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Bachelor thesis

2017   Anita Bär

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Functional analysis of Tb927.9.2900, a protein involved in pre-18S rRNA processing in *Trypanosoma brucei*

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Anita Bär

Supervisor: RNDr. Drahomíra Faktorová, Ph. D.

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Annotation

The aim of this thesis was to perform functional analysis of Tb927.9.2900 protein in *Trypanosoma brucei* and to verify whether this protein is involved in processing of pre-18S ribosomal RNA.

Declaration

I hereby declare that I have worked on my Bachelor thesis independently and used only the sources listed in the bibliography.

I hereby declare that, in accordance with Article 47b of Act No. 111/1998 in the valid wording, I agree with the publication of my Bachelor thesis, in full to be kept in the Faculty of Science archive, in electronic form in publicly accessible part of the STAG database operated by the University of South Bohemia in České Budějovice accessible through its web pages. Further, I agree to the electronic publication of the comments of my supervisor and thesis opponents and the record of the proceedings and results of the thesis defence in accordance with aforementioned Act No. 111/1998. I also agree to the comparison of the text of my thesis with the Theses.cz thesis database operated by the National Registry of University Theses and a plagiarism detection system.

České Budějovice, 11.08.2017

Anita Bär

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Contents

1   INTRODUCTION

1.1   The kinetoplastids

Trypanosomes are parasitic protozoans which belong to the eukaryotic subgroup Excavata, phylum Euglenozoa, order Kinetoplastida, a group named after the kinetoplast. The kinetoplast is a network of mitochondrial DNA (kDNA) consisting of thousands circular DNA molecules (Shapiro et al. 1995). Some other representative species of kinetoplastids are for example *Leishmania*, *Bodo*, *Phytomonas* or *Cryptobia*. The subgroup Trypanosomatida is the best studied group of kinetoplastids due to the fact that several of its members are responsible for various fatal diseases. These include the Human African Trypanosomiasis (HAT), also known as sleeping sickness, and Chagas disease caused by the *Trypanosoma* species as well as leishmaniasis caused by the *Leishmania* species. The research on these parasitic protists is therefore needed in order to provide clues for drug targeting.

1.2   Human African trypanosomiasis and the parasite's life cycle

One of the most studied parasitic protists from the order Kinetoplastida, genus *Trypanosoma* is *Trypanosoma brucei*. There are three subspecies of *T. brucei*: *T. b. brucei*, *T. b. gambiense* and *T. b. rhodesiense*. Only the latter two pose a threat to human health and cause African sleeping sickness, while *T. b. brucei* is susceptible to lysis by the trypanosome lytic factor-1 (TLF-1) (Stephens et al. 2012).

The complex life cycle of *T. brucei* includes different hosts and requires the cells to undergo morphological changes (Figure 1). The hosts are comprised of the insect vector, the tsetse fly (*Glossina spp.*), and a range of mammals (human, cattle, camel).
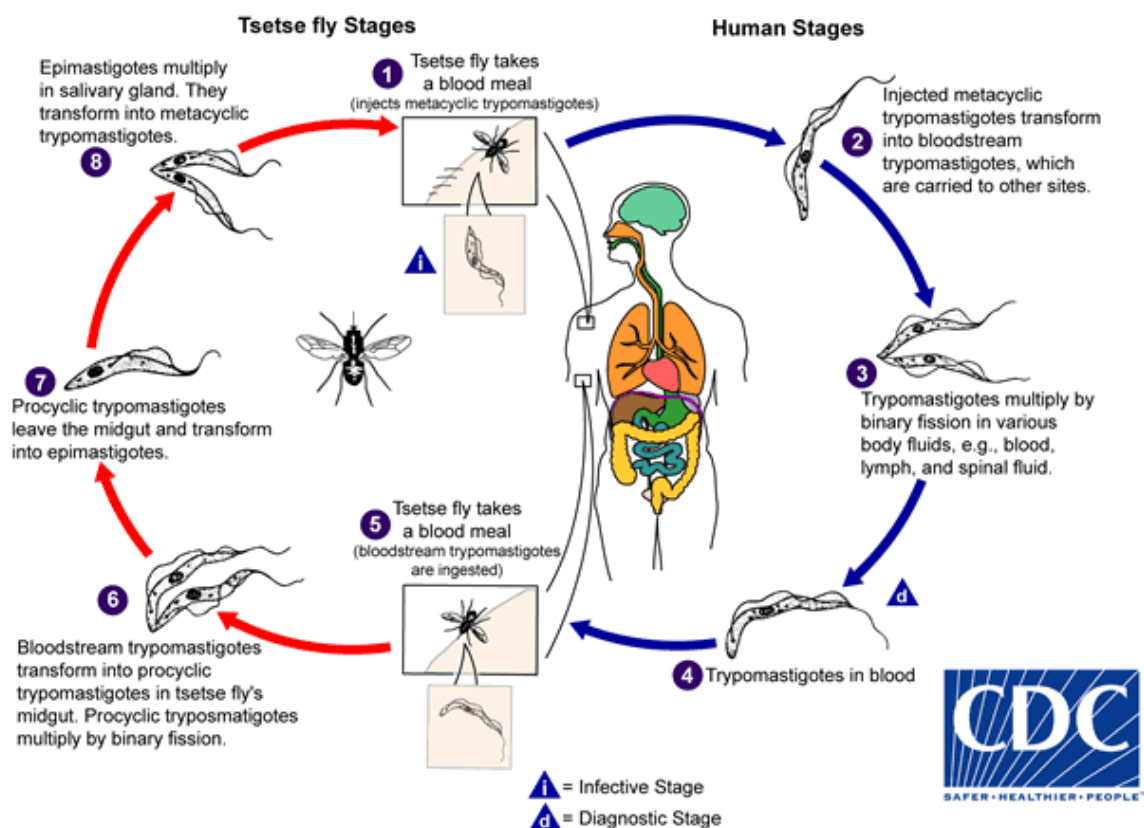
Figure 1 : Life cycle of Trypanosoma brucei (https://www.cdc.gov/parasites/sleepingsickness/biology.html).

After the infected vector bites the mammal in the course of its meal, the non-dividing metacyclic form of trypanosomes enters the bloodstream. These are preadapted for survival in the vertebrate host thanks to their coatings with variant surface glycoproteins (VSG) (Barry et al. 1998). This surface is the key feature to evading the host's immune response and its constant change in composition allows the parasite to stay undetected by the antibodies produced by the immune system. Subsequently the trypanosomes undergo morphological changes to their typical slender trypomastigote bloodstream form. These spread then from the site of infection through lymph vessels and lymph nodes into the bloodstream. There they divide by binary fission and further disperse throughout the body. The energy metabolism of these trypomastigotes is based on glycolysis of the glycose present in the blood of the mammal host (Bringaud et al. 2006). HAT symptoms occur in two stages. One to three weeks after the infection, the symptoms of the first stage start developing. They include fever, headache, joint pain and fatigue. Once the parasite invades the central nervous system by crossing the blood-brain barrier, the second stage of HAT begins. In this stage individuals suffer

from a disrupted sleep cycle, a prominent symptom that is responsible for the name 'sleeping sickness' (Lundkvist et al. 2004). Furthermore, limb paralysis, confusion and other sensory, motor and psychiatric disturbances evolve in the patient. At the peak of parasite density, a trypanosome factor accumulates, inducing the transition of the slender proliferative forms to stumpy non-proliferative forms (Matthews et al. 2004). These newly formed trypomastigotes are ready to be taken up by an uninfected tsetse fly. Upon ingestion by a blood meal, in the fly's midgut a transformation of the parasite occurs. This includes the loss of the VSG coat and subsequent gain of procyclins on its surface (Matthews et al. 2004), cell morphology changes, metabolic changes (Bringaud et al. 2006) and the entry from division arrest into proliferation. These modifications give rise to the procyclic form of the trypanosome which multiply by binary fission. Subsequently, the trypomastigotes travel to the fly's salivary gland, where they transform into epimastigotes. The epimastigotes undergo epi-trypo cell division by asymmetrically dividing into the metacyclic trypomastigote form, which is then again infective for mammals (Ooi 2013). This concludes its life cycle.

T. b. rhodesiense causes the acute form of the disease, termed the East African trypanosomiasis, where death can occur within months. *T. b. gambiense* is responsible for the most infections in humans and is spread throughout west and central Africa. It causes the chronic form of HAT, termed West African trypanosomiasis, that has a slower onset than the acute form and can last several years before death. Treatment of HAT, especially when the disease has progressed to the second stage, involves toxic medications that have severe side effects and can result in death of the patient. HAT is a threat to over half a billion people living in Africa. New treatments are sought for, given that the development of vaccinations is very unlikely due to the ability of the parasites to change their VSGs (Pays et al. 2004).

1.3   *Trypanosoma brucei brucei*

In contrast to *T. b. gambiense* and *T. b. rhodesiense*, *T. b. brucei* (here further called T. *brucei*) causes nagana in cattle and is not infective for human, but it is still closely related to the other subspecies and therefore can be used in many studies instead of them to elucidate their cell biology. Due to the large number of applications and methods that can be used and its easy cultivation in laboratories, *T. brucei* turn out to be a excellent model organism.

T. brucei is a unicellular organism of size ranging between 8 and 50 μm. It has elongated shape, streamlined at both ends (Figure 2). A very characteristic feature is the flagellum, which originates from the parabasal body and ends in the whip-like free flagellum. Additionally, a fin-like extension of the membrane
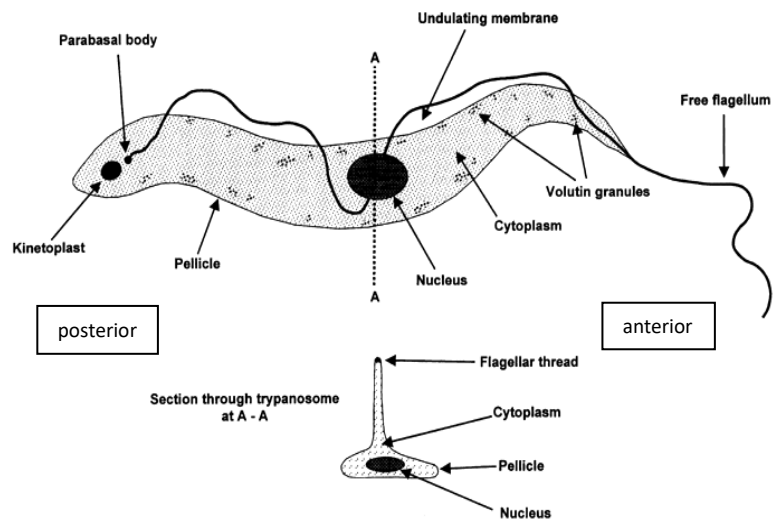

Figure 2: Illustration of fundamental features of Trypanosoma (Uilenberg et al. 1998).

(undulating membrane) is running across its body, which is associated with the flagellum.

The pellicle (cell membrane) of the protist is flexible enough to ensure movement. All these characteristics grant *T. brucei* motility in fluids of the host, like blood. The cytoplasm contains organelles like the nucleus, Golgi and endoplasmatic reticulum. There may be also seen volutin granules in the cytoplasm. These structures could have various origins, like food reserves or as a result of an immune reaction of the host (Ormerod 1958). A robust, tightly spaced microtubule corset of the cytoskeleton protects the parasite from damage as it limits access to the cell body, maintaining the cell shape (Sherwin et al. 1989). The position of one of the trypanosome's most distinctive feature, the kinetoplast, varies with the different stage of the life cycle and species. It is associated with the parabasal body, which is connected to the basal body by a fibril or a thread. Cells with a kinetoplast of posterior position in respect to the nucleus are termed trypomastigotes, while cells with their kinetoplast positioned anterior to the nucleus are termed epimastigotes (Ooi 2013). The single large mitochondrion which contains the kinetoplast is stretched out from the posterior to the anterior of the cell. Its structure differs in the different stages of the life cycle of the parasite, helping it to adjust to different environments by changing its mode of metabolism.

The two forms cultivated and used in most of the experiments, just as in this project, are the procyclic and the bloodstream form. The differences between them are their cell morphogenesis and cytokinesis, their cytoskeletons, the kinetoplast positioning, modes of metabolism and different environments needed for survival (Wheeler et al. 2013). This means

that the cells have different requirements for cultivation. One of them is the temperature that they are kept in. The procyclic form of *T. brucei* grows in temperature of 27°C, while the bloodstream form needs 37°C + 5% CO_2. This is due to the formerly described environments that they exist in naturally, one being in the midgut of the tsetse fly and other in the mammalian host. Furthermore, the medium in which the trypanosomes are grown is different, as a result of different metabolism modes. The mitochondrion of the bloodstream stage is smaller in size and has few cristae, since it relies on the glucose present in the blood of its host (Bringaud et al. 2006). Whereas the mitochondrion of the procyclic stage is fully functional and has many cristae, relying rather on the amino acids present in the insect's guts.

