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Elucidating the subunit composition of tRNA-guanine transglycosylase in *Trypanosoma brucei*

Bachelor thesis

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Annotation

The aim of this thesis was to elucidate the relationship and/or interaction between two tRNA-guanine transglycosylase paralogs (putative subunits of the TGT enzyme) identified in *Trypanosoma brucei*. The overexpression of these proteins was tested in a rescue experiment in a *Schizosaccharomyces pombe* experimental system.

Declaration

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

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1 INTRODUCTION

1.1 Trypanosoma brucei

Trypanosoma brucei is a unicellular flagellate parasite that belongs to the eukaryotic supergroup Excavata, phylum Euglenozoa, order Kinetoplastida. *Trypanosoma brucei* also belongs to a larger group of strictly parasitic trypanosomatids.

Three subspecies of *Trypanosoma brucei* have been described, two of them are major human pathogens. *Trypanosoma brucei gambiense* causes chronic sleeping sickness or trypanosomiasis in west and central sub-Saharan Africa. *Trypanosoma brucei rhodesiense* causes acute sleeping sickness in southeastern Africa. *Trypanosoma brucei brucei* causes nagana in livestock (Barrett et al. 2003).

Apart from the African *Trypanosoma brucei*, human trypanosomatid pathogens also include the South American *Trypanosoma cruzi*, the causing agent of Chagas' disease, and *Leishmania* spp., causing a range of diseases in the tropics and subtropics. Trypanosomatids belong to one of the earliest diverging groups in the eukaryotic domain (Barrett et al. 2003).

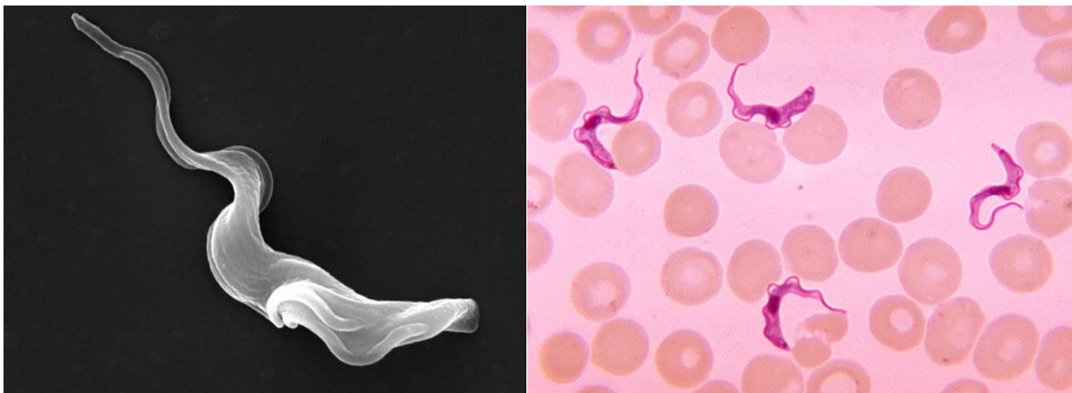

Figure 1 (A): Scanning electron micrograph of a *Trypanosoma brucei* (Wellcome Trust Sanger Institute 2016). (B): Blood smear taken from an individual with trypanosomiasis (Wellcome Trust Sanger Institute 2016).

The life cycle of *Trypanosoma brucei* is complex and includes several differentiated parasitic forms which infect alternately the carrier of the disease, the *Glossina* spp. fly also known as tsetse fly (order Diptera), and the mammalian host. While the infection level in tsetse is generally quite low, the untreated infection of the mammalian host is fatal (Holmes 2013).

The life cycle of *Trypanosoma brucei* is illustrated in Figure 2. When the infected tsetse fly takes a bloodmeal, the **metacyclic trypomastigote** forms of the parasite are transferred to the mammal's lymphatic system and bloodstream. The infection of the bloodstream is carried out by differentiated long slender proliferating forms. Through the blood vessel endothelium, the parasites eventually invade the extravascular tissues including the central nervous system. A number of the long slender forms differentiate into short stumpy forms which are adapted to survive in a tsetse fly. They enter the fly's digestive system during a blood meal on the infected mammalian host and differentiate into **procyclic trypomastigotes** in the midgut of the fly. After proliferation, the procyclic trypomastigotes migrate extensively from the midgut by way of peritrophic matrix, along the foregut, to the proventriculus of the fly. In the proventriculus, the procyclic trypomastigotes undergo an asymmetric division resulting in one long epimastigote and one short epimastigote. The short **epimastigotes** further migrate through the mouthparts and salivary duct of the fly into the salivary gland where they attach to the salivary gland epithelium. They replicate and differentiate into the metacyclic trypomastigotes that are freely distributed in the salivary gland lumen and adapted to survive in a mammalian host (Langousis & Hill 2014). The cycle in the fly takes approximately three weeks (Centers for Disease Control and Prevention 2015).

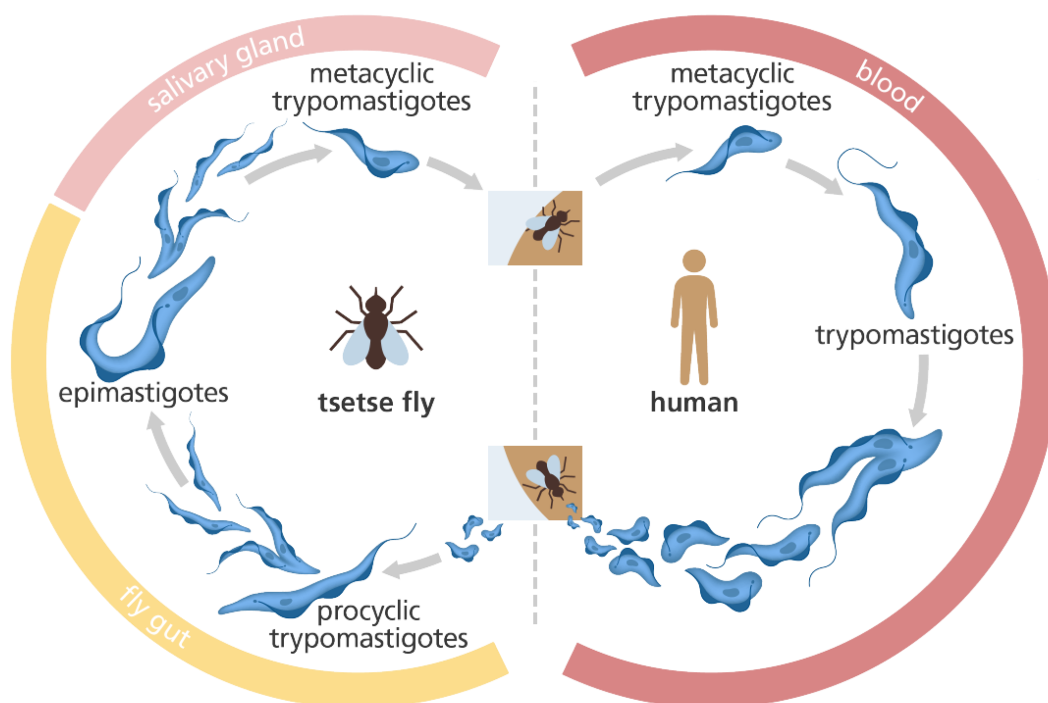

Figure 2: Generalized life cycle of *Trypanosoma brucei* (Wellcome Trust Sanger Institute 2016).

