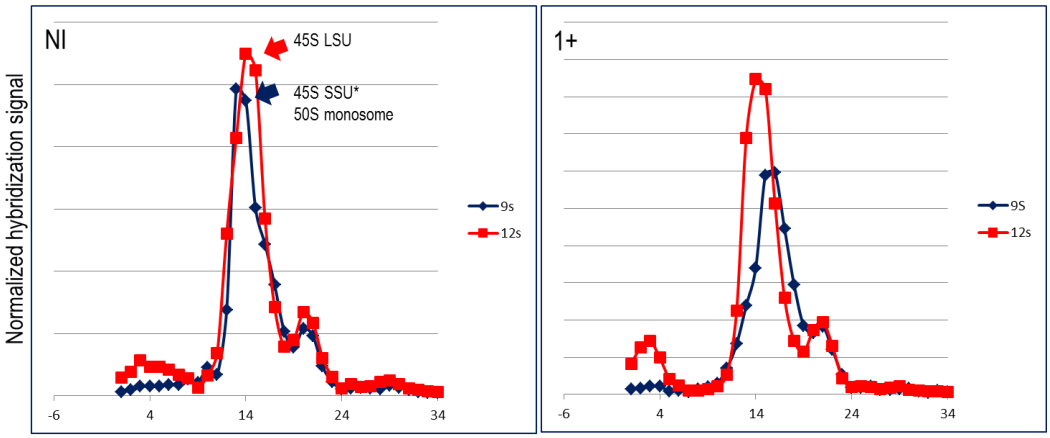

4+
5+
Normalized hybridization signal
12s
9s
Fraction number
-1 4 9 14 19 24 29 34

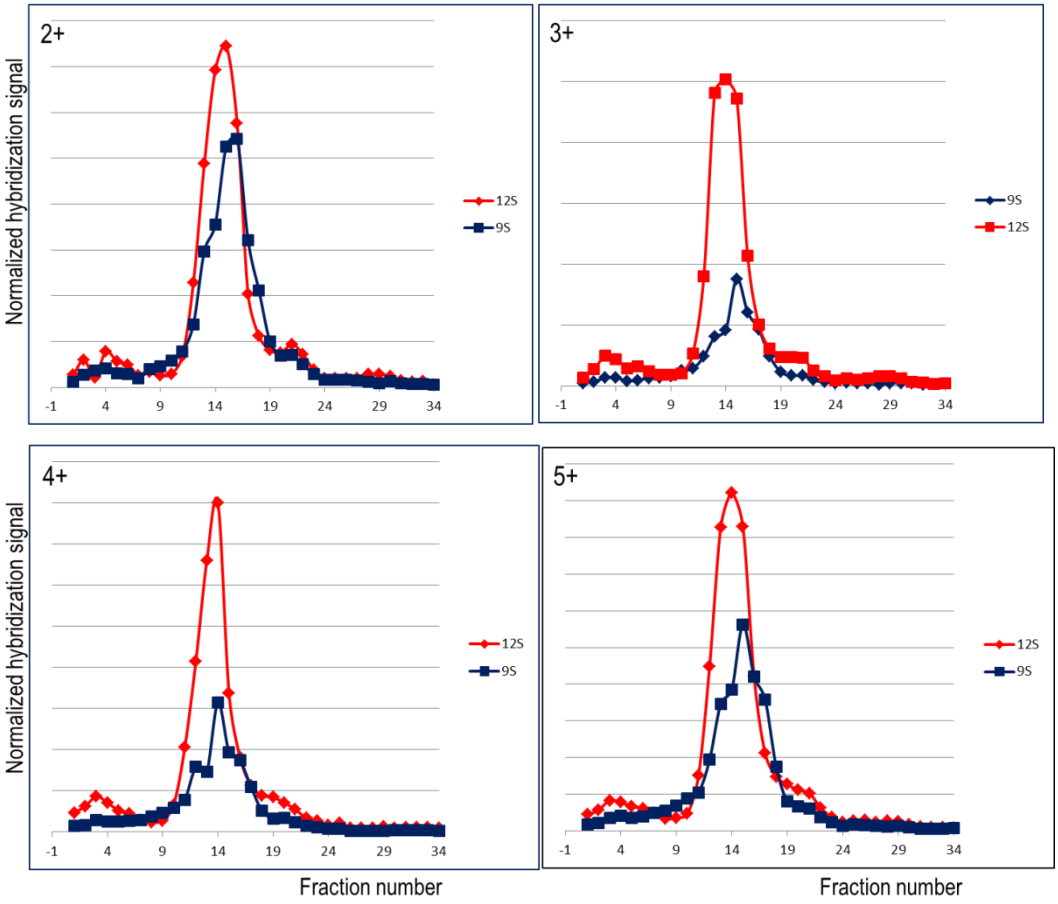
"Rhod"


NI
45S LSU
45S SSU*
50S monosome
1+
2+
3+
Normalized hybridization signal
9s
12S
0 10 20 30 40

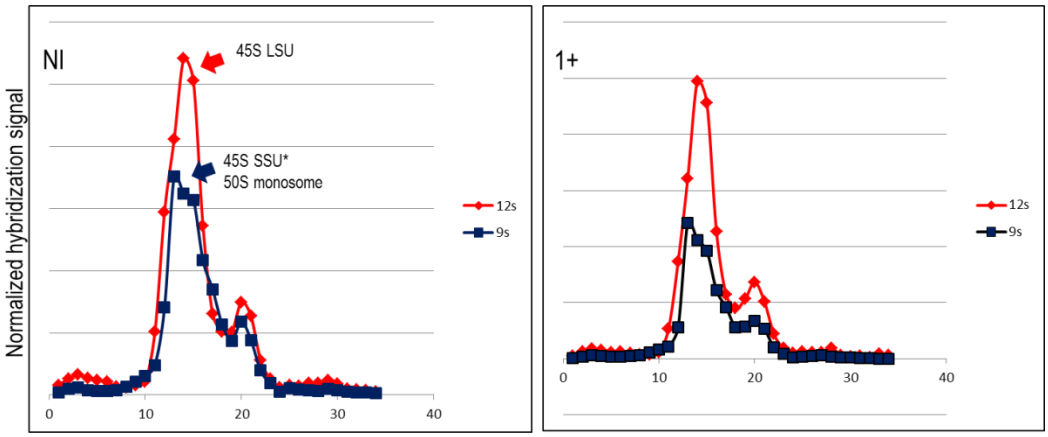

4+
5+
Normalized hybridization signal
9S
12S
0
10
20
30
40
Fraction number

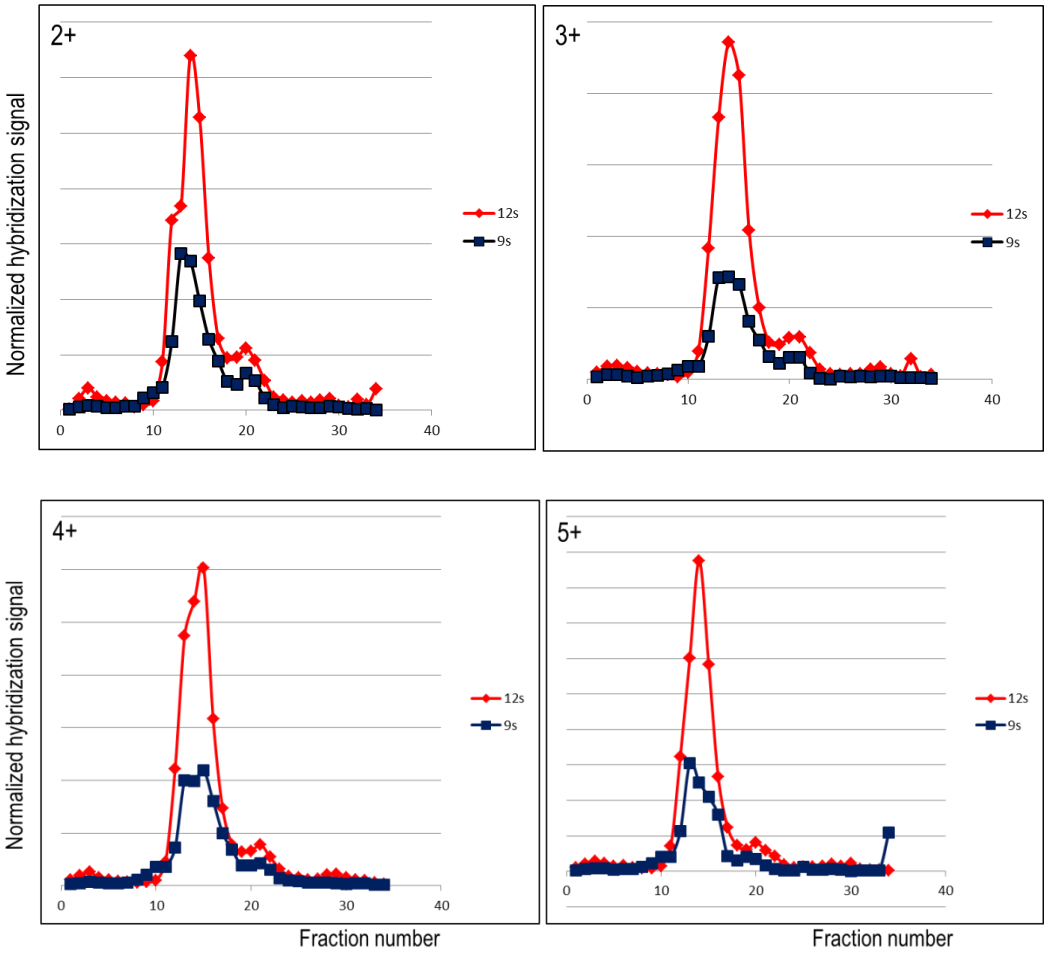
“200 kDa“


NI
45S LSU
45S SSU*
50S monosome
1+
Normalized hybridization signal
9s
12s
-6
4
14
24
34