Just as for other model organisms, like the fruit fly *Drosophila melanogaster*, the bacterium *Escherichia coli* or the plant *Arabidopsis thaliana*, there is a variety of genetic tools that are available for the manipulation of this parasitic protozoan. The most prominent being RNA interference (RNAi) and generation of gene knock-outs, therefore allowing investigations of functions and properties of the trypanosome's proteins. Another reason for *T. brucei* being a good model organism is the fact that its genome has been fully sequenced and can be accessed easily on the internet (http://tritrypdb.org/tritrypdb/).

1.4 RNA interference

Studying the function of individual genes is crucial to understanding the roles of individual proteins and elucidating specific processes in cells. In *T. brucei,* two different approaches can be used to do that - methods based on homologous recombination (gene knock-outs, gene tagging) and the RNA interference (RNAi). The discovery of the cellular mechanism called RNA interference was a methodological breakthrough as it allowed to produce phenotypes in the cells faster than with homologous recombination methods and enabled the study of essential genes.

RNAi occurs naturally in cells and it is a tool for the control of the expression of protein-coding genes in a cell. Eukaryotes use RNAi as a natural defense against viruses (Clayton 2004) and mobile genetic elements, like transposons (Tabara et al. 1999). In 1998 Andrew Fire and his colleagues discovered that by injection of sense and antisense strands of RNA into the nematode *Caenorhabditis elegans,* its gene expression could be manipulated (Fire et al. 1998).

In 2006 this discovery earned its creators the Nobel Prize in Medicine or Physiology. Later in 1998 Huân Ngô et al. discovered that it is possible to use RNAi in *Trypanosoma brucei*. In their experiment, they electroporated double-stranded RNA molecules (dsRNA) into the cells, which resulted in strong phenotypes. Unfortunately, the effects were only transient.

In the process of RNAi, a dsRNA is bound by the endoribonuclease DICER, which cleaves it into double-stranded fragments of about 20 base pairs (bp). These fragments are called small interfering RNAs (siRNAs) and they interact with a ribonucleoprotein, the RNA-induced silencing complex (RISC). RISC unwinds the dsRNA and allows the created guide strand to bind to its complementary sequence on a messenger RNA (mRNA). After binding to the corresponding mRNA, the RISC-induced nuclease activity degrades the mRNA strand. Therefore, the corresponding gene expression is silenced, since the respective mRNA cannot be translated into a protein. By knocking down the expression, the function of the protein can be examined.

In trypanosomes, exogenous dsRNA is recognized and cleaved by TbDcl1, a DICER-like protein, into siRNAs of 25 bp. These are then unwound by TbAGO-1, a member of the argonaute (AGO) family, so that the exposed ssRNA in the AGO1-siRNA complex can be recognized by a specific mRNA. Subsequently, the

Figure 3: Scheme of the RNAi pathway in trypanosomatids (Balaña-Fouce & Reguera 2007).

mRNA is cleaved before it can be transcribed (Balaña-Fouce & Reguera 2007).

RNAi became a powerful tool for the investigation of genes, after ways to create cell lines that produce dsRNA directed against specific mRNA upon induction with a substance were found. One way of achieving this, is the incorporation of a vector with a tetracycline-inducible promoter and two copies of the target gene in sense and antisense orientation, which are

separated by a stuffer fragment of about 700 bp (Shi et al. 2000). This results in a hairpin loop dsRNA. Another approach uses a vector that contains the target gene flanked by head-to-head T7 promoters that are also inducible by tetracycline (LaCount et al. 2000). The result is the production of complementary RNA molecules that hybridize into a linear dsRNA molecule. These methods make it possible to continuously synthesize dsRNA in transgenic *T. brucei* that constitutively express T7 RNA polymerase and the tetracycline repressor (Wirtz et al. 1998). The transcription of the dsRNA starts by addition of tetracycline, which results in release of the tetracycline repressors.

While RNAi provides a fast and efficient way to study effects of genes on the viability and growth of cells and processes in the cells, there are also disadvantages to this method. Even though the protein is downregulated, it might never completely vanish, but rather become undetectable. The efficiency of RNAi is at approximately >80% of mRNA reduction (Motyka & Englund 2004). Therefore, knocking out a gene completely poses a 'cleaner' approach, although this is not possible for essential genes.

1.5   rRNA processing

Ribosomes are crucial components of the cell. They translate the messenger RNAs into proteins. Generally, ribosomes from bacteria, archaea and eukaryotes are very similar to each other and have the same function in every organism. Observed differences are in their sizes, sequences and the ratios of rRNA to protein.

Eukaryotic ribosomes - 80S (S̲ means Svedberg unit, a non-metric unit for sedimentation rate) are composed of two parts, the large 60S subunit (LSU) and the small 40S subunit (SSU). Generally, they contain more than 70 ribosomal proteins and 4 rRNAs, which together constitute the typical shape of the ribosome. The LSU rRNAs consist of 25S/28S, 5.8S and 5S rRNA, the SSU consists of 18S rRNA.

The rRNA processing and ribosome biosynthesis is a complex essential process (Figure 4) that begins in the nucleolus, continues in the nucleoplasm and is completed in the cytoplasm (Tschochner & Hurt 2003).
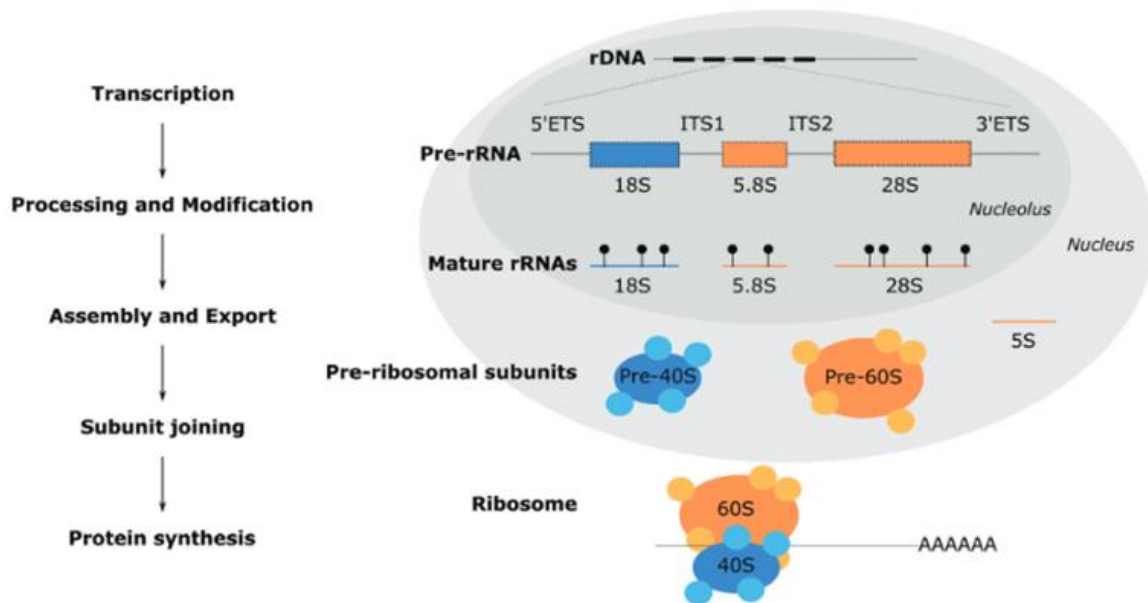
Figure 4: Scheme of ribosome biosynthesis in yeast (Ogawa & Baserga 2017).

In yeast, the ribosomal DNA (rDNA), the DNA that codes for the rRNA precursors, is organized in repeated clusters. The precursor for 25S/28S, 18S and 5.8S rRNA is transcribed by RNA Polymerase I together in one piece as the 35S pre-rRNA in the nucleolus, while the precursor for 5S rRNA by RNA Polymerase III in the nucleoplasm. The 35S precursor is polycistronic and has the structure 5'-[ETS/18S/ITS1/5.8S/ITS2/28S/ETS]-3'. ITS and ETS are internal and external transcribed spacers, respectively. After transcription, the transcript is modified by pseudouridylation and 2'-O-ribose methylation. Pathway to maturation of rRNA is a complex process that involves several small nucleolar RNAs (snoRNA) as well as and many proteins including endonucleases and exonucleases (Venema et al. 1999).

In evolutionary early divergent eukaryotes, like trypanosomes, many processes in the cell exhibit special features. It is not therefore surprising that it is also true for rRNA processing. LSU is fragmented to 2 large fragments (LSUα and LSUβ) and 4 small rRNAs (srRNA1-4), and 18S rRNA is very large compared to 18S rRNAs of other organisms (Campbell et al. 1987, White et al. 1986). The differences between the rRNA processing in yeasts and trypanosomes are shown in Figure 5.
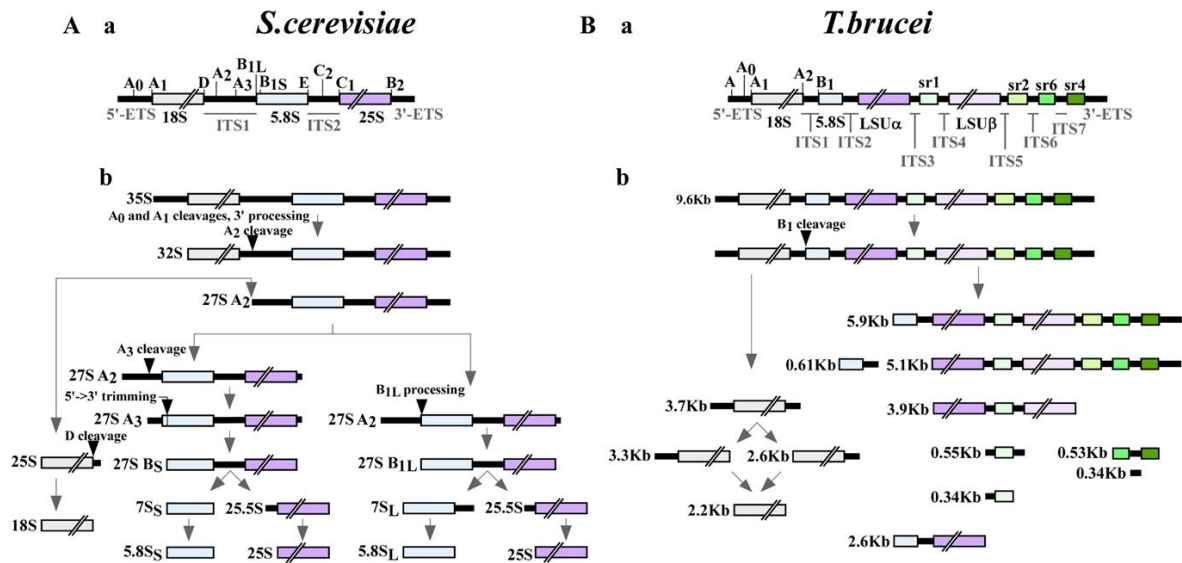
Figure 5: The differences between the rRNA processing steps in yeast and trypanosomes (Michaeli 2011).

For example, the processing of pre-rRNA in yeasts is initiated by cleavage of 5´ETS, while in trypanosomes first step involves the separation of 18S rRNA from the rest of the transcript. More than 140 snoRNAs were found in trypanosomes to be involved in this machinery (Gupta et al. 2010), however our knowledge of protein factors that play role in rRNA processing and maturation is very limited (Michaeli 2011, Fleming et al. 2016).