Trypanosoma brucei is widely studied not only because of its considerable impact on human (livestock) health but also because of its remarkable biology including the dixenous life cycle, the single mitochondrion or the single flagellum of the cell. Although many details of the biology of trypanosomes and the diseases they cause have been clearly established, sleeping sickness has not been effectively tackled. This is partly because of the extensive antigenic variation of the parasites' surface coat of glycoproteins that is present during the whole mammalian part of the cycle. The development of an effective vaccine has thus been prevented. In addition, the few drugs available for treatment are unsatisfactory and often have considerable side-effects. Significant progress has been achieved through the control of tsetse flies but these measures are expensive and re-infestation occurs frequently. Therefore, trypanosomes and the search for a more complex solution of trypanosomiasis treatment remain among researchers' priorities (Holmes 2013).

1.2 tRNA maturation and post-transcriptional modifications

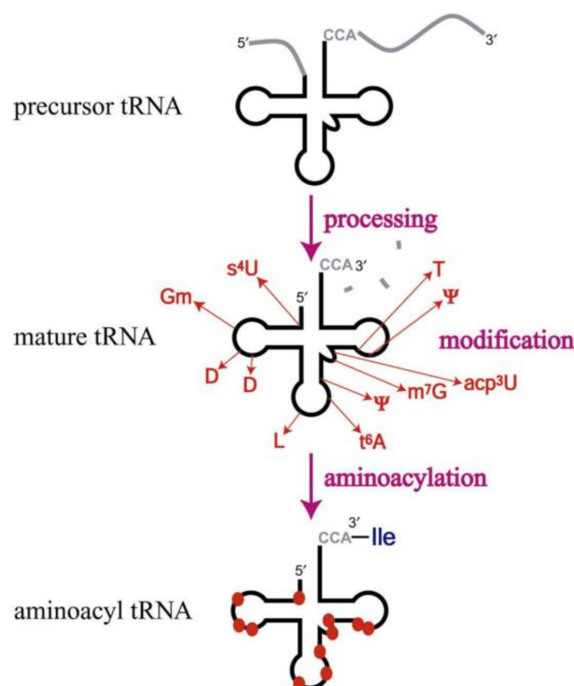

Figure 3: tRNA maturation: processing, post-transcriptional modification and aminoacylation (Nakanishi & Nureki 2005).

To fulfill its crucial function of translating the mRNA codons to amino acids in a ribosome, tRNA transcripts must be processed into the standard length and post-transcriptionally modified. Processing to the standard length includes cleaving of the 5' leader extension and the 3' trailer extension of the original transcript (Figure 3). Many nucleotides in various portions of the precursor tRNA undergo modification by various modification enzymes, the tRNA molecule thus becoming mature. Mature tRNAs are specifically recognized by aminoacyl-tRNA synthetases, and the corresponding amino acid is attached onto the 3'-CCA terminus (Nakanishi & Nureki 2005).

Modifications are found at approximately 12% of all tRNA residues, i.e. a regular tRNA bears approximately 8 modifications (Phizicky & Alfonzo 2010). Around 90 different tRNA modifications have been described so far (Duechler et al. 2016), out of which there are 18 "universal" modifications that occur in tRNA in all three domains of life (Jackman & Alfonzo 2013). They include relatively simple chemical changes such as the addition of one or two methyl groups to various positions of the nucleotide bases and/or ribose sugars, replacement of oxygen with sulfur, isomerization of the uridine base to pseudouridine or reduction of the uridine base to dihydrouridine, or addition of other relatively small chemical functional groups, e.g. acetylation or threonylation. On the other hand, modifications found in only two of the three domains (e.g. in Archaea and Eukarya) or only one domain are chemically complex, in some cases, the added functional groups being greater in size than the original purine or pyrimidine ring (Jackman & Alfonzo 2013). The overall amount of modifications appears to increase with the evolutionary development of an organism (Vinayak & Pathak 2010).

Modifications of tRNA can be divided into two categories based on how they affect tRNA function. Modifications located in the core regions (D- and TΨC-loops) contribute towards stabilizing the highly conserved L-shaped tertiary structure, e.g. by enhancing stacking interactions (Nakanishi & Nureki 2005), and also act as identity determinants for tRNA interacting proteins (Fergus et al. 2015). The two loops, which form the "elbow" of the L-shaped tRNA molecule, take their names from essential modifications: residues 16 and 17 of the D-loop are typically modified into dihydrouridines (D) and T and Ψ denote the ribothymidine and pseudouridine modifications of the TΨC-loop (Zhang & Ferré-D'Amaré 2016).

Modifications occurring within the anticodon loop determine codon pairing, and therefore influence translational accuracy and protein production. They also play a role in recognition

by the cognate aminoacyl-tRNA synthetases (Kirchner & Ignatova 2015). Positions that are prone to be modified at a high frequency are referred to as “modification hotspots” and include positions 34, 37 and 32 (Manickam et al. 2016).

The overall most frequent modifications at position 34 are typically associated with altering (increasing or decreasing) the diversity of codon recognition (Manickam et al. 2016). In his wobble hypothesis, Crick (1966) proposed an unusual pairing in the third position, which allows e.g. the pairing of U_{34} in the anticodon with A or G in the codon. Addition of 5-methylene derivatives, usually combined with 2-thiolation, as in the mnm^5s^2U modification is thought to restrict the decoding to the canonical A (Nishimura 1979). An unconventional U-G pair is thought to require protonation of U_{34} (Takai & Yokoyama 2003).

Modifications at position 37, the nucleotide immediately 3’ of the anticodon, tune the stability of codon-anticodon interactions (Kirchner & Ignatova 2015).

1.3 Queuosine tRNA modification

One of the most remarkable and elaborate modifications at the translationally very important wobble position 34 in eubacterial and eukaryotic tRNAs is the modification of guanosine into queuosine (Q) (Fergus et al. 2015).