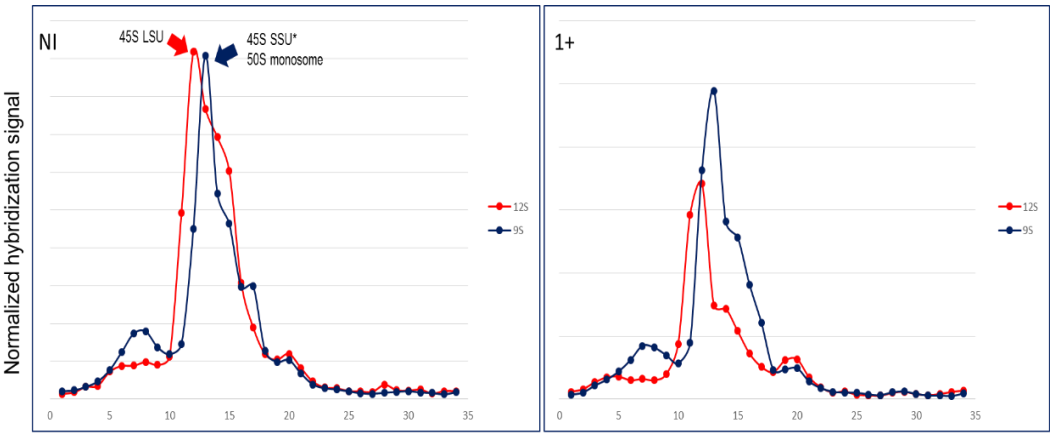

2+
3+
4+
5+
Normalized hybridization signal
Fraction number
12S
9S
-1
4
9
14
19
24
29
34

"S17"


NI
45S LSU
45S SSU*
50S monosome
1+
Normalized hybridization signal
12s
9s
0
10
20
30
40


2+
3+
4+
5+
Normalized hybridization signal
Normalized hybridization signal
12s
9s
0
10
20
30
40
Fraction number
Fraction number

"L3"


NI
45S LSU
45S SSU*
50S monosome
1+
Normalized hybridization signal
12S
9S
0
5
10
15
20
25
30
35

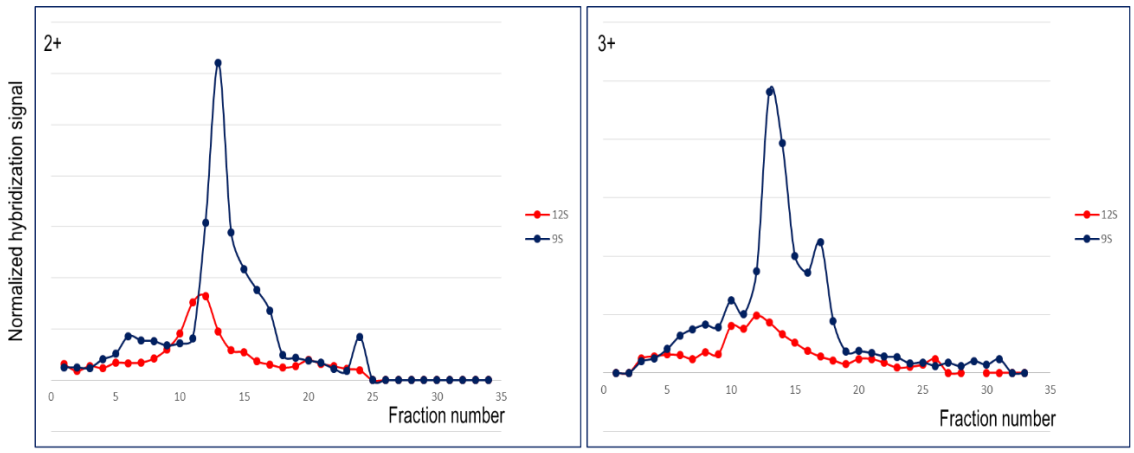


Figure 3.4.1.: Sedimentation profile of 9S and 12S rRNAs before and 1-5 days after RNAi ablation of "PPR29", "Rhod", "200 kDa", "S17" and "L3" mRNAs. The soluble fractions of PS *T. brucei* mitochondria was separated on 7-30% sucrose gradient and fractionated onto 34-35 fractions. Isolated RNA was probed with radioactive labeled oligonucleotides specific for 9S and 12S rRNAs.

3.5. Preparing constructs in pLEW-79 for Tandem Affinity Purification (TAP-Tag)

TAP-tagging is very efficient method for studying protein-protein interactions. The TAP-tag is composed of a calmodulin binding peptide (CBP), TEV protease cleavage site and Protein A, all of them fused in tandem to the C-terminus of a targeted protein. CBP binds calmodulin, while Protein A binds IgG and this double advantage is used in the purification process.

For this technique were chosen three different proteins which are parts of different complexes: "PPR29" is an exclusive subunit of the SSU* complex, Tb927.8.4860 "KRIPP10" and Tb.927.8.6080 "KRIPP11" were identified earlier by mass spectrometry of TAP-tagged SSU complexes and are likely components of the SSU* complex as well (Aphasizheva et al, 2011). Moreover, each protein contains a PPR domain, with possible function in mRNA processing or other function involving a specific RNA binding. The goal of this experiment was to gain a further insight into the composition of the SSU* complexes.

3.5.1. Methods

3.5.1.1. Cloning to pLEW79

- Primer designing for polymerase chain reaction (PCR)

A HindIII restriction site was added to 5'end of the forward (Fw) primer and the XbaI site was added to the reverse (Rev) primer (Table 3.5.1., restriction sites are indicated in bold)

PPR29	
Fw	CAC**AAGCTT**ATGTTCCGTCGTGCGATTCCCCTACTCTCT
Rev	AAA**TCTAGA**TGCCTCCTGTAGCCAGAGCACCCGCATCC

KRIPP10	
Fw	CAC**AAGCTT**ATGCTGTCGCACGCCGTCCCCCGTCTGCGC
Rev	AAA**TCTAGA**TACCAATTGCTCCCCAGGCGAGCGACCAAA

Table 3.5.1.: Primers designed for cloning the "PPR29" and 'KRIPP10" in the vector pLEW79 designed for TAP tagging. The restriction sites are indicated in bold. HidIII was used for forward primer and XbaI was used for reverse primer.

We received the "KRIPP11" construct from our collaborating laboratory (Ruslan Aphasizhev)

- Program for PCR reaction:

Step	Temperature	Time
Denaturation	96 ˚C	5 min
Denaturation	94 ˚C	1 min
Primer extension	55 ˚C	2 min ~ 1min for 1 kBp
Polymerase	72 ˚C	1.5 min
Polymerase	72 ˚C	10 min
Hold at	4 ˚C	

- Ligation of PCR insert and pLEW79 vector (fig.3.5.1.) in the ratio 2:1 and transformation of *E.coli* DH5α (Invitrigen) according to manufacturer's protocol
- After restriction verification and sequencing, the constructs were transfected to PS *T. brucei*

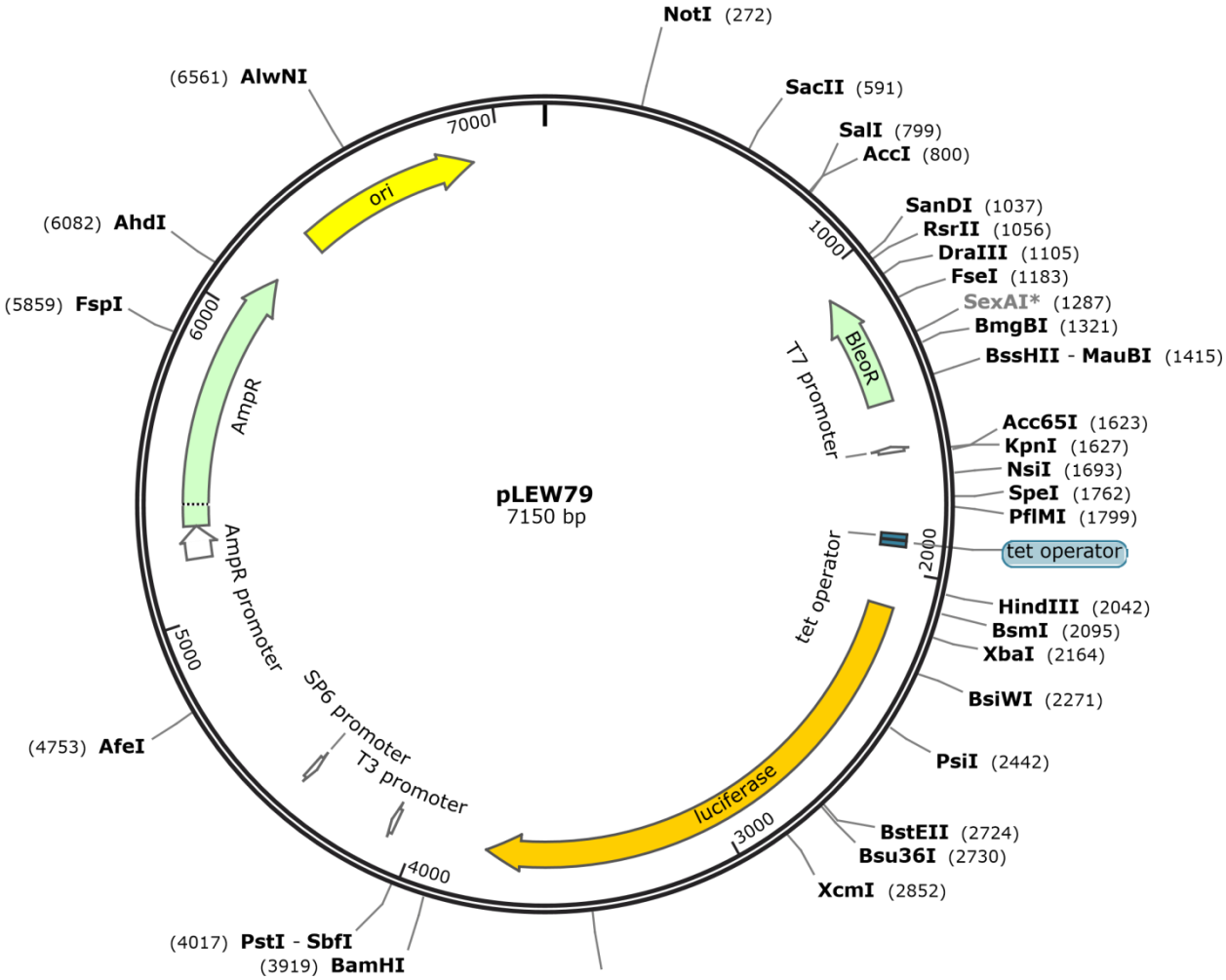


Figure 3.5.1.: Map of the pLEW79 vector.