1.6   Protein complexes involved in rRNA processing

Large protein complexes are crucial for direction of rRNA biogenesis. Late stages of SSU and LSU maturations are described well, while relatively little is known about early stages of 18S processing. In the beginning of this process, a large ribonucleoprotein (RNP) complex, called SSU processome, is assembled (Dragon 2002). It is constituted from numerous ribosome assembly factors, like UtpA, UtpB, UtpC and U3 small nucleolar RNP. The SSU processome is an intermediate product of the SSU maturation process and so far, it is best described in yeast. This large RNP complex is involved in early stage cleavages of the transcript at positions A_0, A_1 and A_2 (Bernstein et al. 2004, Dragon 2002). The first subcomplex that associates with pre-rRNA is UtpA. It is a heptameric multi-protein complex consisting of Utp4, Utp5, Utp8, Utp9, Utp10, Utp15 and Utp17 (Pöll et al. 2014, Sun et al. 2017). At the start of the transcription of the rRNA, Utp8, Utp9 and Utp17 bind to nascent pre-rRNA at its 5' end, whereas Utp10, Utp4,

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Utp5 and Utp15 interact with nucleotides in the 5' ETS region further downstream (Fig. 6a). Furthermore, other subunits of UtpA and UtpB bind to the 5' ETS, subsequently recruiting the U3 small nucleolar RNP (U3 snoRNP) that also binds to the 5' ETS (Fig. 6b-c). Finally, as the transcription goes on, the processome is assembled with a folded 18S rRNA with Utp13 bound in proximity of its 3' end (Fig. 6d-e) (Hunziker et al. 2016). Depletion experiments with proteins from the UtpA complex hint at its role as the initiator of pre-ribosome assembly by binding to nascent pre-rRNA and recruiting UtpB and the U3 snoRNA (Pérez-Fernández 2007).
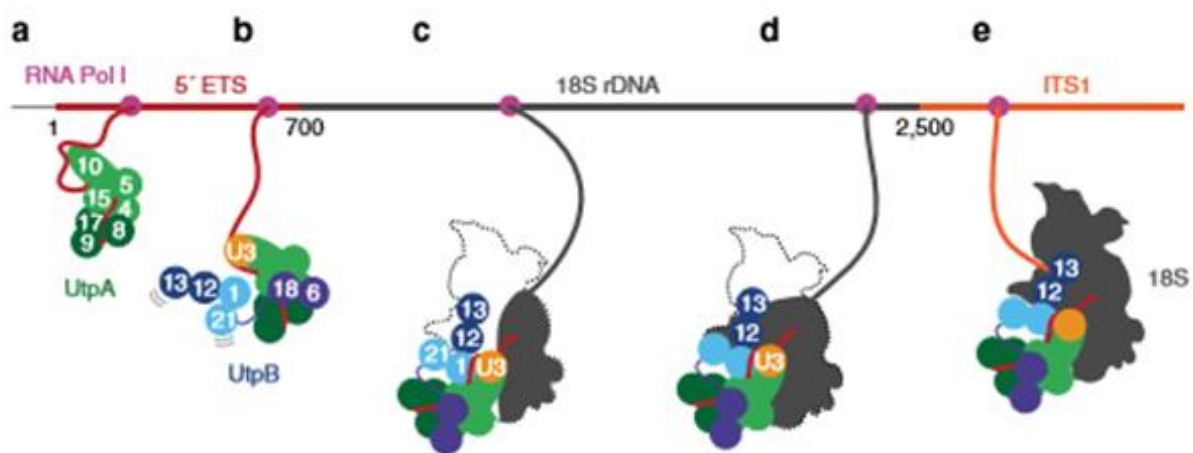

Figure 6: Schematic model of early co-transcriptional events in eukaryotic ribosome biogenesis in yeasts (Hunziker et al. 2016).

Our knowledge of protein factors participating in rRNA processing and ribosome biogenesis in *T. brucei* is still very limited, only few studies were published so far (Jensen et al. 2003, Barth et al. 2008, Sakyiama et al. 2013, Umaer et al. 2014). Recently, several orthologs of processome factors were identified by a bioinformatic survey using BLASTP against *T. brucei* genome (Michaeli 2011).

We have decided to start to fill the enormous gap in study of this essential process. No experimental data are available so far about the very beginning steps of rRNA processing in *T. brucei*. The goal of our study is therefore to perform functional analysis of ortholog of one of the proteins belonging to UtpA subcomplex, called Utp10 in yeast and BAP28 in humans.

1.7   Utp10/Bap28 protein in various organisms

What is known so far about this protein in various organisms? It is a large, conserved protein, that was so far mainly studied in yeasts.

In *Saccharomyces cerevisiae* **Utp10** (U̲ t̲hree p̲rotein, NP_012426.1) is an essential, large 200kDa (1769aa) protein. Utp10 consists of repeated, mainly α-helical motifs, also called HEAT-repeats (H̲untington, e̲longation A̲ subunit, T̲OR) and therefore belongs to the HEATR1/UTP10 protein family (Dragon et al. 2002, Krogan et al. 2004, Dez et al. 2007).

It was purified as component of a large nucleolar ribonucleoprotein complex (mentioned above as SSU processome) that specifically associated with the U3 snoRNA (Dragon et al. 2002). U3 snoRNAs are ubiquitous in eukaryotes and are required for nucleolar processing of pre-18S ribosomal RNA. It is speculated that U3 snoRNP recruitment to the processome may be enhanced by a Utp10-U3 snoRNA 3' domain interaction (Hunziker et al. 2016). Genetic depletion of Utp10 resulted in inhibition of the early pre-rRNA processing steps in 18S rRNA maturation and accumulation of its precursors, although it had only mild effects on rRNA transcription or 25S or 5.8S rRNA synthesis (Dez et al. 2007).

In 2006, Azuma et al. described a mutation in bap28 allele in **zebrafish (*Danio rerio*)** that affects development of the early nervous system. They have identified that this locus is encoding **Bap28** (NP_956194.1), a vertebrate homolog of Utp10 protein. This protein has 242.2 kDa (2159aa). Mutation in bap28 leads also to compromised 18S rRNA maturation and accumulation of its precursors (Azuma et al. 2006).

Another 242.4 kDa (2144aa) protein ortholog was found in **human** - **BAP28** protein (NP_060542.4). Even though the function of mammalian BAP28 protein has not been characterized yet, it can be speculated that it is involved in the same process as in the other organisms.

1.8 Facts about the TbUTP10 (Tb927.9.2900) protein in *T. brucei*

Using NCBI Protein BLAST we have identified the ortholog of Utp10/Bap28 in *Trypanosoma brucei* - Tb927.9.2900 (earlier Tb09.160.1560), which we term here **TbUTP10**.

TbUTP10 is much larger than in the proteins in other organisms - 287kDa (2631aa). Its position is on chromosome 9 (position: 653905-661800) and its isoelectric point is at 6.18. Conserved domains between TbUTP10 and its orthologs in yeast, human and zebrafish suggest that the protein might exhibit similar function (Figure 7). Complete alignment is shown in the Supplementary Figure 1.

Figure 7: Comparison of the N-terminus of TbUTP10 and its orthologs in yeast, human and zebrafish. Complete alignment is shown in Supplementary Figure 1.

The goal of my work was to perform functional analysis of TbUTP10 in *T. brucei.* I used following 4 cell lines that were previously prepared in our lab by D. Faktorová:

RNAi cell lines (in PF and BF):

TbUTP10 gene was *in situ* C-terminally tagged by yellow fluorescent protein (YFP) and Ty tag. This tagging was performed using pPOTv2 vector, provided by Sam Dean (Dean et al. 2015) in *T. brucei* procyclics wild type (wt) cell line 29-13 and bloodstreams wild type cell line 427.

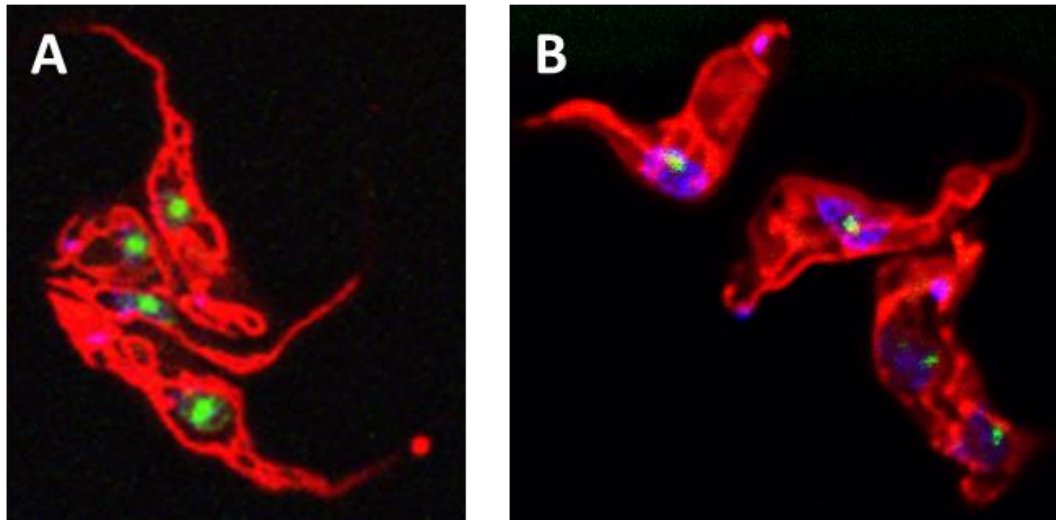In both cell lines TbUTP10 protein was shown to be localized in the nucleolus (Figure 8).

Figure 8: Localisation of TbUTP10 in the nucleolus (in green). Confocal microscopy of living cells: A) procyclics; B) bloodstreams. Cells were stained with 1μl 20μM Mitotracker (mitochondria in red) and 2 drops of Hoechst (DNA in blue), incubated for 15min, spun down and resuspended in IMDM medium. The cells were subsequently embedded in 1% agarose and observed (Faktorová, unpublished data).

In order to prepare RNAi cell lines, TbUTP10 procyclic form (PF) and bloodstream form (BF) cell lines expressing YFP and Ty tag were electroporated with p2T7-177 vector containing a ~500 bp part of TbUTP10 gene. Clones were selected using phleomycin and RNA interference of TbUTP10 was later induced by addition of tetracycline.

Two clones of PF (clone 1 and 4) and clone 2 of BF (already selected by D. Faktorová) RNAi knockdown cell lines were studied in this work.

Single knock-out cell line (in BF):

Additionally, a BF TbUTP10 single knock-out cell line (sKO-Hygro) was used. It was prepared by PCR approach in wt 427, in which one allele of TbUTP10 was replaced by a hygromycin resistance marker. This cell line was also prepared by D. Faktorová and verified by PCR. It is of note that three independent attempts failed to prepare a TbUTP10 double knock-out cell line, suggesting that TbUTP10 is most likely essential for the cells.

2   AIM OF THE THESIS

The aim of my thesis was to perform a functional analysis of TbUTP10 protein and to verify whether this protein in *Trypanosoma brucei* is also involved in processing of pre-18S rRNA like its described orthologs in other organisms. To confirm that, I used *T. brucei* cell lines previously prepared in our lab - RNAi knockdown cell lines of TbUTP10 in procyclic form (PF) and bloodstream form (BF), as well as a bloodstream TbUTP10 single knock-out cell line (sKO-Hygro).

The specific aims of this thesis were:

1) To check the viability of TbUTP10 RNAi cell lines in PF and BF stages of *Trypanosoma brucei* and in TbUTP10 single knock-out (sKO-Hygro) of the BF stage.

2) To verify the downregulation of TbUTP10 protein in RNAi cell lines of both stages using Western blot analysis.

3) To check whether a depletion of TbUTP10 protein will lead to the accumulation of 18S rRNA precursors and decrease of final product (processed 18S rRNA) in all TbUTP10 cell lines using Northern blot analysis.

3   MATERIALS AND METHODS

3.1   Used cell lines and cultivation

The trypanosomes were cultivated in tissue culture flasks (TPP) in liquid medium. The procyclic stage was grown in SDM-79 medium at 27°C. The bloodstream stage of the *T. brucei* cells was grown in HMI-9 medium with 10% fetal bovine serum (FBS) at 37°C with 5% CO_2 content.

Additionally, antibiotics were added to the different media according the used cell line (see Table 1 and 2).

Used T. brucei strains	Added antibiotics
PF wt (SMOX P9)	Puromycin
PF TbUTP10 YFP+Ty tagged RNAi cell lines (clone 1&4)	Puromycin, Hygromycin, Phleomycin
BF wt (427)	G418
BF TbUTP10 YFP+Ty tagged RNAi cell line (clone 2)	G418, Puromycin, Hygromycin, Phleomycin
BF sKO-Hygro	G418, Puromycin, Hygromycin

Table 1: Antibiotics mixture compositions for different cell lines.

Used antibiotics	Concentrations in medium	
	PF	BF
G418	-	2.5 µg/ml
Puromycin	0.5 µg/ml	0.1 µg/ml
Phleomycin	2.5 µg/ml	2.5 µg/ml
Hygromycin	25 µg/ml	2 µg/ml
Tetracycline (for RNAi inductions only)	1 µg/ml	1 µg/ml

Table 2: Concentrations of all used antibiotics in the respective media.

3.2 Cryoprotection of the cells

In order to conserve cells for further experiments and to have backups in case the cultures become contaminated, freezing of culture samples was performed. 800 µl of the cells in the exponential phase were transferred into labelled cryotubes and mixed with 200 µl of 50% glycerol. These tubes were then placed into a cryo-container filled with isopropanol, which was then placed into a -80°C freezer. If the cells were not needed in the course of a few days, the frozen tubes were transferred into liquid nitrogen storage. This way the cells can be stored for a long period.

To start a culture from the frozen cells, the tube with the desired sample is removed from the storage and left to thaw until its content is liquid again. Subsequently, it is transferred into a small flask with about 5 ml of the corresponding medium and is left to grow to an appropriate concentration before further dilution.