Direct tRNA sequencing methods determined that the queuosine modification can be found only in eubacterial and eukaryotic tRNAs that contain a **$G_{34}U_{35}N_{36}$** anticodon sequence where N stands for any of the four canonical bases (Nishimura 1983). The **Q_{34} modified** tRNA acceptors can efficiently decode either **NAU** or **NAC** codons (Figure 4A), whereas the unmodified G_{34}-containing tRNAs preferably recognize NAC (Meier et al. 1985). The synonymous NAC/U codons correspond to the amino acids **asparagine** (AAC/U), **histidine** (CAC/U), **aspartic acid** (GAC/U) and **tyrosine** (UAC/U). These specific tRNAs are often referred to as “the Q-family” tRNAs (Vinayak & Pathak 2010).

The queuosine nucleoside was first identified in 1968 in hydroxylate extracts of $tRNA^{Tyr}$ from *E. coli* (Goodman et al. 1968). Q is a hypermodified analog of guanosine and its base form is denoted as queuine (q). Queuosine consists of a 7-deazaguanosine base wherein the purine nitrogen at position seven is substituted with a carbon (Figure 4B in blue). An amino-methyl side chain (in green) connects the 7-deazaguanosine core to a cyclopentenediol moiety (in orange) (Fergus et al. 2015).

In higher eukaryotes, the cyclopentene-diol ring is further modified by sugar molecules. As shows Figure 4C, galactose modification occurs in $tRNA^{Tyr}$, whereas mannose can be appended to the queuosine of $tRNA^{Asp}$ (Fergus et al. 2015). Such sugar derivatives of Q were denoted Q* (Kasai et al. 1976; Okada et al. 1977). In contrast to eukaryotes, eubacteria do not produce sugar-modified queuosine. However, the cyclopentene hydroxyl groups of eubacterial $tRNA^{Asp}$ can be modified by the addition of a glutamic acid residue to the C4'' or C5'' position (Fergus et al. 2015).

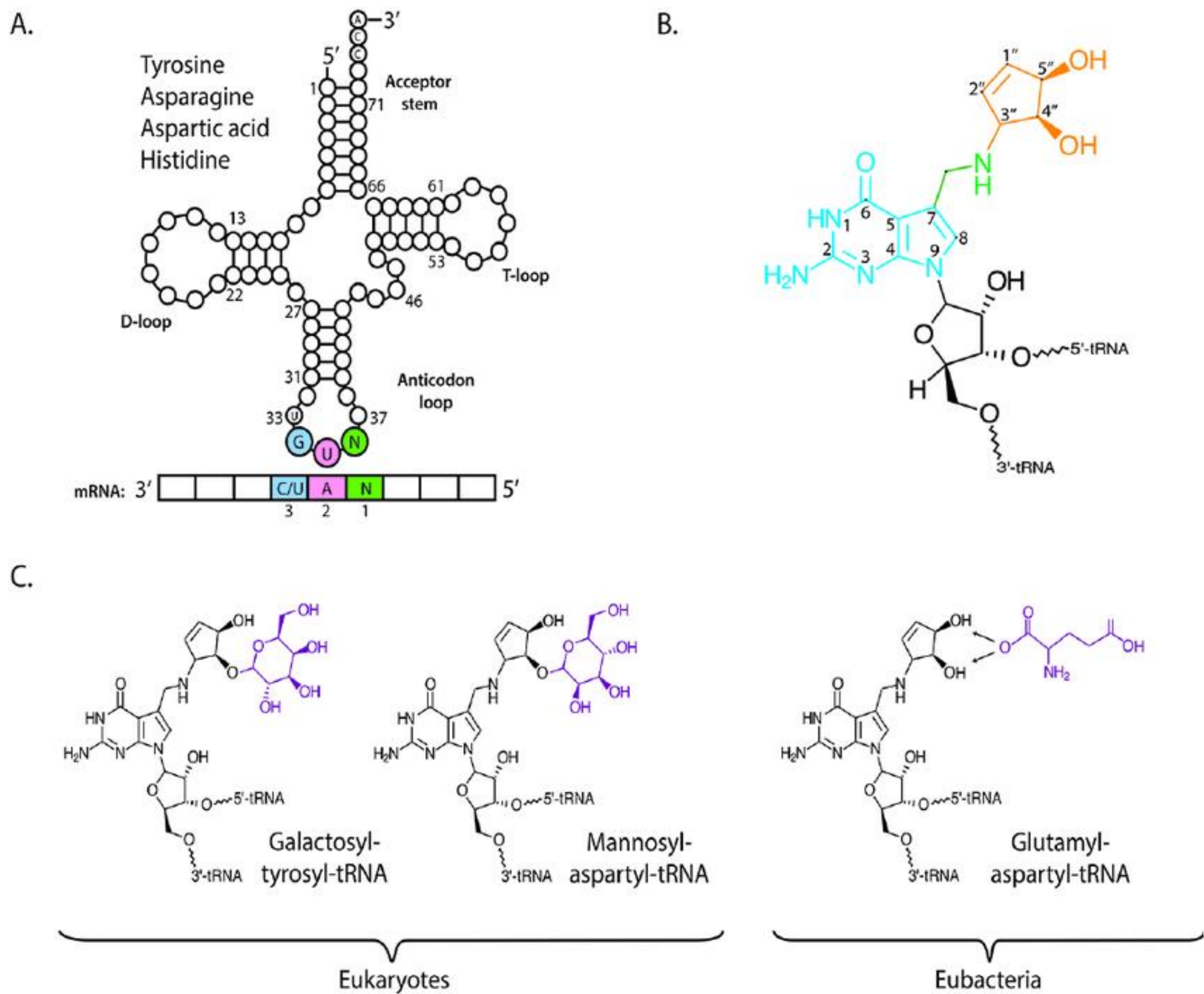

Figure 4 (A): Structure of a $G_{34}U_{35}N_{36}$ tRNA. (B): Chemical structure of the basic queuosine molecule: 7-(3,4-*trans*-4,5-*cis*-dihydroxy-1-cyclopenten-3-yl-aminomethyl)-7-deazaguanosine. (C): Examples of further modifications of queuosine (Fergus et al. 2015).

1.4 Biosynthesis of queuosine

Although the queuosine modification is evolutionarily conserved and present throughout the living system (with certain exceptions of e.g. *Saccharomyces cerevisiae*, *Candida albicans* and others), the modification process itself differs significantly in Bacteria and Eukaryotes (Vinayak & Pathak 2010; Zallot et al. 2014).