3.5.1.2. Transfection of pLEW79 Construct to PS *T. brucei*

- Not I restriction endonuclease
- Bio-Rad Gene Pulser
- 0.4 cm electroporation cuvettes

Buffers:

Cytomix
120 mM KCl
0.15 mM $CaCl_2$
10 mM potassium phosphate

25 mM HEPES
2 mM EDTA
5 mM Mg Cl_2
Adjust pH to 7.6

Phosphate sucrose buffer
277 mM sucrose
1 mM $MgCl_2$
7 mM potassium phosphate
Adjust pH to 7.4

LD (limited dilution)
medium (SDM79 with 20% FBS serum)

EM buffer
3:1 mixture of Cytomix and Phosphate Sucrose buffer

3.5.1.3. Preparation of Plasmids and Cell Lines

- Seed 25-30ml 1-2 x 10^6 cells/ml and cultivate overnight at 27 ˚C
- Digest 10-12 μg of plasmid DNA with 5 units of Not I restriction endonuclease in a 100 μl reaction volume at 37 ˚C
- Precipitate the DNA with 300 μl of ethanol for 20 minutes in dry ice. Do not add extra salt or extract with organic solvents. Wash the pellet with 70% ethanol, air-dry in the laminar hood, and resuspend in 100 μl of sterile water

3.5.1.4. Electroporation

- Use 1.4 x 10^7 cells per transfection
- centrifuge the cells 1500 rpm at 4 ˚C for 5 min
- Wash the cells with EM buffer and repeat centrifugation

- Resuspend cells in EM buffer at 4 x 10^7/ml.
- Transfer the DNA solution into a chilled 4 mm electroporation cuvette. Use sterile water for the control transfection
- Add 450 μl of cell solution to each cuvette
- Set the electroporator to 1,500 V and 25 F. Electroporate cells with two pulses with a 10 second interval
- Chill on ice for 2 min
- Transfer cells to a 25 cm^2 culture flask with 10 ml of 29-13 medium pre-warmed to 27 ˚C and incubate with mild agitation at 27˚C for 24 h
- Add blasticidin (10 μg/ml final), G418 (15 μg/ml final) and hygromycin (50 μg/ml final) 24 h post-transfection
- Transfer 2 ml of cells into 3 wells of 24 well plate and incubate in 27 ˚C in CO_2 incubator until culture will change color to yellow
- Transfer cells into 25 mm^2 flask with 8 ml of SDM supplemented with blasticidin to final concentration 10 μg/ml, G418 (15 μg/ml final) and hygromycin (50 μg/ml final)
- and continue stabilization of cell line at 27 ˚C with agitation

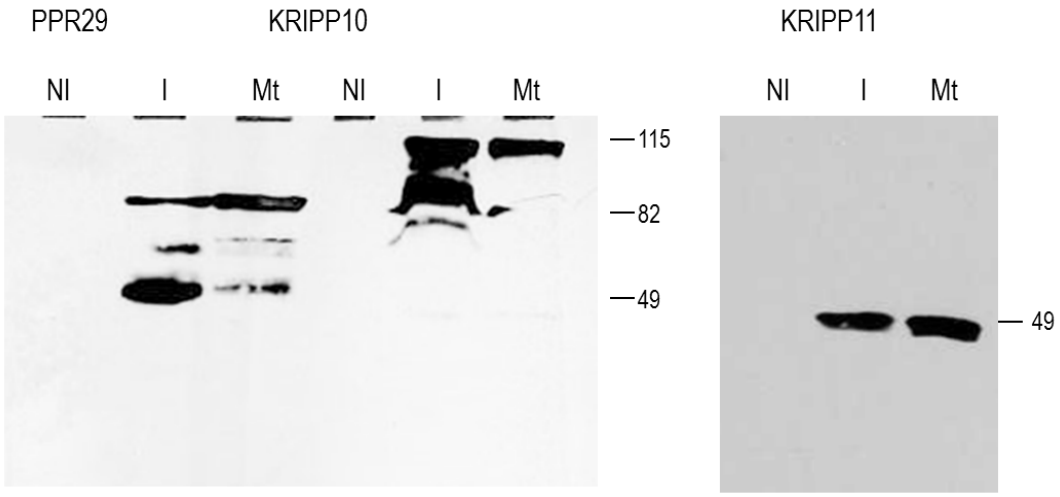
3.5.1.5. Cloning by limiting Dilution

- Add 100 μl of LD medium into wells 2-12 (all rows) of a 96-well flat-bottom plate
- Add 100 μl of LD medium into wells B1, D1, F1 and H1
- Add 300 μl of culture into wells A1, C1, E1 and G1
- Transfer 100 μl of culture from A1 to B1, C1 to D1, E1 to F1 and G1 to H1
- Perform 1:1 serial dilutions (100 μl) from lane 1 through lane 12
- Starting from lane 12, add 100 ul of LD medium to each well to achieve 200 μl of total volume
- Incubate the 96-well plate at 27 ˚C in humidified incubator with 5% CO_2
- Monitor growth under an inverted microscope. Expect to see cell growth in 10-14 days
- When the well with highest dilution becomes confluent and the next dilution remains clear, transfer the entire 200 μl - culture into 3 ml of LD medium in a 25 cm^2 flask and incubate upright without agitation for 24 h
- Start agitation (100 rpm) and continue until cell density reaches 5-10 x 10^6 cells/ml

- Add 3 ml of 29-13 medium with drugs, continue incubation for another 24 h
- Stabilize culture by diluting to 10^6 cells/ml 2-4-fold every 24 h until division time is consistent

3.5.2. Results

Protein expression was induced by adding tetracycline (to 1 μg/ml final concentration) to the growing cell culture. Non-induced (NI) and induced (I) cells were harvested 3 days after the induction of over-expression. Because all investigated proteins are found in mitochondria of *T. brucei,* our main goal was to ascertain that the expressed proteins get properly imported into this organelle, thus mitochondria from induced cells were isolated. Whole cell (I) or mitochondrial (Mt) lysates from approximately 2 x 10^6 cells were analyzed on the 12% acrylamide gel and transferred to PVDF Hybond membrane (GE Healthcare). PAP reagent (Peroxidase-Antiperoxidase soluble complex, Sigma-Aldrich) recognizing Protein A on the TAP tag was used for protein detection in concentration 1:5000. TAP tag is about 20 kDa, thus the expected sizes of our proteins are: '"PPR29" - 50 kDa; 'KRIPP10' - 86.5 kDa and "KRIPP11" - 47 kDa. The results clearly showed that no proteins expression was taking place in the NI cells. The 'PPR29" expression cell line showed a massive expression in whole cell lysate, however in mitochondria the protein amount was relatively low. Expression of "KRIPP11" was very clear without any additional non-specific products and the mitochondria showed the same amount of expressed protein as the cell lysate. "KRIPP10" showed some unspecific signal probably representing a degradation product in the whole cell lysate, but this non-specific signal was absent in the mitochondrial lysate, which contained the same level of expressed "KRIPP10" as the cell lysate (fig.3.5.1.). No further experiments were conducted by us with these cell lines so far due to time limitations.



PPR29+TAP tag – 50kDa

KRIPP11+TAP tag – 47 kDa

KRIPP10+TAP tag – 96,6 kDa

Figure 3.5.1.: Western analysis of cell lysates of the non-induced (NI) cells and cells harvested 3 days after RNAi-induction of over-expression of selected TAP-tagged proteins. The whole cell lysate (NI; I) and mitochondrial lysate (Mt) isolated from *T. brucei* PS were separated on 12% acrylamide gel, transferred onto PVDF membrane (GE Healthcare) and probed with PAP-reagent (Sigma Aldrich) that specifically recognize the Protein A on the TAP tag.

3.6. Preparation of constructs in pET101 for bacterial over-expression and antibodies production

For the preparation of antibodies, we chose proteins Tb927.8.4860 “KRIPP10“, Tb.927.8.6080 “KRIPP11“ and Tb927.7.2490 “KRIPP15“, representing the SSU* complex. Having specific antibodies may be essential for obtaining more information about the protein complex composition and its subcellular localization, and indeed represents a useful tools for many experiments which might be applied in further investigation of the function of this complex.