3.3 Growth curves

In order to show effects of the RNAi and therefore the impact of the protein on the vitality of cells, growth curves were performed to show how much the absence of TbUTP10 affects the survivability of the different clones of *T. brucei* by comparing induced cultures with non-induced cultures.

The cells were kept in the logarithmic phase of growth and the concentrations of cultures were measured for 8 (BF) to 10 (PF) days. Additionally, dilutions to the same concentration were performed every day. In procyclics, the experiment was also conducted with different media to elucidate possible effects of reduced glucose in the medium on the cell growth. For that experiment, clones 1 and 4 were induced in SDM79 and SDM80 (without glucose).

Procedure:

1. The experiment was started by diluting a healthy culture to 3×10^6 cells/ml for procyclics and to 2×10^5 cells/ml for bloodstreams.
2. The vessels which were intended to contain the induced culture were mixed with the appropriate amount of tetracycline (see Table 2).

3. After exactly 24 hours the concentrations of the cells were measured three times each with the Z2 Particle Counter (Beckman Coulter Inc.).

4. The volume needed for dilution back to the original concentration was calculated and replaced with fresh medium. Additionally, an appropriate amount of tetracycline was added to the induced cell cultures only.

3.4 Western blot

In order to show the decrease of the TbUTP10 protein in the cells after induction and therefore show the effect of the RNAi, Western blots were performed. Proteins from induced cultures were sampled for 10 days and compared to proteins from non-induced cultures, as well as a wild type culture.

3.4.1 Preparation of the samples

Used chemicals:

2x Laemmli Sample Buffer	
62mM	Tris-HCl pH 6.8
2%	SDS
25%	glycerol
0.01%	Bromophenol blue
5%	β-mercaptoethanol

10x PBS pH 7.4	
1.37 M	NaCl
27 mM	KCl
100 mM	Na_2HPO_4
18 mM	KH_2PO_4
For 1x PBS, dilute with milliQ water	

Table 3: Composition of the 2x Laemmli Sample Buffer and 10x PBS solution.

Procedure:

1. Cultures of desired cell lines were established and grown to exponential phase and RNAi was started by adding tetracycline.

2. Two days after induction, the cell concentration of each culture was measured and the volume to be taken out in order to extract $1x10^8$ cells was calculated. This volume was then transferred into a falcon tube. To the remaining cell cultures, fresh medium and the corresponding amount of tetracycline was added. For induced cultures, this procedure was repeated at days 4, 6, 8 and 10 after induction. Wild type and non-induced culture samples were collected only once.

3. The cells were centrifuged for 10 minutes, 1800g, 4°C. After that procedure, the samples needed to be kept on ice until the end.

4. Supernatant was removed without disturbing the pellet of cells.

5. The pellet was resuspended in 500 µl 1xPBS and then transferred into a labelled Eppendorf tube.

6. Another centrifugation for 5 minutes, 1800g, 4°C was performed.

7. The supernatant was removed, the pellet was resuspended in 100 µl 1xPBS and mixed with 100 µl 2xSB.

8. The samples were heated at 100°C for 10 minutes.

9. Finally, a short spin of the samples was performed before running the samples on the gel or storing them in a -20°C freezer until needed.

3.4.2 Preparation of a gradient gel

Used chemicals:

30% Acrylamide, 1M Tris pH 8.8, 1M Tris pH 6.8, 10% SDS, 10% APS, TEMED, milliQ water, butanol

Procedure:

Because the protein of interest is very large (290kDa), a gradient gel needs to be prepared to ensure that even the largest proteins will enter the gel. In a gradient gel, the concentration of acrylamide increases throughout the gel, meaning that towards the bottom of the gel the pore size decreases linearly. In this experiment, the two concentrations of acrylamide used to create that gradient were 3% and 10% (see Figure 9).

1. Firstly, all parts of the gel casting form were thoroughly cleaned with ethanol and rinsed with distilled water. The parts were then assembled and it was checked if the assembly was not leaky by adding some water.

2. The gradient-maker was cleaned and checked for blockages. It should be checked that valves are closed before adding anything. In the outermost chamber, the 3% mixture was prepared by adding 350 µl of acrylamide, 1.31 ml Tris 8.8, 35 µl SDS and 1.77 ml milliQ water. The other chamber was filled with 1.15 ml acrylamide, 1.31 ml Tris 8.8, 35 µl SDS

and 962 µl milliQ water. A small stirring magnet was added to the chamber with the 10% mixture and the gradient-maker was placed on a magnetic stirrer, stirring the mixture. The whole assembly was put in an elevated position relative to the cassette and the pipette tip was inserted between the glass plates of the gel casting form.

3. Right before pouring the gel, 35 µl APS and 1.4 µl TEMED were added to each chamber and then mixed well.

4. At first, the first valve was opened, so that a thin layer of the 10% mixture formed at the bottom of the gel casting form. Very soon after that, the second valve was opened so the gradient started to form while pouring of the gel. The flow rate of the solutions was adjusted to be fast enough to not let them polymerize too early and slow enough not to cause turbulences that would disrupt the gradient.

5. After completion, 50 µl of butanol were added on top of the gel to ensure an even gel front. Immediately after that, the gradient-maker was flushed to avoid polymerization inside the system.

6. Once the gel was solid, the butanol was poured off and the gel was rinsed with milliQ water.

7. The 3% stacking gel was prepared by mixing 0.6 ml of acrylamide, 0.75 ml Tris 6.8, 4.46 ml milliQ water and 60 µl SDS. Right before pouring 60 µl APS and 6 µl TEMED were added (enough for two gels).

8. A 1.5mm comb was inserted so that no bubbles formed at the wells.

If it is desired to use the gels at a later time, storing them at 4°C in a plastic bag with a wetted paper towel is a possibility. Before using the stored gels, they should be left to reach room temperature again.
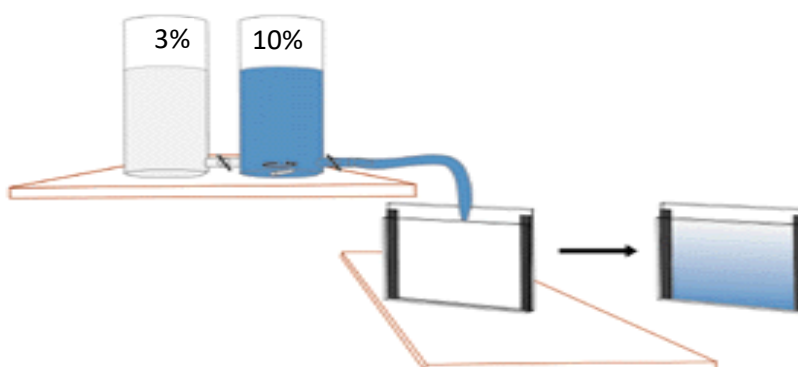

Figure 9: Setup of the gradient-maker and gel casting assembly. Adapted from
https://www.nationaldiagnostics.com/electrophoresis/article/casting-gradient-gels

3.4.3 Running of the SDS-PAGE gel

Used chemicals:

	5x Running buffer
25mM	Tris
192mM	Glycine
0.1%	SDS

Table 4: Composition of 5x Running buffer.

Marker: Precision Plus Protein^{TM} Standards (Bio-Rad; Catalog #161-0373)

Procedure:

1. The gel was placed into the electrophoretic apparatus.
2. Running buffer was prepared by diluting 5x running buffer to 1x running buffer and poured into the electrophoretic apparatus up to the designated mark.
3. The wells were washed with a syringe, then 20 µl of the samples (corresponding to $1x10^7$ cells) and 7 µl of the marker were loaded onto the gel.
4. The gel was run at 90 V until the stacking gel was exceeded, then at 130 V till completion.

3.4.4 Protein transfer to the membrane

Used chemicals:

	Blotting buffer
20 Vol.%	Methanol
38.6 mM	Glycine
48 mM	Tris
1.3 mM	SDS

Table 5: Composition of Blotting buffer.

Procedure:

In this experiment, wet blotting was the method of choice.

1. Two thick filter papers and a PVDF membrane (GE Healthcare; #10600023) were cut to the fitting size. Directly touching the membrane must be avoided at any time!
2. The membrane was soaked in methanol to activate it.

3. The 'blotting sandwich' was assembled in the following order: white panel, sponge, filter paper, membrane, gel, filter paper, sponge, black panel. All components were soaked in blotting buffer to prevent drying out. To ensure that there were no bubbles between the filters, gel and the membrane, a glass rod was rolled across the filter paper on top to remove them.

4. The assembly was clamped together tightly and put into the blotting apparatus so that the gel ended up facing the cathode and the membrane was facing the anode.

5. Before starting, a stirring magnet, as well as cooling units were added and blotting buffer was poured into the blotting apparatus up to the designated mark.

6. After placing the whole assembly on a stirrer and ensuring proper stirring, the blot was performed at 200 mA for 2 hours.

3.4.5 Membrane treatment

Used chemicals:

Used antibodies			Company	Ordering number
anti-TY	primary	monoclonal	Sigma-Aldrich	SAB4800032
anti-GFP	primary	monoclonal	Sigma-Aldrich	A-11120
anti-tubulin	primary	monoclonal	Sigma-Aldrich	T5168
anti-mouse (IgG-peroxidase; produced in rabbit)	secondary	polyclonal	Sigma-Aldrich	A9044

Table 6: Information about used antibodies.

Blocking solution: dry nonfat milk, 1xPBS with 0.05% Tween-20

Clarity™ Western ECL Substrate (Bio-Rad; #170-5061): Luminol, Peroxide

Procedure:

1. For each application, the blocking solution was prepared freshly by dissolving 5 g of dry nonfat milk per 100 ml 1xPBS Tween-20. The membrane was removed from the blotting assembly and immersed in the blocking solution overnight at 4°C.

2. The membrane was transferred into a 50 ml falcon tube so that the side with the marker and proteins faced the inside of the tube. The appropriate amount of primary antibody

was added to 3 ml of the milk solution and incubated with the membrane for 2 hours (up to overnight) on a rotating shaker. For the anti-Ty and anti-GFP antibody the ratio is 1:2000, for anti-tubulin it is 1:5000.

3. The milk was discarded or frozen for further use and the membrane was washed by adding 5 ml 1xPBS Tween-20 and rotating it for 5 minutes. This procedure was repeated 5 times.

4. The incubation with the secondary antibody was performed by adding the appropriate amount of the antibody to 3 ml of milk and leaving it for 1 hour on a rotating shaker. The secondary antibody in this experiment was anti-mouse in every case, with a dilution ratio of 1:1000.

5. The washing procedure was repeated.

6. A piece of sheet protector bigger than the membrane was cut off so that it was opened at three sides.

7. The membrane was placed inside and the reagents from the ECL kit were mixed in a 1:1 ratio in a tube to a total volume of 1 ml. This mixture was then spread on the membrane and left to incubate for 5-10 minutes. Before developing, excess liquid, as well as bubbles, were removed.

8. Pictures were taken using Chemidoc MP imager (Bio-Rad) at different exposure times and the best picture was saved.

3.5   Northern blot

Since TbUTP10 is expected to play a role in rRNA biosynthesis, Northern blots were performed to study the influence of its depletion on the pre-18S rRNA processing. Therefore, wild type cultures were compared to induced (PF and BF) and single knock-out (BF) cultures.

3.5.1 Preparation of the samples

Used chemicals:

1xPBS, TRI Reagent (MRC; #TR118)

Procedure:

1. Cultures of desired cell lines were established and grown to exponential phase and RNAi was started by adding tetracycline.

2. One day after induction, the cell concentration of each culture was measured and the volume to be taken out in order to extract $1x10^8$ cells was calculated. This volume was then transferred into a falcon tube. To the remaining cell cultures, fresh medium and the corresponding amount of tetracycline was added. For induced cultures, this procedure was repeated at days 2, 3 and 4 after induction. Wild type and single knock-out culture samples were collected only once.

3. The cells were centrifuged for 10 minutes, 1800g, 4°C. After that procedure, the samples needed to be kept on ice until the end.

4. Supernatant was removed without disturbing the pellet of cells.

5. The pellet was resuspended in 500 µl 1xPBS and then transferred into a labelled Eppendorf tube.

6. Another centrifugation for 5 minutes, 1800g, 4°C was performed.

7. Once again as much supernatant as possible was removed, the pellet was resuspended in 1 ml TRI Reagent and stored in a -80°C freezer before RNA extraction.

3.5.2 RNA extraction

Used chemicals:

RNaseZap (Ambion; #AM9780), chloroform, iso-propanol, ice-cold 75% ethanol, milliQ water

Procedure:

1. After making sure that the working surface, equipment and gloves were clean and treated with RNaseZap, the procedure was started by letting the tubes with RNA thaw at room temperature for 5 minutes.