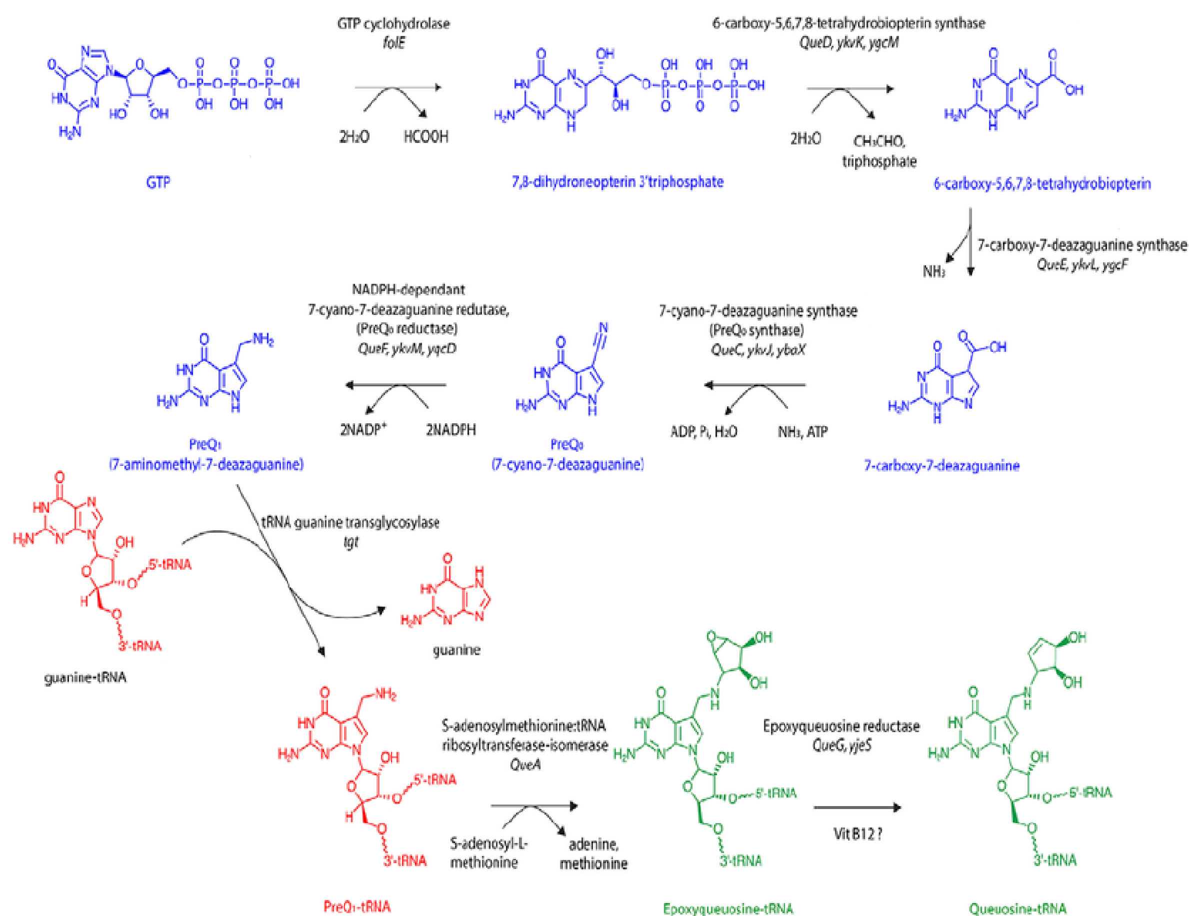

Figure 5: The enzymatic pathway of queuosine biosynthesis in Bacteria (Fergus et al. 2015).

Only bacteria are capable of producing queuosine *de novo*. The process can be divided into two phases. The enzymatic pathway begins with guanosine triphosphate (GTP), from which the precursor of Q, 7-aminomethyl-7-deazaguanine or preQ$_1$, is produced by way of five enzymatic steps (Figure 5 in blue). In the second phase, preQ$_1$ is reversibly incorporated in the wobble position 34 by tRNA-guanine transglycosylase (TGT or TGTase) in a base-exchange reaction (Figure 5 in red). In this *trans*-glycosylation reaction, the N-C glycosyl bond is broken by a non-energy dependent mechanism that is unique to the TGT enzyme, without cleaving the phosphodiester backbone. Two more enzymatic steps ensure the modification of preQ$_1$-

tRNA into the Q-tRNA *in situ* (Figure 5 in green). The Q-modified tRNA fulfills normal cellular function until its turnover. Bacteria salvage neither queuosine nor its base denoted as queuine (q). Prokaryotic Q degradation products are lost to the surrounding environment as metabolic waste (Vinayak & Pathak 2010; Fergus et al. 2015).

Unlike prokaryotes, eukaryotes are not able to synthesize any of the Q precursors (preQ_1 or queuine). Animals, plants and fungi acquire queuine as a micronutrient from the environment, diet or intestinal microbiota. Apart from that, they employ a salvage system to ensure a consistent supply of queuine (Vinayak & Pathak 2010).
The Q pathway in eukaryotes significantly differs from the prokaryotic one and can be divided into three steps. First, the free q base is taken up by the cell from the environment. Afterwards, **this completely modified** base is directly incorporated in the Q-family tRNAs by eukaryotic TGTase in a **single-step** enzymatic reaction (Figure 6). Lastly, Q-tRNA turnover is followed by salvage of q from the degradation products: the queuosine nucleoside, queuosine-5'-phosphate and queuosine-3'-phosphate (Figure 7). However, the mechanism of the salvage remains unknown. In *S. pombe*, the DUF2419 protein was recently identified to have a role in Q salvage (Zallot et al. 2014).

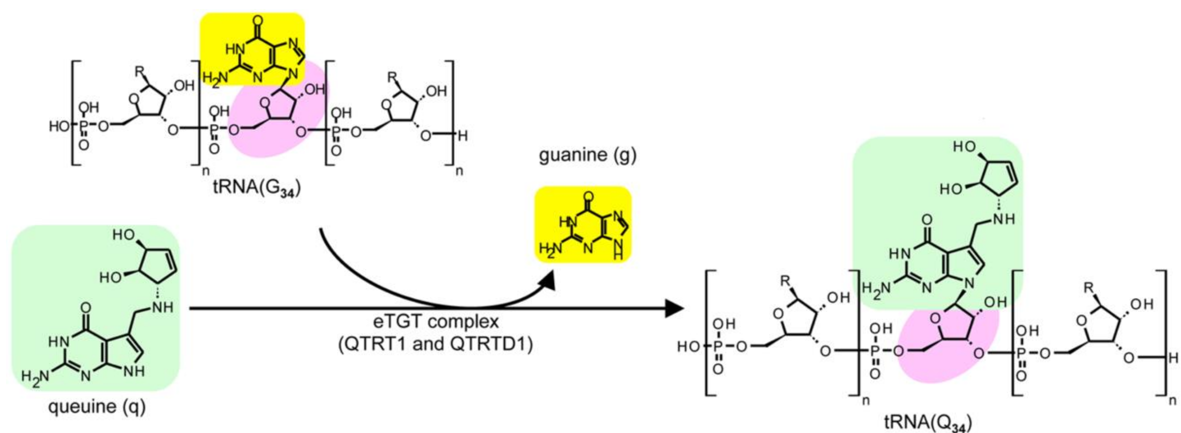

Figure 6: The single-step enzymatic replacement of guanine with queuine in eukaryotes (Zallot et al. 2014).