3.6.1. Methods

3.6.1.1. Cloning

A tetranucleotide CACC followed by an ATG start codon was added to 5'end of each forward (Fw) primer. The CACC nucleotides, base pair with the overhang sequence, GTGG, in pET TOPO® vector. For the reverse (Rev) primer we started just one codon upstream of the stop codon (Tab. 3.6.1.1.).

KRIPP15	
Fw	**CACCATG**CTTCAAAAGGCAGGCCGCCAC
Rev	AACAGCGCTGTTGCCGTTCCACTTCTTAAC

KRIPP10	
Fw	**CACCATG**CTGTCGCACGCCGTCCCCCGTC
Rev	TACCAATTGCTCCCCAGGCGAGCGACC

KRIPP11	
Fw	**CACCATG**CACCCGCGATTGTCGGGGCAG
Rev	ACCACGAGGTAAAGTGGGGGGCAGCG

Table 3.6.1.: Primers designed for "KRIPP10", "KRIPP11" and "KRIPP15" for protein expression and antibody production on pET101 vector.

- Use Phusion proof read polymerase (New England Biolabs) which does not add A 's on the end of the PCR product
- Transformation was done by following the Champion™ pET101 Directional TOPO® Expression manual (Invitrogen)
- Pepared construct were sequenced to verify that ORF was maintained and to avoid any possile protein mutation
- Prepared plasmid with sequenced insert were transfected into *E. coli* BL21 Star which are suitable for protein expression
- The success of the transformation was verified by restriction reaction and sequencing of prepared constructs

3.6.1.2. Overexpression

- The BL-21 cells containing prepared pET101 construct were cultured in 3 ml of LB medium agitate at 37 °C overnight
- The cell culture was diluted to 20 ml of fresh LB medium to the final concentration OD 600 approximately 0.2 and grow on the shaker at 37 °C till reach OD 0.4
- The culture was divided onto two half, the first part was induced by (IPTG+) to the final concentration 1mM, and the second half was used as a control (IPTG-)
- Grow the cell culture at 37 °C
- 1ml of the cells culture was harvested for KRIPP10 0.5 h, 1 h, 1.5 h and 2 h after over-expression induction, for "KRIPP11" at 2 h and 5 h after protein expression and for "KRIPP15" at 6 h and 24 h after the IPTG induction
- Cells were pelleted and resuspended in Sample Buffer
- Lysates were separated on 12% acrylamide gel and blotted to PVDF Hybond membrane (GE Healthcare)
- Probe the membrane with V5 antibody diluted to 1:5000 (V5 antibody detect V5 epitope on C terminus of pET101 vector)

3.6.1.3. Solubilization Assay (Following Champion™ pET101 Directional TOPO® Expression manual (Invitrogen)

- Cell cultrure of BL-21 with prepred plasmid was grown and induced with IPTG to final concentration 1mM like described in above „overexpression" and agiteted for KRIPP10 1h; 5 h for KRIPP11 and 24 h for KRIPP15 at 37 °C
- Pellet from 2 ml of culture was dissolved in "Lysis buffer"
- Add lysozyme to final concentration 1mg/ml and keep 30 min on ice
- The samples were 3 X frozen in liquid N_2 and thaw at 42 °C
- Spin 20 min at 4 °C for 16100 g
- The supernatant represents soluble fraction
- The pellet represents insoluble fraction
- Resuspend both in Sample Buffer
- Separate the soluble and insoluble fractions on the 12% acrylamide gel

- The gel was transfer on polyvinylidene difluoride (PVDF) membrane (GE Healthcare)
- Proceed with Western blot analysis using V5 antibody recognizing V5 epitope on the C terminus of PET101

Buffers:

SB sample buffer
4% SDS, glycerol
125 mM Tris-HCl
10% β-mercapthoethanol
0.004% bromphenol blue

Lysis Buffer
50 mM buffer KH_2PO_4 a K_2HPO_4
400 mM NaCl
100 mM KCl
10% glycerol
0.5 % Triton-X
10 mM imidazole
Adjust pH to 7.8

3.6.2. Results

Initially, sequences of genes cloned to the pET101 vector were verified by comparison with the *T. brucei* genome database. The genes did not contain any amino acid substitutions, nor were there shifts in their open reading frames. All three PCR-amplified fragments were cloned and transfected into the *E. coli* strain, BL-21, for expression. The selected colonies were seeded into the LB medium with ampicillin and at an optical density (OD_{600} ~3) of 0.4, and the expression of the cloned genes was induced with IPTG added to a final concentration of 1 mM. Samples of the cell lysates, taken at different intervals after the induction were analyzed in 12% SDS polyacrylamide gels. Non-induced (-IPTG) cells were used as a control. Proteins were then transferred to the PVDF membrane for Western blot analysis. Using the primary antibody V5 (expression constructs in the pET101 vectors contain a V5 and 6xHis tags on the C terminus) and mouse secondary antibody allows for specific detection of the target over-expressed proteins (Fig. 3.6.1). This procedure verified that a successful synthesis of targeted proteins was achieved in *E. coli*.

Before the over-expression of the targeted proteins in a big volume and its subsequent purification, it was necessary to determine whether the proteins are localized in the cytoplasm or in the inclusion bodies of bacterial cells. Induced and non-induced *E. coli* cells collected after 24 h of growth at 37 °C were lysed by

lysozyme and disrupted by repeated freezing in liquid nitrogen and subsequent thawing at 42 °C. The collected fractions, representing a pellet (P) and a supernatant (S), were analyzed by SDS - PAGE, which showed that all three proteins were insoluble (fig. 3.6.1.). This finding indicates that the expressed proteins are packed in the inclusion bodies.

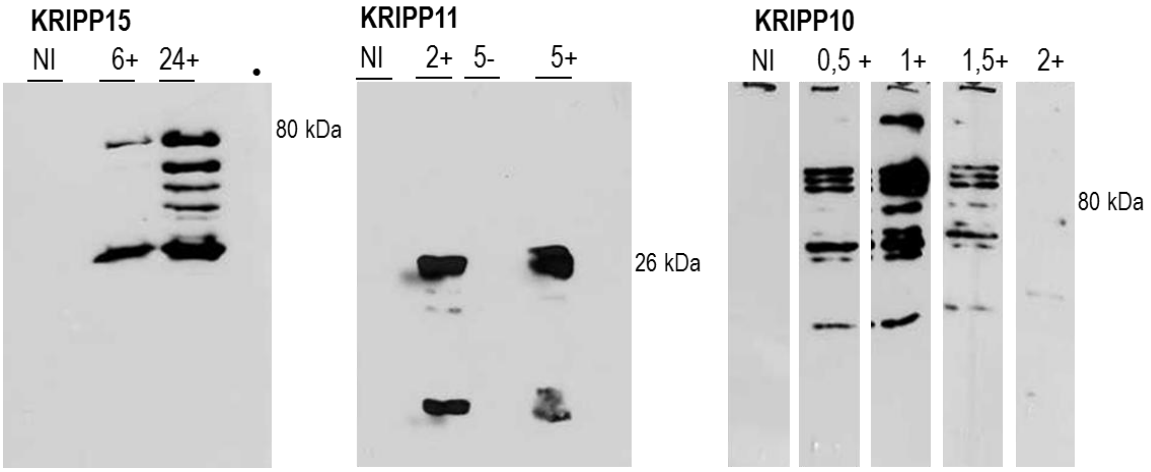


Figure 3.6.1.: Western analysis of lysates from induced (+) and non-induced (NI) cells collected after 6 and 24 h for “KRIPP15“; 2 and 5 h for “KRIPP11“ and 0.5 h, 1 h, 1.5 h and 2 h for “KRIPP10“ growth at 37 °C were loaded on a 12% acrylamide gel, transferred to PVDF membrane and probed with V5 antibody. The antibody binds to the protein at the expected size of 80 kDa (KRIPP15) 27 kDa (KRIPP11) and 77 kDa (KRIPP10) corresponding to the expected size.

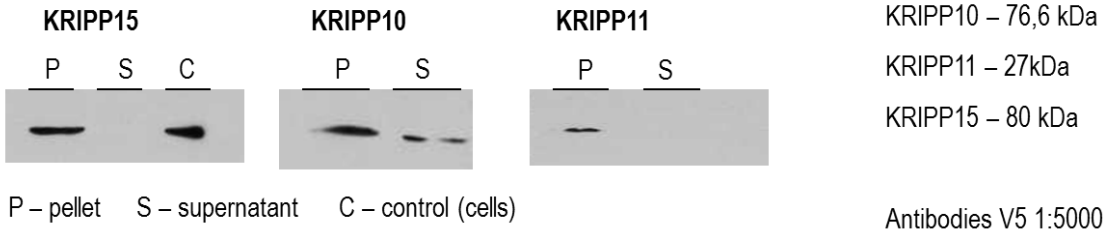


Figure 3.6.2.: Testing of “KRIPP15“, “KRIPP11“ and “KRIPP10“ proteins solubility. Induced cells harvested after 24 hour for “KRIPP15“; 5 h for “KRIPP11“ and 1 hour for “KRIPP10“ over-expression induction by IPTG. Pellet (P) represents insoluble fraction and supernatant (S) correspond as soluble fraction and cell lysate (C) were separated on 12% acrylamide gel and probed with V5 antibody specificly recognizing C

terminus of expressed protein. The expected size of the proteins are 80 kDa for KRIPP15, 27 kDa for KRIPP10 and 77 kDa for KRIPP11. Only small fraction of KRIPP10 expressed protein was detected in soluble fraction but the main part was insoluble. KRIPP15 and KRIPP11 were detected only in pellet, meaning that they are insoluble.