2. To each tube 200 µl chloroform was added.

3. After mixing by shaking the tubes, they were left at room temperature for 10 minutes.

4. The samples were centrifuged for 15 minutes at 12.000g, 4°C.

5. The upper phases were transferred into new labeled tubes. It is of utmost importance not to disturb the interphase.

6. To the new tubes, 500 µl isopropanol were added. Then they were kept for 10 minutes at room temperature.

7. The samples were centrifuged for 15 minutes at 12.000g, 4°C. A pellet of RNA should have formed at the bottom of the tube.

8. The supernatant was removed and washed with 1 ml ice-cold ethanol.

9. The tubes were centrifuged for 5 minutes at 7500g, 4°C.

10. The supernatant was removed and the pellet was left to dry by leaving the lying tube open for approximately 15 minutes. Completely drying out the pellet is not recommended, since this makes it difficult to resuspend it.

11. The pellets are resuspended in 20 µl milliQ water for bloodstreams and in 50 µl milliQ water for procyclics.

12. The RNA samples were incubated for 10 minutes at 60°C.

13. Finally, the purity and concentration were checked on the NanoDrop (Thermo Scientific).

14. The samples were stored in a -20°C freezer if they were going to be used in the course of a week, or stored in a -80°C freezer for longer storage.

3.5.3 RNA gel electrophoresis

Chemicals used:

10x MOPS, agarose, milliQ water, formaldehyde 37%, RNA ladder (Promega; #G3191), loading dye (6x MassRuler: Thermo Scientific; #R0621), RNase Zap (Ambion; #AM9780)

	2x Sample buffer			10x MOPS (100 ml)
600 µl	Formamide		40 ml	0.5M MOPS pH 7.0
210 µl	Formaldehyde 37%		1.67 ml	3M sodium acetate
125 µl	10x MOPS		2 ml	0.5M EDTA pH 8.0
5 µl	Ethidium bromide (10mg/ml)		56.33 ml	milliQ water

Table 7: Compositions of 2x Sample buffer and 10x MOPS.

Procedure:

1. The working space in the fume hood was thoroughly cleaned and treated with RNase Zap, as well as all of the equipment for running of the gel. Furthermore, the gloves were also treated with RNase Zap.

2. The 1% agarose gel was prepared by dissolving 1 g of agarose in 72 ml milliQ water, using a microwave to heat the mixture up. After the mixture was not hot anymore, 10 ml of 10x MOPS and 18 ml of 37% formaldehyde were added.

3. The gel mixture was poured into the apparatus, a 10-well comb was added and left to solidify, which took approximately 30 minutes.

4. Appropriate volumes for taking out 10 µg of RNA were calculated according to the measured concentrations, the RNA sample tubes were thawed and the RNAs were transferred into tubes with the double volume of 2x sample buffer. The same was done with 3 µl of the ladder.

5. The samples and the ladder were then incubated at 65°C for 10 minutes.

6. After heating, the tubes were put on ice for three minutes.

7. For the running buffer, 10x MOPS was diluted to 1x MOPS with milliQ water. The running buffer was then poured into the apparatus till the mark.

8. Prior to loading, 5 µl loading dye were added to the samples.

9. The gel was run at 60 V for about 3 hours. Every 30 minutes, the buffer was shaken.

10. The separated RNA was visualized and a picture was taken using the Chemidoc MP imager (Bio-Rad).

3.5.4 Transfer to the membrane

Chemicals used:

20xSSC (for 1 l)	
175.2 g	NaCl
88.23 g	Sodium citrate

Table 8: Composition of 20xSSC solution.

Procedure:

1. ZETA probe membrane was cut into the size of the gel. Four pieces of filter paper the same size as the membrane and one piece of the shape and size of the glass plate with additional 2 cm overlaps on each side were cut out. Additionally, paper towels the same size as the membrane were cut out to yield a stack that was approximately 10 cm thick.
2. The 20xSSC was diluted to 5xSSC.
3. First, the big filter paper was placed on the glass plate, then the small filter paper was put in the center of it. 5xSSC was poured on top of every layer. Next was the gel, the membrane and the three remaining filter papers. Bubbles were removed by rolling with a glass stick.
4. The bath was filled with 5xSSC, the paper towels were placed on the membrane and a glass plate with weight was put on top of it.
5. Using saran wrap, the bath was covered on the free edges of the filter paper.
6. The RNA gel was blotted onto the membrane overnight.
7. The gel with the membrane on top of it was put looked at under UV. The ladder was marked on the membrane by cutting out arrows and the membrane was labelled with a pencil on the RNA side.
8. After the membrane dried, it was UV-crosslinked at 120 mJoules for 1 minute in the UV Stralinker 1800^{TM}. Subsequently, the membrane is stored at room temperature.

3.5.5 Probe preparation for labeling

Chemicals used:

50 μM Oligos (Sigma, see Table 14 for sequences), PNK (polynucleotide kinase) & 10x PNK buffer (New England Biolabs; #M0201S), [gamma-^{32}P]-ATP isotope

Procedure:

1. In a tube 1 μl oligo, 1 μl PNK, 1 μl 10x PNK buffer, 1 μl [gamma-^{32}P]-ATP isotope and 6 μl water were mixed and incubated at 37°C for up to one hour.
2. After incubation, 50 μl milliQ water were added to the tube.
3. The reaction was purified by an illustra^{TM} MicroSpin^{TM} G-25 Column (GE Healthcare; #27-5325-01) according to the instructions.

4. The probes were then stored in the fridge. One tube was enough for two membranes.

3.5.6 Oligonucleotide hybridization and detection

Chemicals used:

	Hybridization solution (0.5l)
125 ml	20x SSC
10 ml	1M Pi pH 7.2
35 g	SDS
5 ml	100x Denhards solution
5 ml	Salmon sperm DNA (100 mg/ml)
add up to final volume	milliQ water

Table 9: Composition of Hybridization solution.

100x Denhards solution
2% Ficoll 400
2% Polyvinylpyrrolidone
2% Bovine serum albumine

Table 10: Composition of 100x Denhards solution.

	Oligo wash 1 (0.5 l)
75 ml	20x SSC
125 ml	20% SDS
12.5 ml	NaH_2PO_4 pH 7.5
add up to final volume	milliQ water
add 15 ml 100x Denhards solution per 135 ml Oligo wash 1	

Table 11: Composition of Oligo wash 1.

	Oligo wash 2 (1 l)
50 ml	20x SSC
50 ml	20% SDS
add up to final volume	milliQ water

Table 12: Composition of Oligo wash 2.

	Stripping solution (1 l)
5 ml	20x SSC
5 ml	20% SDS
add up to final volume	milliQ water

Table 13: Composition of Stripping solution.

Procedure:

Prehybridization:

1. The membrane was put into a glass tube with the RNA side facing the inside of the tube.
2. 5-10 ml of hybridization solution were added to the tube, which was then placed into an oven for 30 minutes at 50°C.

Hybridization:

1. The desired probe was removed from the fridge, placed on a heating block heated to 100°C for 5 minutes. As soon as the probe was placed on the block, it was switched off.
2. Subsequently, the probe was placed on ice for 1 minute.
3. The half of the probe was added into each tube from the prehybridization step. The volume of probe prepared was enough for two membranes.
4. Hybridization took place overnight in the oven at 50°C.

Washing and developing of the membrane:

1. The probe was poured out into a tube and stored in the fridge till disposal.
2. 5-10 ml oligo wash 1 were added to the tube and it was rotated at 50°C for 20 minutes. This step was repeated a second time.
3. 5-10 ml oligo wash 2 were added to the tube and it was rotated at 50°C for 20 minutes.
4. The membrane was wrapped into cling wrap with a slightly wetted piece of filter paper on the side without the RNA.
5. The phosphoimaging screen (GE Healthcare^{TM}) was illuminated to erase the signal.
6. The wrapped membrane was put into the cassette, covered with the screen and exposed from 4 hours to 4 days, depending on the probe and desired intensity of the signal.
7. The screen was developed with the Typhoon Scanner 9410.
8. The membrane is removed from the wrap and stripped twice in stripping solution in a waterbath at 80°C for 20 minutes. With a Geiger counter, it should be checked if there is some residual radioactivity, so that the stripping must be repeated.
9. The stripped membrane was stored in fresh stripping solution at room temperature for possible reprobing.

4 RESULTS

4.1 Growth curves experiment

To determine the influence of TbUTP10 on the viability of *T. brucei*, growth curve experiments on different cultures of procyclic and bloodstream forms were conducted.

4.1.1 Procyclic form

The growth of non-induced versus induced TbUTP10 RNAi cultures of clone 1 and clone 4 was investigated in the course of 10 days. RNAi was induced by addition of tetracycline. At the same time every day, cell density measurements were performed and dilutions to the starting cell density were done. The actual growth curves were calculated by summing up every day's growth in a diagram, taking into account the dilutions. The result is a continuous curve that depicts the summed-up growth of a culture.

Also, the effect on growth in the medium without glucose (SDM 80) was investigated in comparison to the standard glucose-containing SDM79 medium. The lack of glucose in SDM80 fully activates the mitochondrion of the cell. Therefore, the possible effect of the absence of glucose on enhancement of phenotype was investigated by comparing non-induced and induced cultures (Figures 10 – 13).
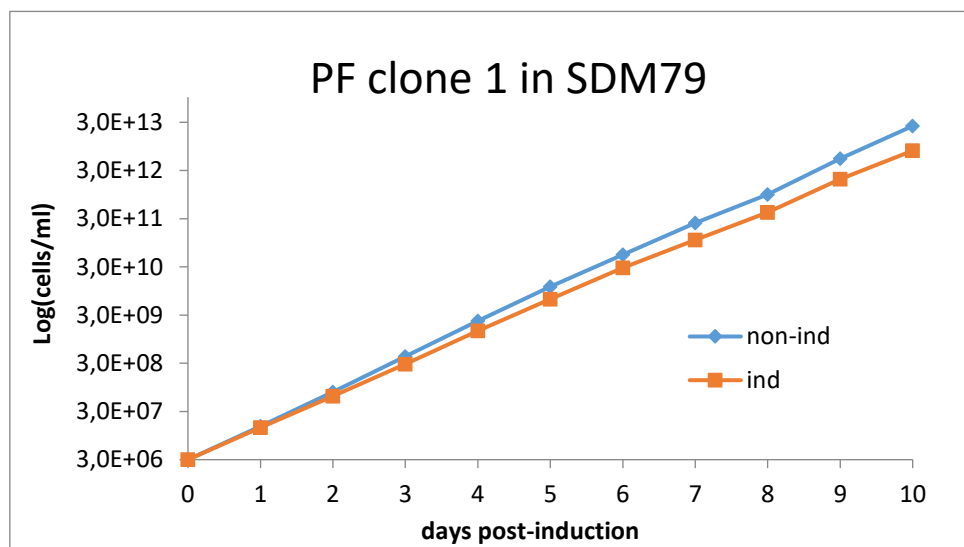
Figure 10: The growth curves of PF TbUTP10 RNAi clone 1 non-induced versus induced in SDM79 medium.
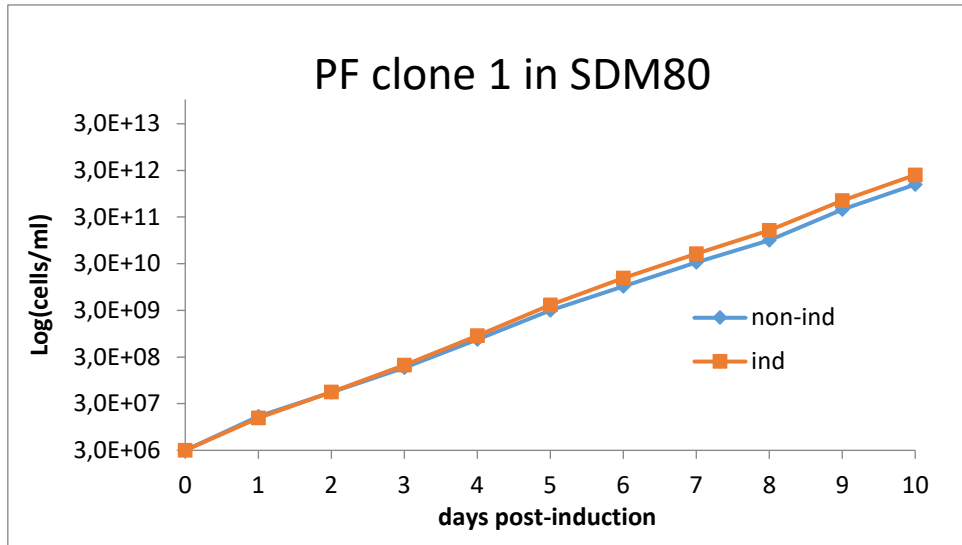
Figure 11: The growth curves of PF TbUTP10 RNAi clone 1 non-induced versus induced in SDM80 medium.
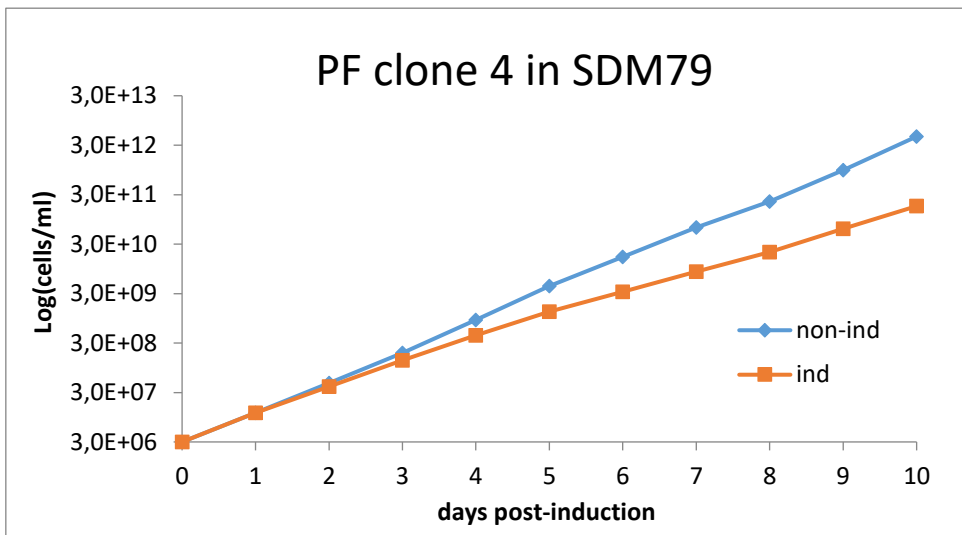
Figure 12: The growth curves of PF TbUTP10 RNAi clone 4 non-induced versus induced in SDM79 medium.
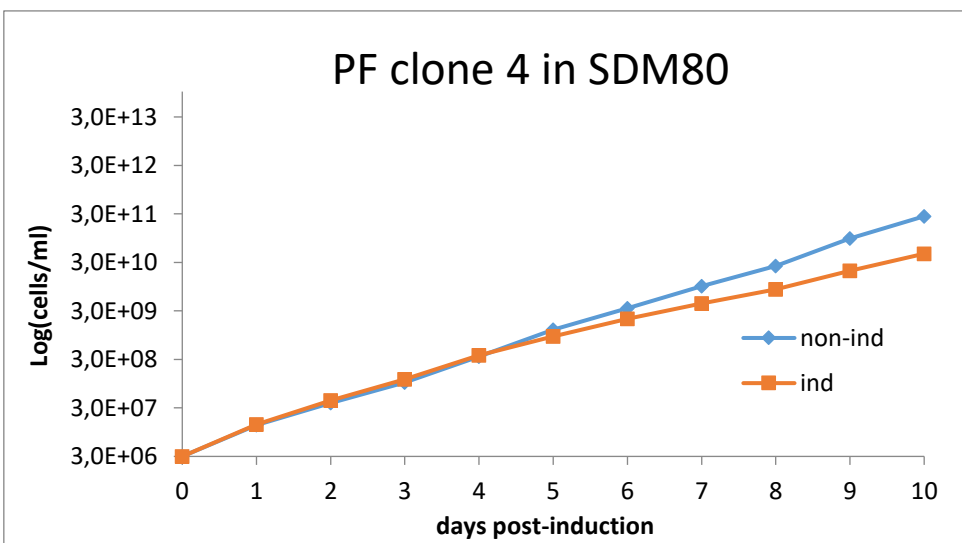
Figure 13: The growth curves of PF TbUTP10 RNAi clone4 non-induced versus induced in SDM80 medium.