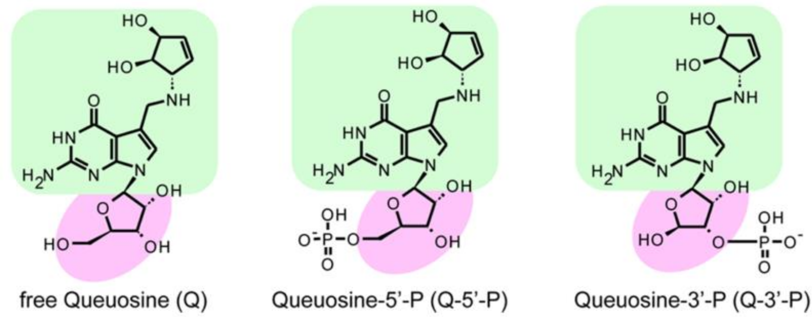

Figure 7: Queuine salvage precursors (Zallot et al. 2014).

1.5 tRNA-guanine transglycosylase

The key *trans*-glycosylation reaction of the Q modification process is catalyzed by the bacterial or eukaryotic enzyme tRNA-guanine transglycosylase (TGT or TGTase) (Chen et al. 2010).

The eubacterial TGT (bTGT) is a single protein species, that has been shown to oligomerize. For example in the bacterium *Zymomonas mobilis*, the formation of a 2:1 complex of the enzyme and tRNA respectively has been identified (Chen et al. 2011). In addition, the crystal structure of the *Z. mobilis* TGT has been determined and revealed the enzyme to be an irregular $(\beta/\alpha)_8$ TIM barrel with a C-terminal zinc-binding subdomain (Romier et al. 1996).

In contrast to the **homodimeric** bTGT, the eukaryotic TGT (eTGT) has been shown to function as a **heterodimer** in mice, humans and other types of eukaryotic cells (summarized in Table 1), except for the plant (wheat germ) cells where it is homodimeric (Walden et al. 1982). The mouse TGT has been proven to consist of two different subunits, a queuine tRNA-ribosyltransferase 1 catalytic subunit (QTRT1), a homolog of the bTGT enzyme, and a queuine tRNA-ribosyltransferase domain-containing 1 subunit (QTRTD1) (Boland et al. 2009). Chen (2010) provides evidence that the human TGT also consists of two different subunits in a 1:1 ratio that have been named hQTRT1 and hQTRTD1. Individually, neither of them was functional when overexpressed in *E. coli* (Chen et al. 2010). It has been originally proposed that the QTRTD1 subunit could act as a queuine salvage enzyme and that the eukaryotic heterodimer could have evolved from the prokaryotic homodimer, QTRT1 specializing in the catalytic activity and QTRTD1 in the salvage of its substrate (Chen et al. 2010).

Type of cells	TGT composition	Size of the subunits (kDa)	Reference
Escherichia coli	Homodimeric	42,6	UniProt database
Zymomonas mobilis	Homodimeric	43	Romier et al. 1996
Wheat germ	Homodimeric	68	Walden et al. 1982
Schizosaccharomyces pombe	Heterodimeric	45 (QTRT1) and 73.2 (QTRTD1)	UniProt database
Caenorhabditis elegans	Heterodimeric	45.2 (QTRT1) and 42 (QTRTD1)	UniProt database
Drosophila melanogaster	Heterodimeric	47.7 (QTRT1) and 46.6 (QTRTD1)	UniProt database
Mouse	Heterodimeric	44 (QTRT1) and 10 (QTRTD1)	UniProt database
Rat liver	Heterodimeric	60 (QTRT1) and 34.5 (QTRTD1)	Morris et al. 1995
Rabbit erythrocytes	Heterodimeric	60 (QTRT1) and 43 (QTRTD1)	Howes & Farkas 1978
Bovine liver	Heterodimeric	66 (QTRT1) and 32 (QTRTD1)	Slany & Müller 1995
Human	Heterodimeric	45.5 (QTRT1) and 47.4 (QTRTD1)	Chen et al. 2010

Table 1: Overview of TGT subunits composition and size in different types of cells.

1.6 Queuosine in *Trypanosoma brucei* and the *Schizosaccharomyces pombe* experimental system

Using the BLAST search, *T. brucei* orthologs of the human QTRT1 and QTRTD1 were identified in the kinetoplastid genome database (tritrypdb.org). The *T. brucei* TbTGT1 shows all the conserved aspartates required for the transglycolase activity. In contrast, TbTGT2 doesn’t possess any catalytic Asp residues. The cysteins and histidine responsible for zinc binding are present in both sequences. It is therefore speculated that, similar to its human or mouse counterparts, TbTGT1 might possess the catalytic function, whereas TbTGT2 might be responsible for the stability of the tRNA-TGT complex and possibly also for the salvage of queuine from the environment.

Recently, a homolog of DUF2419, the protein responsible for queuine salvage in other eukaryotes, was annotated in the tritrypdb.org database in *T. brucei* but the function of this protein is not yet clear.

The physiological function of the trypanosomatid TbTGT1 and TbTGT2 putative subunits has been intensively studied in our laboratory. Preliminary data suggest that both proteins are involved in formation of the queuosine modification. Our laboratory’s goal is to elucidate the

possible interaction between the two paralogs as well as to shed more light on the role of the Q modification in *T. brucei*.

In this thesis research project, the interaction between the two proteins was studied by inducing their overexpression in a *Schizosaccharomyces pombe* experimental system.

Schizosaccharomyces pombe, also known as fission yeast, is a unicellular eukaryote which belongs to Ascomycota. This model organism is convenient for genetic manipulation thanks to its undemanding growth conditions, short generation time of 2-4 hours and the commercial availability of the required genetic manipulation components such as plasmids.

S. pombe was chosen as the model organism for this study rather than *S. cerevisiae*, the universal model for tRNA research, because *S. cerevisiae* lacks TGT and consequently, the Q-modified tRNAs. *S. pombe*, on the other hand, contains the genes encoding QTRT1 and QTRTD1 as well as the DUF2419 protein (Zallot et al. 2014).