3.7. Functional Analysis of Tb927.8.2650 and Tb11.02.2710

In colaboration with Jiří Týč, we investigated a potential ribosome association of two proteins which were earlier found by masss-spectrometry in the TAP-isolated mitochondrial ribosomal fraction of *T. brucei* (Zíková et al., 2008). The first is Tb927.8.2650, it is conserved protein which belongs to metallo-beta-lactamase superfamily. Its homologues were found in humans playing a potential role in paroxysmal nonkinesigenic dyskinesia as a myofibrillogenesis regulator 1 (Ghezzi et al, 2009). This protein has a hydrolyse activity and contains a zinc ion binding site. Nearly 40% of proteins containing this functional domain represent transcriptional factors in humans. The remaining 60% of proteins that can bind Zn^{2+} are enzymes involved in ion transport (Andreiny et al., 2006). The second targeted protein is Tb11.02.2710. This protein is also conserved, it contains a well known domain – Rhodopsine (Rossmann-fold NAD(P) binding site). Homologues of this protein were found in human cells as a methyltransferase as well as in yeast as a part of SSU of mitochondrial ribosome. Usually proteins binding copper play a role in exchanging electrons with cytochome.

Moreover, as mentioned earlier, both proteins are probably associated with the SSU of *T. brucei* mitochondrial ribosome. The data, presented in this thesis, have lead us to the hypothesis that SSU* can exist in multiple compositions. Mainly because of the ablation of SSU* subunits was found to influence only some long tailed mRNAs, such as CoI and CyB, but not others, such as edited RPS12, we tentatively concluded that multiple forms of the SSU* with different protein composition can exist in the mitochondrion. To investigate this idea, further analyses would be necessary. However, as a starting point to probe into this hypothesis, we decided to look at the conserved proteins with no known function in trypanosomatids, yet those for which a putative association with the SSU has been shown.

3.7.1. Generation Tb927.8.2650 and Tb11.02.2710 knockdowns cell lines in PS *T. brucei*

Primer designing:

A BamH I restriction site was added to the 5´-end of the forward (Fw) primer and the Xho I site was added to the reverse (Rev) primer (Tab.2.1; sites are indicated in bold).

Tb927.8.2650
Fw: **GGATCC**GATAACCGCGAGGACGATAA
Rev: **CTCGAG**GGAAGTGCTTCTAACAGGCG

Tb11.02.2710
Fw: **GGATCC**AACATTACAAACATGCCTGAC
Rev: **CTCGAG**CCTTCATAAAGGCTGCTTGC

Table 3.7.1. Primers for the PCR amplification of the Tb927.8.2650 and Tb11.02.2710 fragments. In bold are the sequences recognized by the restriction endonucleases BamHI and XhoI.

- Both fragments were cloned by TOPO TA Cloning® kit (Invitrogen) to the TOPO vector and subsequently to the p2T7-177 vector (Fig. 3.7.1.).

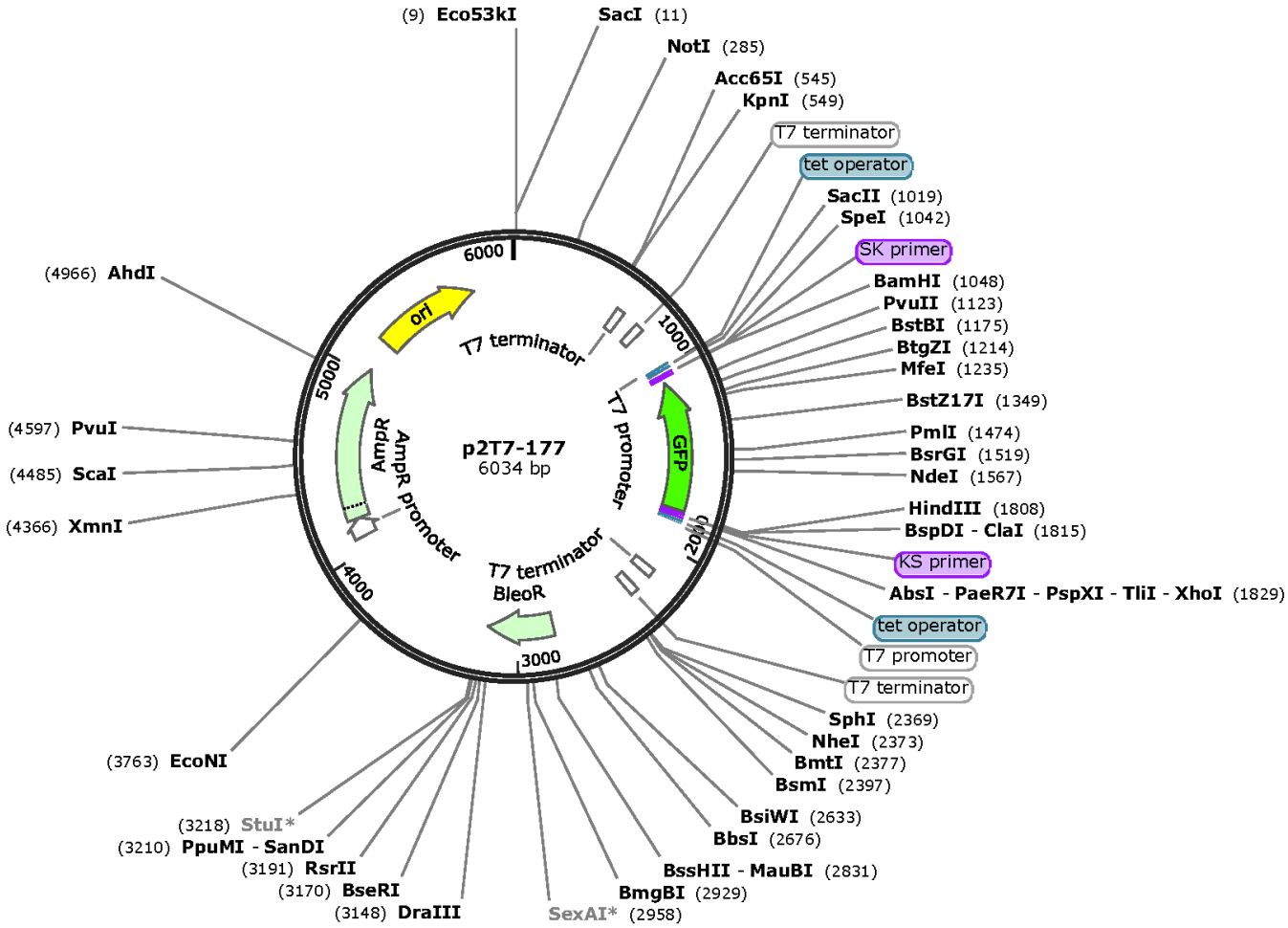


Figure 3.7.1.: Map of p2T7-177 vector.

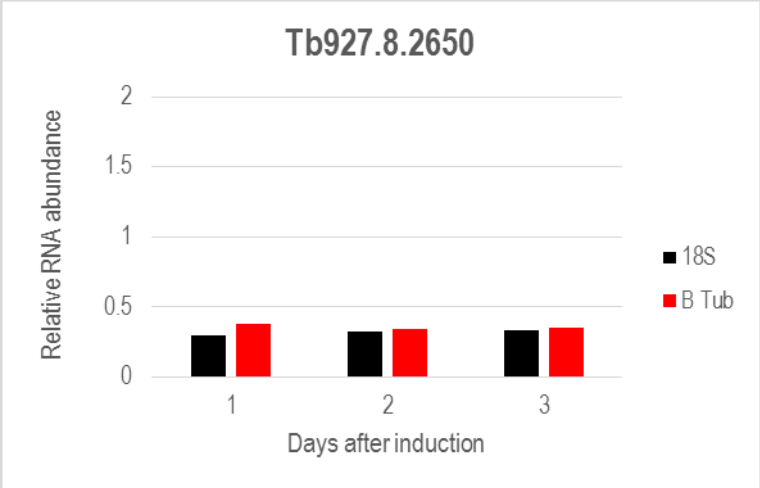
- Clones were verified by sequencing
- PS *T. brucei* cells were transfected and stable cell lines were selected using the same method previously described for pLEW70 constructs on pages 77-79

3.7.2. Results

3.7.2.1. Quantitative Real-time PCR

The prepared constructs were transfected into PS (strain 29-13) cells. After obtaining stable transformants by selection for phleomycin resistance, the cells were induced by tetracycline at a final concentration of 1 μg/ml and collected at 1st, 2nd and 3rd day post-induction for qPCR analysis to verify the down-regulation of each investigated mRNA. As a control, the non-induced cells (tet-) were grown and processed in parallel (Fig. 3.7.1.).

As a template for this assay was used cDNA generated from RNA collected at each data time point. The data were normalized to the measured level of cytoplasmic 18S rRNA and β-tubulin (B-tub), because these mRNAs are not expected to be affected by RNAi, and calculated using the Pfaffl method (Pfaffl et al., 2001). The result show a clear decrease of Tb927.8.2650 and Tb11.02.2710 mRNAs already at first day after the RNAi induction and this down-regulation is consistent throughout the investigated time period.