4.1.2 Bloodstream form

The growth of non-induced versus induced BF TbUTP10 RNAi clone 2 and wild type versus single knock-out (sKO-Hygro) cultures was investigated in the course of 8 days. The procedure for BF cells is almost identical, differing mostly in the media used. The obtained curves are shown in Figures 14 and 15.
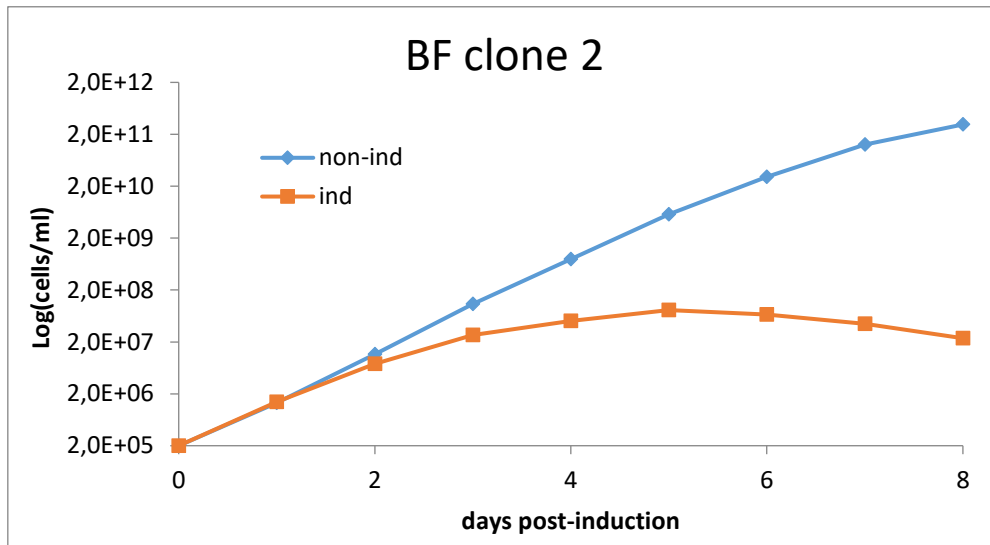
Figure 14: The growth curves of BF TbUTP10 RNAi clone 2 non-induced versus induced cells.
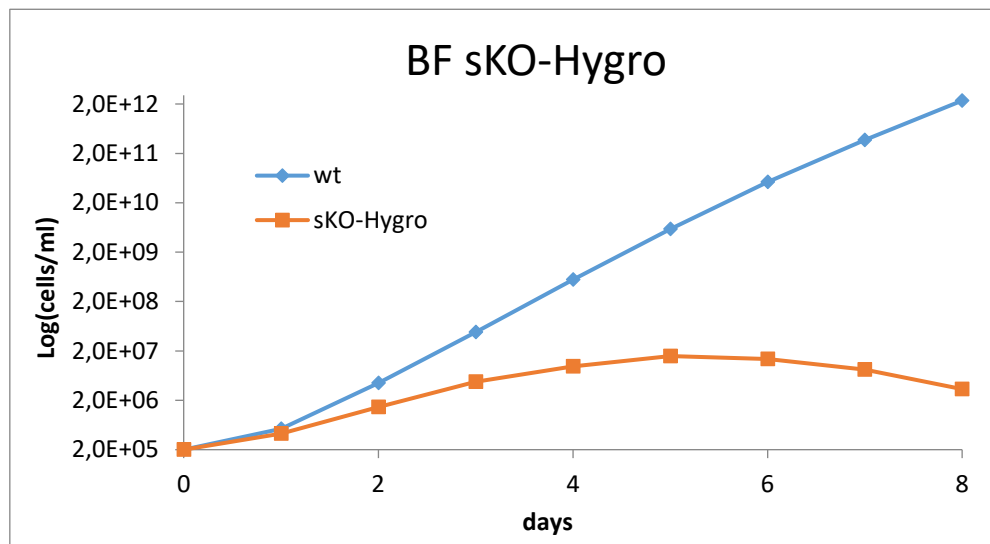
Figure 15: The growth curves of wild type cells versus single knock-out hygromycin cells.

4.1.3 TbUTP10 depletion inhibits growth in both PF and BF

The curves shown above are representative curves, the experiment was repeated three times to ensure reproducibility. In both forms, procyclic and bloodstream, a phenotype is observed, with the exception of PF clone 1. The gradual decrease in cell densities indicates that the depletion of TbUTP10 has an influence on the survivability of the cells.

The PF growth curves clearly show that in comparison to clone 4, clone 1 does not seem to respond to the induction. In contrast, clone 4 shows a clear reduction of cell density after day 4 post-induction. Thus, clone 4 was selected for use in subsequent experiments. Comparing the growth curves recorded in SDM79 to the ones in SDM80, no significant change in phenotype is observed. Therefore, indirectly ruling out a relation of TbUTP10 to mitochondrial functions. Furthermore, decreased glucose in medium seems to affect the overall growth rate of both clones negatively, as it can be seen in Figures 10 to 13.

Observing the BF growth curves, it can be seen that the phenotype of sKO-Hygro is more pronounced than that of clone 2. The growth inhibition of both clones started already on day 2. Even though the decrease is quite intense for both cell lines, the knock-out strategy seems to stronger impair the viability of the cells. Clone 2 and sKO-Hygro cultures completely ceased to grow after day 5, clearly showing that TbUTP10 is indeed an essential protein.

4.2   Western blot analysis

Western blot analysis was performed on both PF and BF RNAi cell lines to investigate a reduction of expression of the TbUTP10 protein. To see an effect of TbUTP10 depletion, tagged cultures were induced and every second day till day 10 a sample with 1×10^7 cells was taken. The expected size of the protein of interest is about 290 kDa. A classical SDS-PAGE gel does not work with proteins which are that large, therefore the samples were separated on a 3-10% acrylamide gradient gel. Wild type culture samples served as negative control because the cell line is not tagged. Since the protein in PF clone 4 and BF clone 2 is tagged with a YFP (yellow fluorescent protein) and Ty tag, anti-Ty and anti-GFP (recognizing the YFP protein) could be used as primary antibodies. The Ty-antibody was preferred because it is more specific. The secondary antibody was anti-mouse conjugated with HRP. The

membranes were developed with ECL substrate and chemiluminescence was used to visualize the protein. Furthermore, the membranes were reprobed with anti-tubulin primary antibody and anti-mouse secondary antibody which was used as loading control.
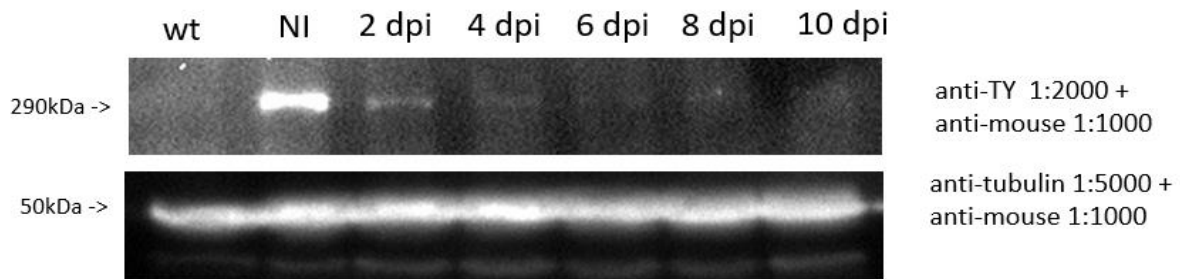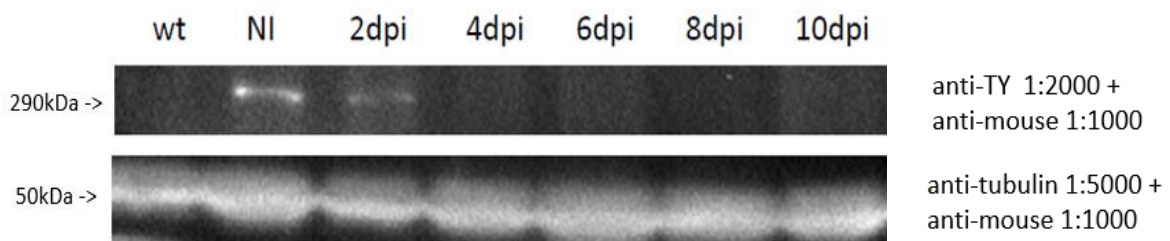

Figure 16: Western blot analysis of PF TbUTP10 RNAi cell line with anti-Ty antibody and anti-tubulin as loading control. wt is the wild type sample, NI is the non-induced sample and 2dpi – 10dpi indicate the days after the start of induction.

Figure 17: Western blot analysis of BF TbUTP10 RNAi cell line with anti-Ty antibody and anti-tubulin as loading control. wt is the wild type sample, NI is the non-induced sample and 2dpi – 10dpi indicate the days after the start of induction.

4.2.1 TbUTP10 RNAi cell lines show depletion of the protein

A clear decrease of the tagged protein can be seen in both clones. After day 4 almost complete depletion of TbUTP10 can be observed in PF and BF induced clones in comparison to the non-induced clones. The conclusion is that RNAi worked, resulting in decreased TbUTP10 expression.