2 WORK AIMS

Using the overexpression of two *T. brucei* tRNA-guanine transglycosylase paralogs in *S. pombe*, the overall aim of this thesis was to elucidate whether both TbTGT putative subunits are responsible for the formation of the queuosine tRNA modification.

1) Preparation of the *S. pombe* overexpression constructs containing TbTGT1 and TbTGT2 ORFs
2) Electroporation of the constructs into *S. pombe*
3) Confirmation of the protein overexpression
4) Determination of the Q-tRNA modification levels

3 MATERIAL AND METHODS

3.1 Preparation of *S. pombe* overexpression constructs

3.1.1 Primer design and PCR

Using the SnapGene software, four primers containing appropriate restriction sites were designed for PCR amplification of the genes encoding two TbTGT putative subunits (Table 2). The nucleotide sequences of TbTGT1 and TbTGT2 were obtained from the TriTryp database (tritrypdb.org) under the accession numbers **Tb927.6.3130** and **Tb927.5.3530**, respectively. For the final yeast constructs, the ORFs were amplified without the start codon because both proteins will be overexpressed with fused N-terminal protein tags.

Name	Direction	Oligonucleotide sequence (5'→3')	T_m [°C]	Restriction site (underlined)
Q1Sp_ZP53F	F	GCTGGATCCCCCGTCGCTACTCAAGCTACTC	58	BamHI
Q1Sp_ZP54R	R	AGCGGATCCTCAGCGGGGGAGCTCAACACTG	58	BamHI
Q2Sp_ZP55F	F	GCTGTCGACCACGGAGTGTATCCTATTTTAG	58	SalI
Q2Sp_ZP56R	R	AGCGGATCCTCACAACTGTGCCAGCACCCAAG	58	BamHI

Table 2: List of the used primers.

The time of the PCR elongation step (Table 4) was set to 1.5 min according to the expected size of the TbTGT1 and TbTGT2 PCR products which is **1239 bp** and **1023 bp**, respectively.

Compound	Volume [µl]
5x Q5 Reaction Buffer	10
10 mM dNTPs	1
Forward Primer	2.5
Reverse Primer	2.5
Template DNA (TbTGT1 or TbTGT2)	1
Q5 High Fidelity DNA Polymerase (NEB#M0491S)	0.5
PCR water	32.5

Table 3: Composition of the PCR reactions.

Step	Temperature [°C]	Time [s]
1. Initial denaturation	98	30
2. Denaturation	98	10
3. Annealing	58	30
4. Elongation	72	90
5. Final elongation	72	300
6. Hold	4	∞
Number of repeats of steps 2.-4.	35	

Table 4: PCR cycles setup.

3.1.2 Gel electrophoresis

A small sample of each PCR product was mixed with 6x loading dye. The length of the two PCR amplicons was verified on a 1% agarose/1x NNB gel using ethidium bromide at a final concentration of 0.1 µg/ml. The separated DNA was visualized by HP Alphaimager and its size compared to a 1 kb Plus DNA ladder (ThermoFisher#10787018). The DNA band of the expected size was cut out under a UV transilluminator and eluted with GenElute™ Gel Extraction Kit (Sigma#NA1111-1KT).

3.1.3 Ligation

The blunt-ends of the two PCR products were A-tailed according to the manufacturer's protocol and individually ligated into bacterial pGEM-T Easy plasmids with complementary T-overhangs (Promega A137A). The reaction was gently mixed, briefly centrifuged and incubated overnight at room temperature.

Compound	Volume [µl]
2x Rapid Ligation Buffer	5
Vector (pGEM-Teasy)	0.5
PCR product	1
T4 DNA ligase (NEB#M0202S)	1
Water	2.5

Table 5: Composition of the ligation reaction.

3.1.4 Bacterial transformation and blue-white screening

From each of the ligation reactions, 5 µl was taken and gently mixed to an aliquot of *E. coli* competent cells. The tubes were incubated on ice for 20 min and then subjected to a heat shock of 42 °C for 30 s. The cells were immediately cooled down on ice for 2 min, 500 µl of nutritionally rich SOC medium was added and the tubes were placed in a 37 °C shaking incubator for 1 hour. After that, the cells were spread onto LB agar plates containing IPTG

(200 mg/ml), X-gal (20 mg/ml) and ampicillin (100 µg/ml) and incubated overnight at 37 °C to generate individual colonies.

The following day, the plates were screened and four white colonies of bacteria were selected. Samples of individual colonies were inoculated into 3 ml of LB broth with ampicillin (0.1 mg/ml) and grown in a 37 °C shaking incubator overnight.

3.1.5 Isolation of plasmid DNA

The minipreps were centrifuged and the LB broth discarded. Plasmid DNA was isolated from the pelleted cells using GeneAll® Hybrid-Q™ Kit (GeneAll#100-102). The DNA was eluted with deionized water and the concentration of the plasmid DNA was measured using NanoDrop Spectrophotometer. The correct sequence of the obtained plasmids was confirmed by sequencing (SEQ*me*).

3.1.6 Plasmid screening by restriction analysis

To confirm the presence and position of the insert in the vectors, the isolated plasmids were digested by restriction endonucleases. The restriction reactions were incubated at 37 °C for 90 min. The results of the restriction were analyzed on a 0.75% agarose gel.

Compound	Volume for TbTGT1 [µl]	Volume for TbTGT2 [µl]
Plasmid DNA	10	10
BamHI (NEB#R3136S)	2	2
SalI-HF (NEB#R3138S)	-	2
10x Cut Smart Buffer	10	10
Water	78	76

Table 6: Composition of the restriction reactions.

The correct sized separated inserts were cut out from the gel under a UV transilluminator, the DNA was purified by GenElute™ Gel Extraction Kit (Sigma#NA1111-1KT) and NanoDrop Spectrophotometer was used to measure the concentration.

3.1.7 *S. pombe* overexpression plasmids

The next step was to clone the amplified TbTGT1 and TbTGT2 inserts into yeast expression plasmids pREP41 and pREP42, respectively (Figure 8). The plasmids were kindly provided by Dr. Martin Převorovský, Faculty of Science, Charles University, Prague, Czech Republic. The size of the plasmids is nearly 9000 bp and they contain selectable nutritional markers for

leucine (pREP41) or uracil (pREP42). Upstream of the inserted cassette, there is nmt1 promotor, 5' UTR, start codon, N-terminal tags and multiple cloning site sequences. Stop codon and 3' UTR are located downstream of the cassette.