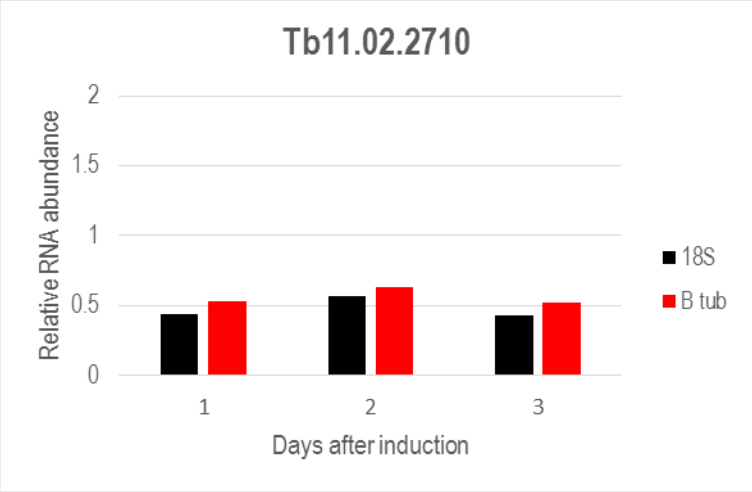
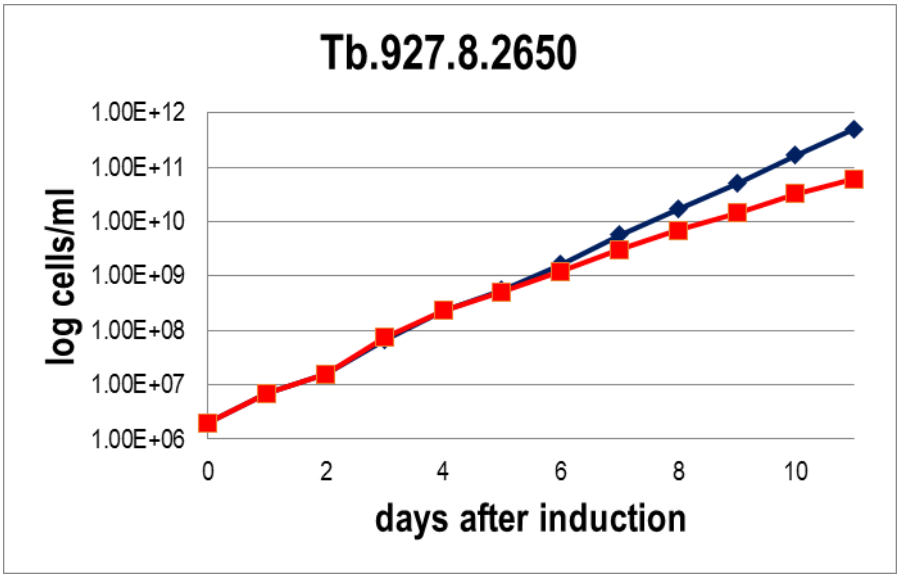


Figure 3.7.1.: Quantitative real time PCR analysis of mRNAs Tb927.8.2650 and Tb11.02.2710 performed on knock-down cell lines of these genes. The RNA levels averaged from three replicates were normalized to 18S rRNA and β-tubulin. The relative RNA abundance is expressed in linear scale.

3.7.2.2. Growth phenotypes of the knock-down cell lines

The stable cell lines were grown for 11 days in the presence or absence of tetracycline. The density (cells/ml) of the cell culture cultures was measured every 24 h after induction (Fig.3.). Inhibition of growth in induced cells starting to be apparent around day 5 of induction as compared to the non-induced cells.



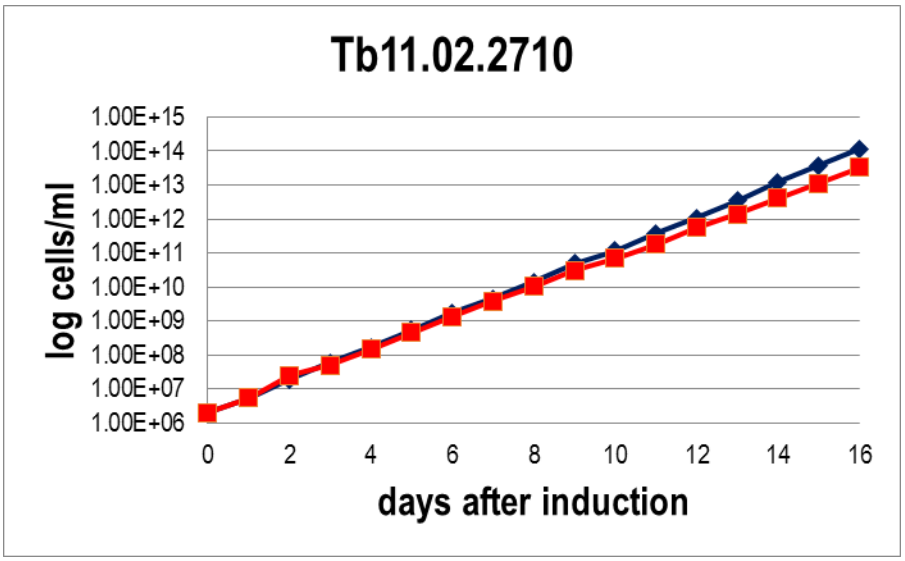
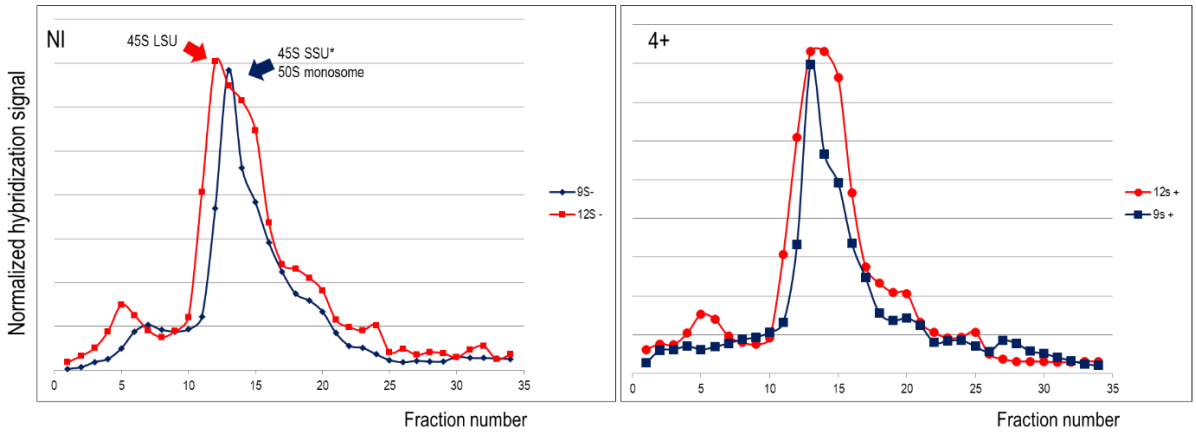


Figure 3.7.2.: Effect of down-regulation of Tb927.8.2650 (A) and Tb11.02.2710 (B) on the cell growth. Graph plotting the density of non-induced (blue lines) and induced (red lines) cells (cell/ml) against days after tetracyclin induction. The cells were diluted every day to the concentration 2 x 10^6 cells/ml. In both cases, the inhibition of growth becomes apparent on the 5th day after RNAi induction.

3.7.2.3. Mitochondrial ribosomal 9S and 12S sedimentation profiles in the knock-down cell lines

Similar to the functional analysis of the SSU* components “PPR29“, “Rhod“ and “200 kDa“, we have also peformed an investigation of the effects of RNAi-mediated ablation of the Tb927.8.2650 and Tb11.02.2710 proteins on sedimentation properties of ribosomal subunits. Because the growth curves showed only slight growth retardation on the 4th day after RNAi silencing of each targeted mRNA, we decided to test the cells harvested in this time point, instead of day 5 when the growth phenotype is fully apparent. This was thought to reduce secondary effects of the RNAi. The harvested cells were lysed, the lysates were loaded on 7-30% sucrose gradient and fractionated under the same conditions as used previously for “PPR29“, “Rhod“, “200 kDa“, S17 and L3. The gradients were fractionated onto 35 (A) or 17 (B) fractions, and isolated RNA was analyzed by slot-blot hybridization as described on pages 67 and 68.

A Tb927.8.2650



B Tb11.02.2710

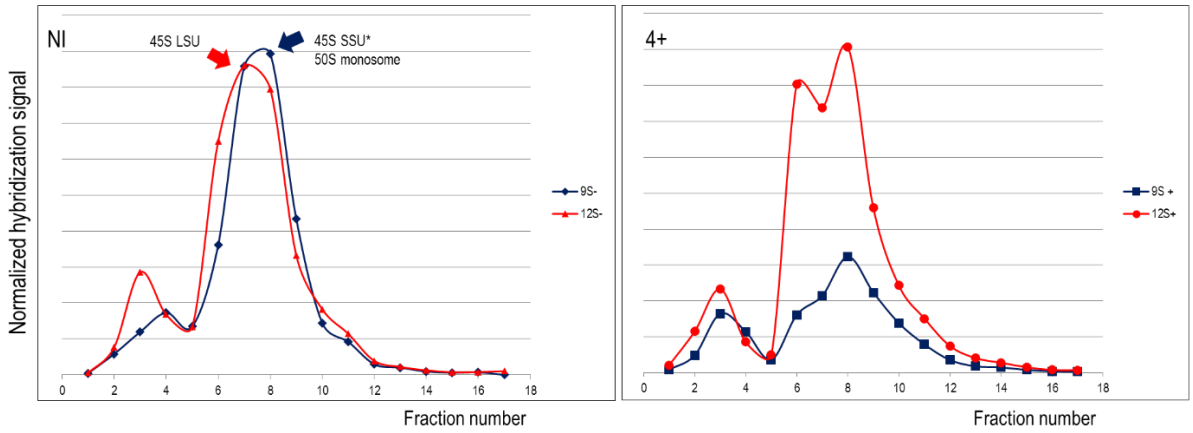


Figure 3.7.3.: Sedimentation profiles of 45S LSU and 45S SSU* ribosomal complexes after Tb927.8.2650 (A) and Tb11.02.2710 (B) RNAi down-regulation. 2 x 10^7 PS *T. brucei* cells harvested before induction (NI) and 4th day after induction (4+), cell lysates were separated on 7-30% sucrose gradient. RNA isolated from each gradient's fraction was visualized by hybridization with radioactively labeled oligonucletiodes recognizing the 9S and 12S rRNAs.