4.3 Northern blot analysis

Since the depletion of the TbUTP10 protein worked, we decided to investigate the effect of decreased TbUTP10 expression on the 18S rRNA biogenesis. The goal was to check, whether and how it affects pre-18S rRNA processing. In case pre-18S rRNA is processed incorrectly when the protein is depleted, an accumulation of precursors is expected. Because Utp10 in yeasts and the other orthologs are involved in processing of pre-18S rRNA, it is expected that TbUTP10 has a similar function.

Three different oligo probes (Table 14 and Figure 18) were used in the Northern blot analysis to see if certain precursors of pre-18S rRNA accumulate and if the final product, the SSU, is not properly processed and is therefore decreased. If TbUTP10 protein is involved in pre-18S rRNA processing, it is expected to see an increase of the full-length precursors (9.6 kb) and other intermediates, and a decrease of the final 2.2 kb product.

The obtained pictures of the bands were quantified with ImageQuant program (GE Healthcare). For the quantification, the intensity of the bands from wild type samples was regarded as 100% and compared to induced and sKO-Hygro samples. Wild type samples were used for the comparison instead of the non-induced samples to prevent the effect of potential RNAi leakage.

Probe	Sequence of the probe
5'ETS	5'-AGTGTAAGCGCGTGATCCGCTGT-3'
SSU	5'-GGCTAAGTCCTTGAAACAAGCA-3'
ITS1	5'-GGTTGCATACTGTGCAATTATACATGC-3'

Table 14: Used oligo probes for the hybridization and their sequences.
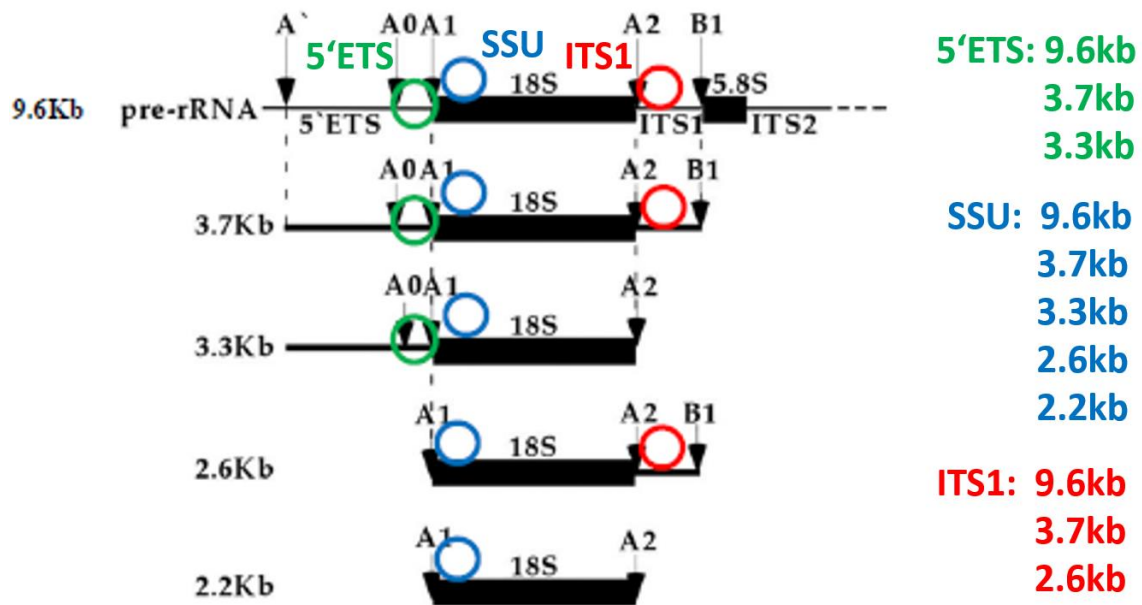
Figure 18: Visualized positions of probes on the precursors in the pre-18S rRNA processing pathway with respective lengths of full-length precursors (9.6 Kb), intermediates and the final product (2.2 kb) (adapted from Barth et al. 2008).

4.3.1 Procyclic form

Total RNA samples of non-induced and induced cultures up to day 4 after induction were separated on a 1% RNA gel, transferred onto a Zeta probe membrane and hybridized subsequently with the before mentioned probes. The pictures obtained after exposure of the membranes to the phosphoimager screen are shown in Figure 19. Different exposure times for the SSU probe were chosen to adjust the intensity of the bands of the precursors (4 days - long exposure) and the final product (4 hours - short exposure). The other probes were exposed for 19 hours.

4.3.2 Bloodstream form

Total RNA samples of sKO-Hygro, wild type and induced cultures up to the fourth day of induction were separated on a 1% RNA gel, transferred onto a Zeta probe membrane and hybridized with the before mentioned probes. The pictures obtained after exposure of the membranes to the phosphoimager screen are shown in Figure 20. Exposure times were the same for the respective probes as for PF.

A

RNA gel

5'ETS probe

ITS1 probe

SSU probe

Figure 19: A) Northern blot analysis of PF wild type (wt) and TbUTP10 RNAi clone 4, induced for up to 4 days (I1 to I4). The colors in the picture correspond to the probes from Figure 18.

B) Band quantification using ImageQuant. Shown are relative intensities of the bands compared to the wild type band.

B
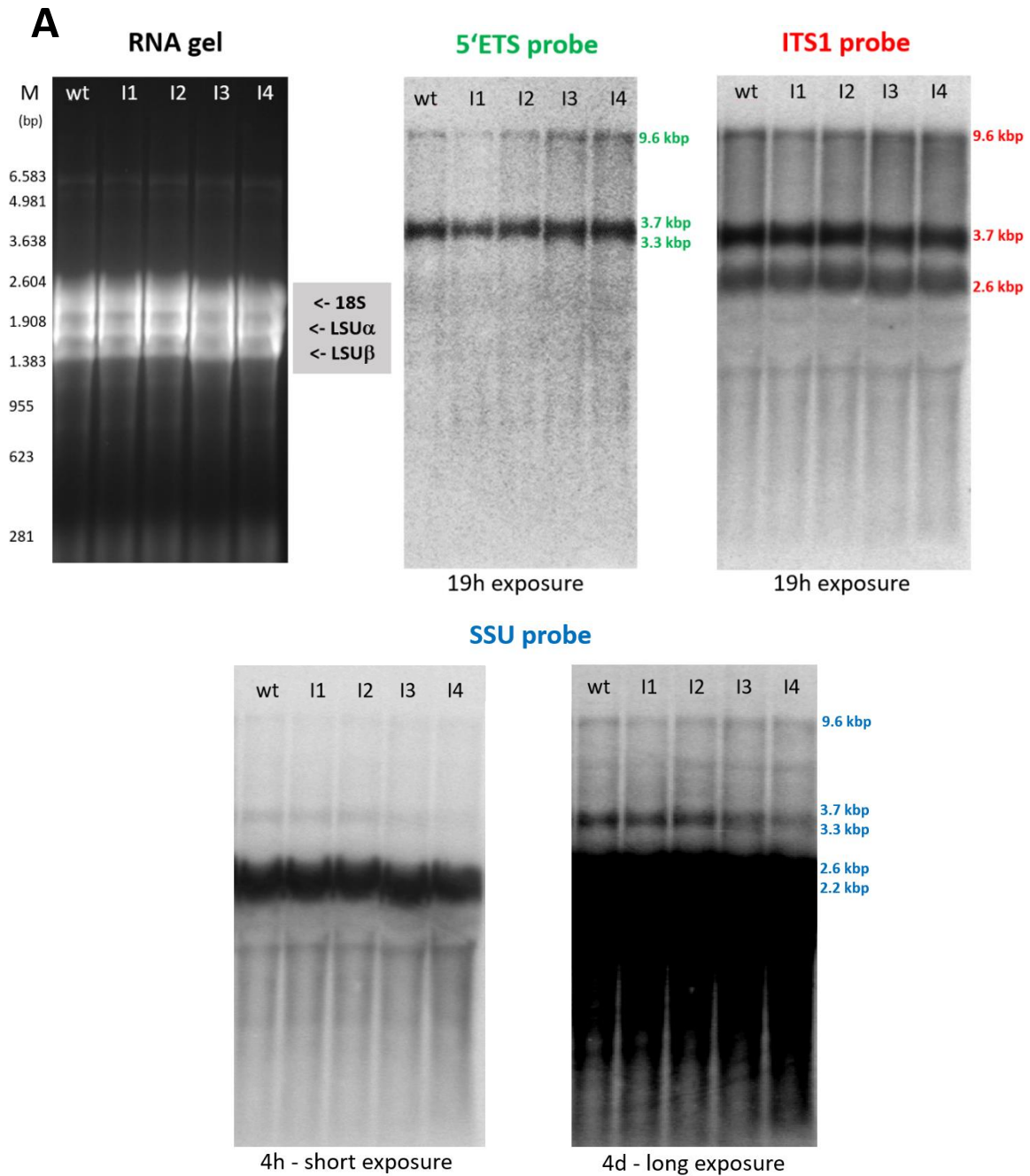
	5'ETS 9.6kb	5'ETS 3.7kb	ITS1 9.6kb	ITS1 3.7kb	ITS1 2.6kb	SSU 9.6kb	SSU 3.7kb	SSU 2.2kb
wt	100%	100%	100%	100%	100%	100%	100%	100%
Ind 1dpi	51.62%	65.40%	69.02%	80.20%	97.39%	90.32%	80.30%	94.89%
Ind 2dpi	96.29%	78.59%	87.47%	85.63%	100.87%	95.96%	92.86%	89.72%
Ind 3dpi	168.55%	105.26%	114.31%	58.99%	92.02%	106.23%	43.65%	87.30%
Ind 4dpi	197.85%	117.85%	108.64%	58.04%	80.39%	120.15%	45.24%	71.84%

A

RNA gel

5'ETS probe — 19h exposure

ITS1 probe — 19h exposure

SSU probe

4h - short exposure

4d - long exposure

B
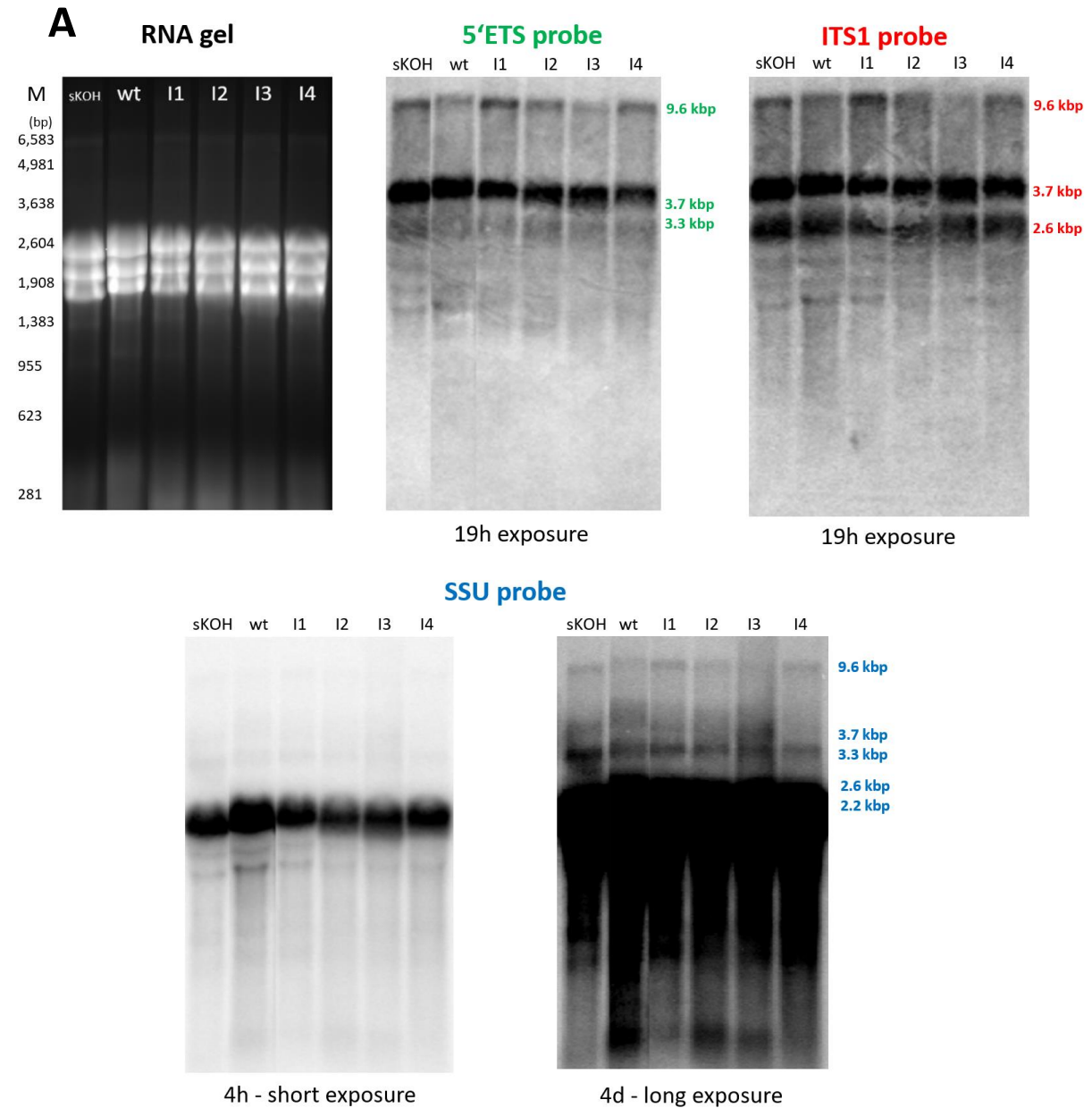
	5'ETS 9.6kb	5'ETS 3.7kb	ITS1 9.6kb	ITS1 3.7kb	ITS1 2.6kb	SSU 9.6kb	SSU 3.7kb	SSU 2.2kb
sKO-Hygro	412.43%	158.26%	111.66%	163.76%	104.42%	153.87%	141.77%	80.86%
wt	100%	100%	100%	100%	100%	100%	100%	100%
Ind 1dpi	324.69%	83.60%	144.43%	82.94%	65.12%	213.17%	91.52%	78.48%
Ind 2dpi	313.16%	78.61%	98.12%	77.41%	63.21%	161.59%	88.24%	75.33%
Ind 3dpi	89.21%	86.29%	81.53%	108.99%	93.07%	218.14%	109.19%	89.58%
Ind 4dpi	241.24%	60.90%	143.36%	90.77%	88.22%	165.92%	66.64%	90.26%