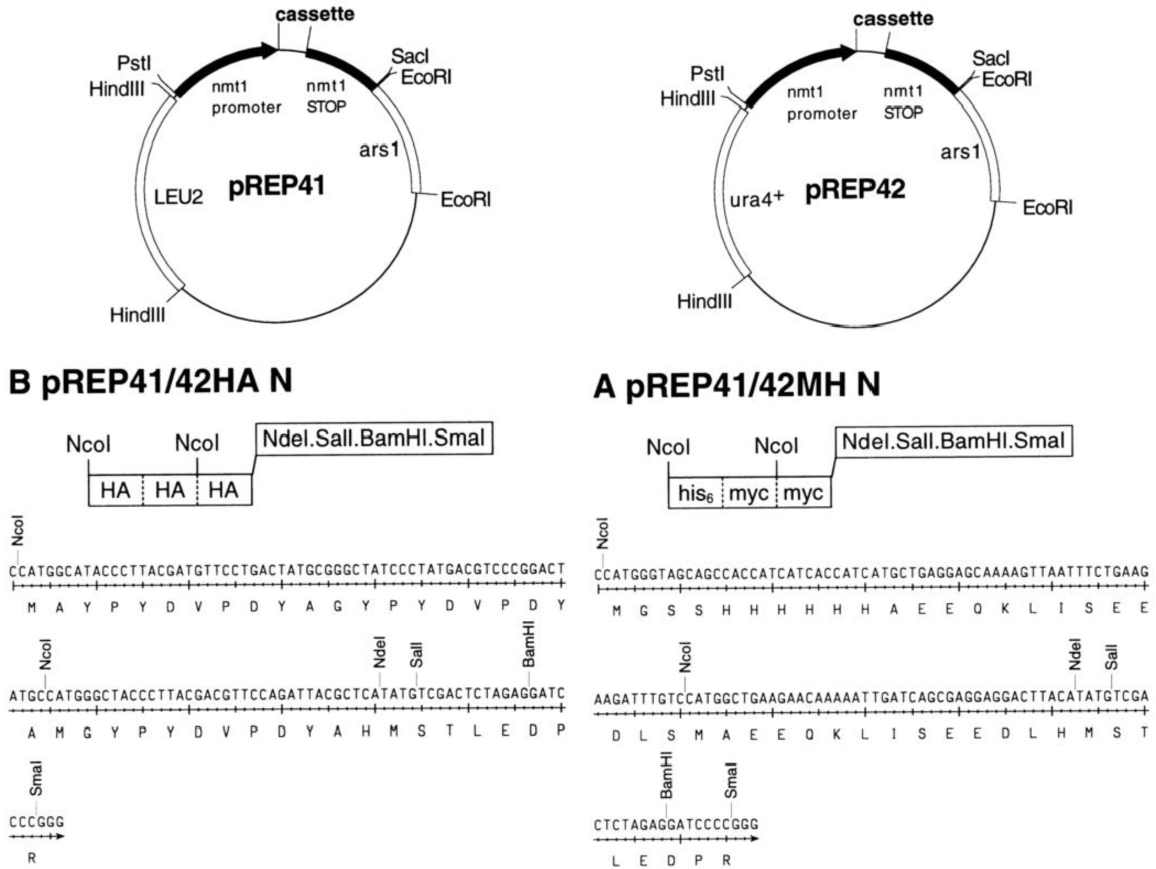

Figure 8 (A): The pREP41 plasmid containing three N-terminal HA tags. (Craven et al. 1998). (B): The pREP42 plasmid containing two N-terminal myc tags. (Craven et al. 1998).

The yeast pREP41 and pREP42 plasmids were digested with the same restriction endonucleases as both TbTGT1 and TbTGT2 inserts. To increase the efficiency of the ligation, the 5' ends of the yeast plasmids were treated with alkaline phosphatase and incubated at 37 °C for 1 hour. Afterwards, the plasmid DNA was purified using GenElute^{TM} PCR Clean-Up Kit (Sigma#NA1020-1KT) and the concentration was measured by NanoDrop Spectrophotometer.

Compound	Volume [µl]
Plasmid DNA	30
10x Cut Smart Buffer	3
Calf Intestine Phosphatase (NEB#M0290S)	1
Water	1

Table 7: Alkaline phosphatase treatment of plasmid DNA.

3.1.8 Bacterial transformation and its confirmation

NEB Ligation calculator (nebiocalculator.neb.com/#!/ligation) was utilized to calculate the amount of the insert needed for the ligation reaction according to the molar vector to insert ratio 1:3. *E. coli* competent cells were transformed with complexes of pREP41+TbTGT1 and pREP42+TbTGT2 and positive clones were confirmed by restriction analysis and sequencing (SEQ*me*).

3.2 Transformation of *S. pombe* by electroporation

S. pombe TGT⁻ yeast cells (BG_H0432) and WT controls (ED668) were thawed and streaked out on YES plates (composition described below, see Chapter *3.4 S. pombe cultivation*). The plates were incubated at 30 °C for 6 days until individual colonies appeared.

50 ml of YES medium (see Chapter 3.4.1) and 50 ml of TGT⁻ YES medium (see Chapter 3.4.2) was aliquoted to Erlenmeyer flasks and inoculated with cells from one colony from each plate. The flasks were incubated at 30 °C in a shaking platform (110 rpm) for approximately 24 hours. The yeast were harvested at OD_{600} = 0.6-0.7, i.e. in the exponential phase.

The cell cultures were harvested by centrifugation (3 000 rpm, 10 min). The WT control cells were washed 3x with 1x PBS and stored at -80 °C for further use.

The transformation protocol was adapted from Prentice (1992). The TGT⁻ cell pellet was washed 3x with 1.2 M sorbitol. Sterile electroporation cuvettes with a 2 mm electrode gap (BTX) were cooled on ice. The cell pellet was resuspended in 500 µl of 1.2 M sorbitol to ensure extremely low conductivity and 1 µg of plasmid DNA (pREP41+TbTGT1 or pREP42+TbTGT2) was added. Immediately, 200 µl of the mix was transferred to a cuvette and pulsed with 2.25 kV, 200 Ω, 25 µF. 1 ml of 1.2 M sorbitol was added directly. 500 µl of the suspension was spread on a TGT⁻ selective plate (see Chapter 3.4.3) and incubated at 30 °C for 6 days.

3.2.1 Colony PCR

The presence of pREP41+TbTGT1 or pREP42+TbTGT2 plasmids in individual *S. pombe* colonies was confirmed by colony PCR.

Compound	Volume [µl]
2x PPP Master Mix (Top-Bio#P124)	12.5
Forward Primer (TbTGT1 or TbTGT2, see table X)	1
Reverse Primer (TbTGT1 or TbTGT2, see table X)	1
Plasmid DNA	1
PCR water	9.5

Table 8: Composition of the PCR reactions.