3.7.3. Conclusion

From the results we collected from the experiments on ablation ofTb927.8.2650 and Tb11.02.2710, we can conlude, that each protein is essential for PS *T. brucei,* and the silencing of Tb11.02.2710 subunit leads to drastic distruction of 45S SSU* complex on day 4 post RNAi induction. The latter observation strongly indicates that this protein can be 1) a structural component of this complex or 2) essential for its biogenesis (or both). Further experiments are necessary to shed more light on this issue.

4. Discussion

Mitochondrial mRNA maturation of *Trypanosoma brucei* is an intensively studied field, however significant gaps remain in our knowledge of the mechanism of recognition and recruitment of mature mRNAs to mitochondrial ribosomes, as well as many other aspects of the translation process. The aim of this thesis is to characterize an unusual complex termed 45S SSU*, which represents a heterodimer of the SSU lobe and the SSU-like part. We speculate that in this complex, the SSU-like lobe might play a role in the process of mitochondrial translation by recognizing long poly(A/U)-tailed of fully edited mRNAs and delivering them to the mitoribosome.

Although the 45S SSU* complex was originally described in *L. tarentolae* (Maslov et al., 2007), all experiments presented in this thesis were performed in *T. brucei*, primarily because of the experimental advantages conferred as a result of the susceptibility of *T. brucei* to RNAi manipulation. Nevertheless, our first step before commencing functional studies of this complex in *Trypanosoma* was to prove its existence in this parasite. No less important was the need to ascertain a way of separating the 45S SSU* from free LSU, which has a similar size and sediments in close proximity to that complex in sucrose gradients.

Using 0.5 M KCl and 1% dodecyl maltoside to lyse cells for gradient sedimentation resulted in a shift of the original ~45S LSU to 40S. These cell lysis conditions and three repetitions of the sedimentation through a 7-30% sucrose gradient minimized the amount of LSU in the preparation, allowing us to isolate the 45S SSU* complex for proteomic analysis (Ridlon at al., 2013). These results, coupled with previous mass spectrometry analysis of the mitoribosomal fraction of *T. brucei* (Zíková et al., 2008) provided the necessary evidence to establish the presence of the 45S SSU* complex in PS *T. brucei*, and the prior mass spectrometry analysis also provided information about the protein composition of this complex. At least 30 orthologs out of the 34 previously described in *L. tarentolae* (Maslov et al. 2007) were also found in *T. brucei*. (Zíková et al., 2008).

To demonstrate that the 45S SSU* and the LSU are two different complexes in *T. brucei*, RNAi was used to individually silence either the L3 subunit (part of the free LSU and the monosome) or the

S17 subunit (a component of the free SSU, the 45S SSU* and the monosome). Cells harvested at 1 to 5 days post RNAi induction were lysed, the lysate was separated on a sucrose gradient and fractionated into 35 fractions. RNA isolated from each fraction was slot-blotted on a nitrocellulose membrane and probed with radioactively labeled oligonucleotides specific for the 9S or 12S mitoribosomal RNAs. The results clearly indicated that silencing of L3 affects both free LSU and monosomes but has no significant impact on the 45S SSU* complex. On the other hand, after ablation of S17 we observed a significant decrease of monosomes, 45S SSU* and free SSU subunits, but no change in the level of LSU was detected. These data demonstrate that targeting of specific proteins affects specific complexes and further show that the 45S SSU* and the LSU are distinct separate complexes.

The accuracy of sucrose gradients used for our purposes was validated by sedimentation of *E. coli* ribosomes under the same conditions as those used for separation of *T. brucei* mitoribosomes. Isolated bacterial ribosomes and trypanosomatids mitoribosomes were sedimented on 7-30% sucrose gradient for 16 h, 17,000 rpm at 4°C and fractionated into 35 fractions. Fractions of the main peaks of ribosomal subcomplexes of *E. coli* were plotted against their known S values and data were fitted in a linear fashion. Subsequentialy, particular fractions of the *T. brucei* ribosomal complexes were superimposed onto this graph in order to calibrate the sucrose gradient and determine exact S values of *T. brucei* mitoribosomal complexes.

Once the presence of the 45S SSU* in *T. brucei* was verified and it was ascertained that this complex is not directly associated with the LSU, but insted represents a distinct and still unexplored entity, we could start our investigation of its function. As was mentioned previously, *T. brucei* is a better model organism for genetic manipulation than *L. tarentolae* because it contains a mechanism for RNA interference, which represents an important tool for monitoring effects of silencing genes. Theoretically, if the SSU* particle plays a role in mRNA processing or mitochondrial translation, the ablation of different SSU* protein components via RNAi should be lethal for the cell, therefore we strongly suspected that the SSU* would be essential for survival of thei pathogenic flagellate.

Three proteins specific for SSU-like lobe were selected for targeting by RNAi based on the presence within each of recognizable functional motifs. Two of these proteins have motifs which are

usually associated with the maintenance of structural stability of protein complexes: the first protein is Tb927.8.5200 (dubbed "200 kDa“) with a coiled-coil motif; the second protein is Tb927.6.4930 (termed "Rhod“) with a rhodanese domain. The third chosen protein is Tb927.5.1790 ("PPR29“), which contains a PPR domain that is anticipated to directly interact with RNA in a sequence-specific manner. Based on the functional motifs, the predicted function of “Rhod“ and “200 kDa“ is most likely involved in structural stability of the 45S SSU* complex, however, if our hypothesis about the recruitment of mature mitochondrial mRNA to mitoribosome is correct, the “PPR29“ containing PPR motif may be functionally very interesting.

After verifying the RNAi silencing of each gene by quantitative PCR, the effect of the RNAi-mediated gene silencing on cell growth was monitored daily after induction of silencing. The inhibition of growth was first observed on day 4 post-induction, indicating that each selected target gene is fundamental for the PS of *T. brucei* and supporting our hypothesis regarding the central importance of the 45S SSU* complex to the viability of these cells (Ridlon et al., 2013).

Because these three components are purported to be a part of the SSU-like lobe, their down-regulation should not have any direct effect on the monosome. Therefore, the S17 and L3 riboproteins were targeted to ascertain that the silencing of L3 affects only the free LSU and ribosome but not the free SSU, SSU* or 45S SSU*, while the S17 down-regulation causes changes in free SSU, SSU*, 45S SSU* and ribosome but does not affect free LSU subunits. Comparing the sedimentation profiles of the RNAi knockdowns of S17 and L3 with those prepared from RNAi knockdowns of “200 kDa“, “Rhod“ and “PPR 29“ provides another means of verification that 45S SSU* is a complex different from the mitochondrial ribosome. The results show that upon the selective ablation of the SSU* the level of monosomes and LSU remain unchanged.

Both the growth retardation phenotype of the “200 kDa“, “Rhod“ and “PPR 29“ knockdowns and the fact that 45S SSU* complex is independent from the mitoribosome attested to the importance of this complex but did not shed light on its function, indicating the need for additional experimental analyses.

The "central dogma" of molecular bigology postulates that DNA is transcribed to mRNA and protein is then synthesized by translation of mature mRNA. With the aim to verify our prediction about the role

of 45S SSU* complexes in mitochondrial translation, our next step would be to analyze the mRNA levels and subsequently changes in amount of translation products after RNAi silencing of the complexes. Because of the connection of SSU* with SSU, we chose a somewhat unconventional approach and looked at the problem in a reverse order. Meaning that in the first step of the functional investigation of 45S SSU* we directly checked the amount of mitochondrial translational products after ablation of the SSU* complexes. We observed that by day 2 post RNAi-induction, the level of mitochondrialy translated proteins was rapidly decreasing in all three RNAi knockdowns. This is consistent with the hypothesis that SSU* subunits influence translation machinery. In the next step we performed quantitative PCR to find out if SSU* ablation is the trigger of the observed translation collapse or rather represents a secondary effect of the gene down-regulation due to depletion of mature mRNAs. We focused mainly on CoI and edited CyB because these are the only two reliably identifiable mitochondrial proteins in these organisms (Maslov and Agrawal, 2012). Major losses of the *de novo* translated products were detected for CoI and CyB but a significant difference in the amount of each protein was also noticed. While the CoI translation product disappeared completely as soon as day 5 post-induction, the quantity of CyB was significantly reduced but still remained detectable. qPCR data validated the concomitant decrease in RNA level for each examined mRNA as well as the dissimilarity between the CoI and CyB mRNA levels.

These data support two contrasting conclusions. The first is the previously hypothesized function of the SSU* in the specific recognition and recruitment of mature mRNAs to mitoribosomes. The second is that the SSU* is directly involved in mRNA processing. This conclusion comes from the observation that the ablation of SSU* complexes affected not only the level of the translational product but also the respective mRNA level. mRNA processing is a multistep process and it is plausible that the SSU* participates in some step of this very complicated but well controlled machinery. We previously hyphothesized that an SSU-like complex specifically recognizes the A/U long tail on mature mRNA, however, no direct interaction between the SSU* components and the proteins responsible for the A/U extension (i.e. RET1 TUTase, KPAP polyA polymerase and KPAF 1 and 2 polyadenylaton factors) has been detected so far. Theoretically, if the levels of long-tailed mRNA (LT) would be decreased but those of short-tailed mRNAs (ST) would not, one could speculate that SSU* ablation does not cause mRNA downregulation directly but instead alleviates the requirement for LT CoI and CyB mRNA in the cell. To prove or disprove this hypothesis, we examined the effect of SSU* component knockdown on

LT and ST CoI, CyB and edited RPS12 mRNA levels. A decrease in CoI and CyB LT mRNA was detected even at day 1 post-induction of each target's RNAi (PPR29, Rhod and 200 kDa proteins), however there were no changes in edited (E) LT or ST RPS12 , and an increase in the ST form of CoI and CyB mRNAs was observed (Ridlon et al., 2013).