Figure 20: A) Northern blot analysis of BF wild type (wt), single knock-out Hygro (sKOH) and TbUTP10 RNAi clone 2, induced for up to 4 days (I1 to I4). The colors in the picture correspond to the probes from Figure 18. B) Band quantification using ImageQuant. Shown are relative intensities of the bands compared to the wild type band.

4.3.3 TbUTP10 influences the processing of pre-18S rRNA

The pictures of the PF membranes show a clear trend. Days 3 and 4 after induction show an increase of full-length precursor of the 5'ETS probe, as well as the intermediates. Bands of the ITS1 probe show an increase of 9.6 kb precursor and a decrease of the smaller precursors. Finally, the SSU probe shows that the largest precursor accumulates after the second day of induction, while the other decreases. The final 2.2 kb product decreases by approximately 30% on day 4, meaning that production of the mature SSU is impaired.

The picture of the BF membrane incubated with 5'-ETS shows an increase in intensity of the full-length precursor and a decrease of the 3.7 kb precursor for the induced samples. The ITS1 bands from induced clone 2 show an increase of full-length precursor and a decrease of the two intermediates on day 4 post-induction. The same can be said for the SSU probe, importantly the 2.2 kb SSU product is again decreased.

The sKO-Hygro sample shows an even more pronounced increase of 9.6 kb 5'ETS precursor and at the same time also an increase in the smaller precursor. Also, the precursors of ITS1 and SSU are increased, while the final product is decreased by about 20%. This means the production of final mature SSU product is impaired in all three TbUTP10 depleted cell lines.

4.4 Polysome profile analysis

Given that TbUTP10 is localized in the nucleolus and the fact that the results of Northern blot analysis confirm that its absence affects the maturation of pre-18S rRNA, we decided to confirm the assumptions by polysome profiling experiment. Since TbUTP10 seems to affect pre-18S rRNA processing, we expected to see a decrease of 40S ribosomal subunit and as a consequence, also of 80S ribosome.

Since this analysis was not possible to be done in our laboratory due to lack of necessary equipment, PF RNAi cell line clone 4 was sent to the lab of Prof. Juan D. Alfonzo (The Ohio State University, Columbus, USA). There, cell lysates of PF non-induced clone 4 and after 6 days of induction were separated by 10 to 50% sucrose gradient centrifugation. The fractions were monitored by absorbance at 254 nm. The resulting peaks are shown in Figure 21. This experiment was repeated three times with the same outcome.
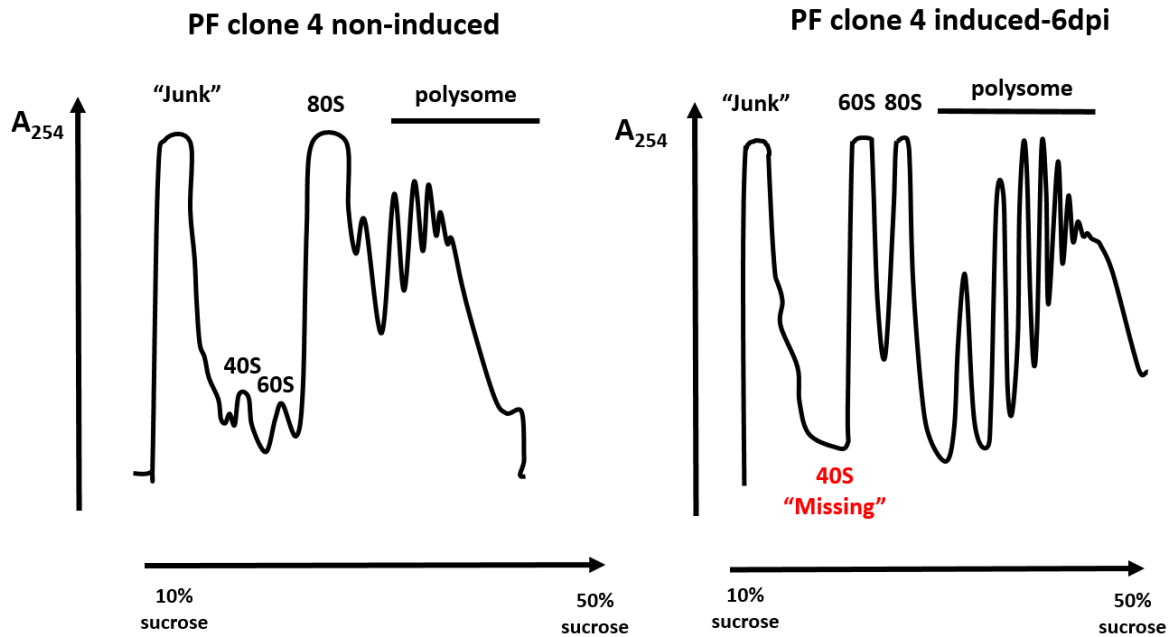
Figure 21: Results of the polysome profile analysis. Peaks corresponding to 40S, 60S, 80S and the polysomes are indicated (performed by Katherine McKenney).

4.4.1 Depletion of TbUTP10 results in an abnormal ribosome profile

The assumption that TbUTP10 is involved in pre-18S rRNA processing is verified by the results of the polysome profiling. The profile of the induced culture sample shows a completely missing peak for the 40S subunit (SSU). Furthermore, it shows that the LSU (60S) is substantially accumulated, while the 80S final product peak is much narrower than in the non-induced sample. This indicates significantly impaired ribosome assembly due to depletion of TbUTP10.

5   DISCUSSION AND CONCLUSIONS

Ribosomes are large ribonucleoprotein complexes that are responsible for protein synthesis, an essential process in all living cells. The biogenesis of ribosomes is a very complex process that involves multistep pre-ribosomal RNA (pre-rRNA) cleavage and modifications that are followed by the assembly of ribosomal proteins around completely processed rRNAs. The large ribosomal subunit (6OS) contains three rRNAs – 25/28S, 5.8S and 5S, while the small subunit (40S) assembles around 18S rRNA. This process is very well studied and conserved throughout the eukaryotes.

T. brucei, the early divergent single cell eukaryote and causative agent of sleeping sickness, exhibits several distinct features in this process. For example - fragmentation of LSU rRNA into 2 big and 4 small pieces (White et al. 1986). Also 4 unique inter-subunit bridges were observed using cryo-electron microscopy of *T. brucei* ribosomes (Hashem et al. 2013).

Another significant difference was observed in pre-rRNA processing (see Figure 5, for recent review see Michaeli 2011). It is surprising that very little is known about the early stages of pre-18S rRNA processing in trypanosomes and that no experimental work was published so far about this topic. The aim of our work was therefore to start to investigate this process.

I have focused on the study of the possible ortholog of yeast Utp10 (200kDa), zebrafish Bap28 (242 kDa) and human BAP28 (242kDa) proteins that we identified using NCBI protein BLAST in the genome of *T. brucei*. The complete alignment of these proteins is shown in Supplementary Figure 1.

Only one candidate was found in *T. brucei* (although with not very significant homology) – the hypothetical, conserved protein Tb927.9.2900. The only conspicuous feature is its large size (290kDa), which indicated that it indeed could be a true ortholog. Therefore, we decided to name it TbUTP10 (http://tritrypdb.org/tritrypdb/app/record/gene/Tb927.9.2900).

Before my project started, the cellular localization of TbUTP10 was shown to be in the nucleolus (Faktorová et al., unpublished). This was recently also confirmed by the TrypTag project, that aims to determine the localizations of all trypanosomal proteins by C-terminal and N-terminal tagging with mNeonGreen (tryptag.org) (Dean et al. 2016).

The goal of this project was to perform its functional analysis and verify the assumption that the TbUTP10 protein plays a role in pre-18S rRNA processing and maturation and therefore 40S ribosome subunit assembly as in the other organisms. Therefore, TbUTP10 was RNAi silenced in procyclic and bloodstream stages of *T. brucei*. Additionally, a single knock-out BF cell line was created.

Even though RNAi does not eliminate all of the protein from the cell, an effect of genetic depletion on the viability of the cultures was observed. It was more pronounced in BF (RNAi induced and sKO-Hygro), which can be attributed to their faster metabolism and shorter doubling time.

PF cells adjust their metabolism in response to carbon source availability. Although in their natural habitat they metabolize L-proline, since it is D-glucose-free, procyclic trypanosomes prefer to utilize D-glucose as their main source of energy (Coustou et al. 2008). If their environment does not contain D-glucose, they metabolize L-proline with their fully activated mitochondrion. Hence, growing them in glucose-free medium (SDM80) does not influence their growth significantly if the depleted protein is not involved in the mitochondrial functions of the cells.

The decrease of the tagged TbUTP10 protein in PF and BF was confirmed via Western blot. On day 4 after RNAi induction the protein almost completely vanished.

Further, Northern blot analysis in both PF and BF clones was performed in order to see the effect of depletion of TbUTP10 protein on processing of pre-18S rRNA and therefore confirm the similar function as in the other organisms. Although the band quantifications did not consistently exhibit increasing intensity in all the intermediates, there is a clear trend of the accumulation of full-length pre-18S rRNA precursor and a decrease of final 18S product (2.2kb). Also, in case of PF clone 4 the accumulation of precursors is clearly seen on day 4 after induction, even though the growth phenotype on that day was not very strong. It can be expected that an even stronger accumulation will be observed at later time points.

Another experiment, although not done in our lab due to lack of the equipment, was the polysome analysis. This experiment was done only on procyclic RNAi cell line clone 4 (non-induced vs induced cells-6dpi), because in the collaborator's lab they do not work with bloodstreams.

The polysome analysis of induced cells showed that the 40S ribosomal subunit peak completely disappeared, while 60S ribosomal subunit signal showed very prominent accumulation. Assuming that there are still some residues of 40S (very abundant subunits) in the cells after the sixth day of induction, some the final 80S product is still assembled. Consequently, the detected signal is much narrower because if 40S was not present at all, the cells would die since ribosomes cannot be assembled anymore. Such an enormous change in the profile suggests that TbUTP10 protein really is essential for the survival of the cell. This was also indirectly indicated by unsuccessful attempts to create TbUTP10 double knock-out cell lines.

In zebrafish, the ortholog of TbUTP10 (Bap28) is involved in processing of pre-18S rRNA and ribosome biosynthesis. It is required in the vertebrate's central nervous system for the cell survival and development. Depletion of this protein by mutation lead to abnormal brain and organ development and subsequent death of the embryo between 6 and 7 days post-fertilization (Azuma et al. 2006). Although the human ortholog BAP28 is named, but not characterized, it is assumed to have a similar function. Still, mutations of the SSU processome components in humans are linked to some diseases. The ribosome assembly defects are confined to specific cell-types and do not affect all tissues. A mutation in a gene responsible for human UTP4 (cirhin) was shown to damage the liver in the progression of a disease, called North American Indian childhood cirrhosis (NAIC). Diseases of mutations of genes for UTP14 copies may include infertility in men, ovarian cancer and scleroderma (Sondalle et al. 2014).

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7. *Supplementary Figure 1: Complete alignment of TbUTP10 with its orthologs. The second aa sequence is Bap28 from zebrafish Danio rerio. The third is BAP28 from human and the fourth is Utp10 from Saccharomyces cerevisiae.*