Another way of discerning whether the SSU* is involved in the translation machinery or the interface between mRNA processing and translation is to explore the effect of its ablation on interactions of LT mRNAs (the form intended for translation) with the 85S translation complex. Because the results of our Northern blot hybridization showed a decrease but not a complete disappearance of LT mRNAs after RNAi silencing, there was a possibility that the mechanism of adding long poly(A/U) tails was not affected by down-regulation of the SSU* by RNAi of its components. Importantly, this aspect of RNA maturation could be fully functional while mRNAs with long poly(A/U) tails could be degraded at a higher rate pre-translation. This degradation could be triggered by an unknown feedback mechanism sensing the failure of the translation process. Conversely, if association of LT mRNAs with the 85S ribosome persists this would suggest that translation is unchanged and completly functional, but the newly synthesised proteins undergo immediate degradation. This could be the reason of why we observed the reduced levels of translation products. To investigate these possiblities, we chose day 3 post RNAi induction as the time point for analyzing the mRNAs binding to mitoribosome. At this time point the retardation of cell growth is prominent, translation still works but slowly collapses, and the amount of CoI and E CyB LT forms shows a noticeable decrease (Ridlon et al., 2013). The reduction of LT forms was significant for both examined mRNAs, lending additional credence to the idea that SSU* complex actually fills the gap in between mRNA maturation and translation.

This conclusion brings us back to the translation products of CoI and CyB and the unresolved question of why the amount of each protein is influenced with different dynamics. It surfaced during the discussion of unpublished data with Ingrid Škodová that the newly synthesized CoI product is entirely incorporated into a cytochrome c oxidase complex. However, the fate of the CyB product is more complicated; most of the newly synthesized CyB is not found incorporated into cytochome bc1 complex, but rather remains in association with other proteins (probably chaperones) as a small sized protein complex. Apparently, the amount of synthesized CyB is excessive and overloads the system. In

any case, association of Cyb with other proteins can cause its preservation or at least a lower rate of degradation, while due to the absence of a similar protection mechanism, any excess CoI, if it exists, is immediately degraded. It remains unclear what causes these differences in the post-translation fate of these two proteins.

A very interesting and also unexpected finding was the accumulation of short tail (ST) mRNAs after the SSU* knock-downs. An explanation for this phenomenon is still lacking, and the issue remains open to speculation. One reasonable possibillity is that the 45S SSU* complex is connected directly to the poly-adenylation/uridylation complex and that SSU* ablation directly affects the process of LT formation on mRNAs. Another possibility is that there is a mechanism that enables a cell to recognize a collapse of mitochondrial translation and stop the production of mature mRNAs destined for translation. However, as mentioned earlier, no interaction between 45S SSU* compplexes and the A/U-tail elongation machinery has been detected so far.

The fact that E RPS12 mRNA was not affected by the SSU* loss-of-function suggests that the chosen protein components of this complex confer the specificity for certain mRNAs (such as CoI and CyB) but not for others (such as edited RPS12). Another alternative is that SSU* exists in several subclasses which differ somewhat in their compositions, and that a particular subclass of SSU* complexes interacts with a certain set of mRNAs. To investigate this idea about the existence of SSU* subcomlexes with different protein compositions, we decided to examine two additional proteins (Tb927.8.2650 and Tb11.02.2710). Both proteins contain binding domains: Tb927.8.2650 has a zinc binding site and Tb11.02.2710 can bind copper. More importantly, both proteins were found in association with the SSU of the mitochondrial ribosome of *T. brucei*. Human homologues were found for both proteins and Tb11.02.2710 even exhibits a yeast homologue that associates with the yeast ribosomal SSU. PS *T. brucei* cell growth was retarded as soon as the 4th day after RNAi silencing of either Tb927.8.2650 or Tb11.02.2710. This time point was therefore used to study effects of the silencing of these proteins on the 45S LSU and 45S SSU*. As such, the sedimentation profiles of 9S and 12S rRNAs demonstrate that ablation of Tb927.8.2650 does not influence either of the 45S complexes, however Tb11.02.2710 down-regulation clearly causes a collapse of 45S SSU* complex. This suggests that Tb11.02.2710 plays an important role in maintaining the mitoribosomal integrity. However, it would require significant additional work, which is outside the scope of this study, to

determine whether this subunit is also esential for mitochondrial translation, and further, whether it is a part of the SSU* complex that significantly regulates other specific mitochondrial mRNAs.

As with any new study, the work presented herein has raised numerous questions and generated hypotheses that await being proven or disproven. However, this research also made a first step toward mapping a so far unknown process in the *T. brucei* mitochondrion – the interface between mRNA processing and mitochondrial translation. Our data point at the lilkely participation of SSU* complexes in this process, but the details of these interactions remain unclear. Thus, the possibility that the SSU* complex is involved in mRNA recruitment to the mitochondrial ribosome still remains hypothetical, however our results support such a contention. In yeast many different proteins are involved in specific recognition of mRNAs intended for translation. Thus, if we speculate that initiation of translтion in trypanosomatids is similiar to yeast, it makes sense that ablation of specific targets (subclasses of the SSU* complexes) would impact only some mRNAs while others remain unaffected. Future work should shed light on this and other questions. Potentially productive approaches include TAP-tag analysis of 45S SSU* subunits, because targeting of specific proteins that are part of the SSU* could provide important information about the protein composition of the complex as well as the possible association of other proteins. Also useful would be to finish the work on production of antibodies against the 45S SSU*, which could be used for monitoring complex composition and integrity and, most importantly, for investigating protein localization.

5. Conclusions

This thesis is mainly focused on translation intiation of mitochondrial mRNAs in *T. brucei*, primarily on uncovering the function of a unique complex termed "45S SSU*" by studying effects of its ablation by RNAi.

Our experimental data clearly proved the presence of this complex in *T. brucei*, as well as the fact that in this parasite, as in *L. tarentolae*, the two most abundant ribosomal complexes are 45S SSU* and 40S-45S LSU. The 50S ribosomes (monosomes) are also present but represent a relatively minor fraction among the ribosomal complexes. Additionally, we have shown that 45S SSU* is a distinct complex and it is independent from the LSU.

The main conclusions based on the results obtained after silencing of the three targeted proteins specific for the SSU* moiety of 45S SSU* complex ("200 kDa", "Rhod" and "PPR 29") by RNAi, are as folows:

1. 45S SSU* complex is present in *T. brucei* and is essential for its viability.
2. The silencing of "200 kDa", "Rhod", "PPR 29" and Tb11.02.2710 triggers a collapse of the 45S SSU* complex on day 2 post RNAi-induction.
3. Mitochondrial translation is inhibited as soon as 2nd day after induction of RNAi silencing of the targeted components; and on 3rd day post RNAi-induction, the translational products, primarily of CoI and CyB, almost completely disappear.
4. A substantial difference between the LT and ST forms of CoI and CyB mRNAs was detected on 1st day after SSU* silencing. The amount of LT mRNAs is drastically reduced, however, the CoI and CyB mRNAs containing ST are accumulating in the organelle. The total steady state level of unedited CoI and edited CyB mRNAs remain unchanged.
5. The amount of associated LT CoI and CyB mRNAs with the 85S translational complex rapidly decreased already on day 3 post induction.
6. In contrary, no difference between the LT and ST mRNA was observed for edited RPS12 molecules. Similarly, the level of LT RPS12 associated with the 85S ribosomal complex remains unaffected.

Based on our experimental data, we summarize that the 45S SSU* complex is an indispensable component of translation initiation of the unedited CoI and edited CyB. However, since its ablation does not show any significant impact on the edited RPS12, the most plausible explanation postulates that this complex is specific for only a subset of mitochondrial mRNAs. We further speculate on the existence of multiple subclasses of the 45S SSU* complexes with different specificities.

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7. List of abbreviation

AA	Amino Acid
BF	Bloodstream Stage of *T. brucei*
Co	Cytochrome oxidase
CyB	Cytochrome reductase subunit B
E	Edited
FBS	Fetal Bovine Serum
fRNAMet-tRNA	formylated Methionine-tRNA
gRNA	guide RNA
HAT	Human African Trypanosomiasis
IF	Initiation Factor
Kb	Kilo bases
kDNA	kinetoplast DNA
KPAF	Kinetoplast Polyadenylation Factor
KPAP1	Kinetoplast Poly (A) Polymerase 1 Complex
LSU	Large Ribosomal Subunit
LT	Long Tail
Mt	Mitochondrial
MRP	Mitochondrial Ribosomal Protein
NI	Non-induced
ND	NADH Dehydrogenase
nt	nucleotide
ORF	Open Reading Frame
OXPHOS	Oxidative Phosphorylation
PPR	Pentatricopeptide Repeat
PF	Procyclic Stage of *T. brucei*
qPCR	quantitatice Real-Time PCR
RET	RNA Editing TUTase
RNAi	RNA interference
rRNA	ribosomal RNA molecule

RPS12	Ribosomal Protein S12
RT	Reverse Transcriptase
S	Svedburg units
SB	Sample Buffer
SSU	Small Ribosomal Subunit
ST	Short Tail
TA	Translation Activator
tet	Tetracycline
tRNA	transfer RNA molecule
TUTase	Terminal Uridylyl Transferase
U	Uridine
UTR	Untranslated Region
Us	Uridines
WT	Wild Type

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