Step	Temperature [°C]	Time [s]
1. Initial denaturation	94	60
2. Denaturation	94	15
3. Annealing	58	15
4. Elongation	72	90
5. Final elongation	72	300
6. Hold	10	∞
Number of repeats of steps 2.-4.	30	

Table 9: PCR cycles setup.

3.3 Induction of the TbTGT proteins overexpression

One TbTGT1 transformant and one TbTGT2 transformant were selected and further cultivated in 50 ml of EMM medium (see Chapter 3.4.3, Table 12). Upon reaching late log phase (OD_{600} = 0.6-0.7), the yeast were harvested by centrifugation (6 000 rpm, 5 min), washed 3x with 1x PBS and protein overexpression of TbTGT1 and TbTGT2 was induced by inoculating the yeast cells in 50 ml of EMM medium **without thiamine**. The cultures were harvested at OD_{600} = 0.6-0.7, centrifuged, washed 3x with 1x PBS and stored at -80 °C for further use.

The **co-expressing *S. pombe* cell line** (TbTGT1+TbTGT2) was created by electroporating the TbTGT1 transformant with pREP42+TbTGT2 plasmid DNA following the above mentioned protocol (Chapter 3.2). The culture was induced and harvested following the above mentioned steps.

3.4 Yeast cultivation

S. pombe prototrophic wild-type strain (ED668), and *S. pombe* TGT knockout strain (BG_H0432) auxotrophic for adenine, uracil and leucine with G418 resistance marker gene were kindly provided by Prof. Valérie de Crécy-Lagard, Department of Microbiology and Cell Science, University of Florida, Gainesville, United States. Wild-type and TGT^{-} control yeast were grown in complex YES medium, TGT^{-} transformants containing the pREP plasmids were grown in selective Edinburgh Minimal Media (see Chapters 3.4.1-3 below). Since eukaryotes obtain queuine from their environment, 2% bactopeptone was added to all media to provide sufficient substrate for the TGT enzyme (Zallot et al. 2014). To repress nmt promotor in the pREP vectors, sterile 15 μM thiamine was added to the media. Protein overexpression was induced by growing TGT^{-} transformants in media without thiamine.

3.4.1 Wild-type strain cultivation

Prototrophic wild-type *S. pombe* were grown in complex medium containing yeast extract, glucose and bactopeptone (Peptone, bacteriological grade, Amresco#J636-100G) dissolved in distilled water. 2.5% bacteriological agar was added to the above mentioned when plates were made. All media were autoclaved before use.

YES medium	
Compound	**Amount**
Yeast extract	5 g/L
Glucose	30 g/L
Bactopeptone	20 g/L
YES medium plates	
Bacteriological agar	25 g/L
Autoclaving	

Table 10: Composition of 1L of the wild-type cultivation and plates medium.

3.4.2 *S. pombe*$^{TGT-}$ cultivation

The TGT knockout strain was grown in YES medium containing yeast extract, glucose and bactopeptone (Peptone, bacteriological grade, Amresco#J636-100G) dissolved in distilled water. When plates were made, 2.5% bacteriological agar was added to the above mentioned. Since the TGT deletion mutant carries G418 resistance, G418 selectable marker (200 mg/ml) was added to the media.

TGT^{-} YES medium	
Compound	**Amount**
Yeast extract	5 g/L
Glucose	30 g/L
Bactopeptone	20 g/L
TGT^{-} YES medium plates	
Bacteriological agar	25 g/L
Autoclaving	
G418	200 mg/ml

Table 11: Composition of 1L of the TGT^{-} cultivation and plates medium.

3.4.3 *S. pombe*$^{TGT-}$ transformants cultivation

The auxotrophic TGT^{-} transformants were grown in Edinburgh Minimal Medium Broth without Dextrose (Formedium, PMD0305) dissolved in distilled water with glucose, bactopeptone (Peptone, bacteriological grade, Amresco#J636-100G) and nutritional selectable markers adenine, uracil or leucine according to the type of the plasmid construct(s). When plates were made, 2.5% bacteriological agar was added to the above mentioned. After autoclaving and preparation of 50 ml growing flasks or before the plates were poured, G418 (200 mg/ml) and thiamine (15 µM) were added.

Compound	TbTGT1 EMM medium	TbTGT2 EMM medium	TbTGT1+2 EMM medium
Edinburgh Minimal Medium	12.3 g	12.3 g	12.3 g
Glucose	20 g	20 g	20 g
Adenine	250 mg	250 mg	250 mg
Uracil	250 mg	-	-
Leucine	-	250 mg	-
Bactopeptone	20 g	20 g	20 g
Plates			
Bacteriological agar	25 g		
Autoclaving			
G418	200 mg/ml		
Thiamine	15 µM		

Table 12: Composition of 1L of the TGT^{-} transformants cultivation and plates medium.

3.5 Isolation of RNA by guanidine extraction

Prior to RNA extraction, the harvested yeast cells were disrupted by extensive sonication. Each tube (yeast cells in 500 µl of Solution D) was sonicated using Ultrasonic Homogenizer with 15 consecutive pulses of 20 kHz ultrasound (the pulser set to 50%), and the procedure was repeated six times.

Total RNA was isolated following the guanidinium thiocyanate-phenol-chloroform extraction protocol (Chomczynski & Sacchi 1987). Yeast cells were resuspended in 500 µl of Solution D (Table 13), 50 µl of 2 M NaOAc (pH 4), 500 µl of H_2O saturated phenol, and 150 µl of chloroform/isoamyl alcohol (24:1).

Solution D	
Compound	**Concentration**
Guanidine isothiocyanate extract	4 M
Sodium citrate (pH 7)	25 mM
Sarcosyl	0.5%
Mercaptoethanol amine	0.1 M

Table 13: Composition of Solution D (Chomczynski & Sacchi 1987).

The samples were vortexed for 10 min and placed on ice for 10 min. To separate the phases, the tubes were centrifuged at 12 000 rpm at 8 °C for 15 min. The upper aqueous phase containing RNA was transferred to a fresh tube and equal volume of isopropanol was added together with 1 µl of glycogen (20 mg/ml). The samples were stored in -20 °C for at least 20 min and afterwards, centrifuged at maximum speed at 8 °C for 30 min. The supernatant was discarded and the pellets were washed with 70% ethanol, i.e. centrifuged for 5 min at maximum speed and after the ethanol had been discarded, air-dried for 10 min in room temperature. 200 µl of water was added to the pellets without stirring and the tubes were incubated for 10 min in room temperature. The extraction was repeated with half of the sample volume of phenol (Tris-HCl, pH 8) and half of the sample volume of chloroform/IAA. The samples were vortexed for 10 min, cooled on ice for 10 min, centrifuged at 12 000 rpm at 8 °C for 15 min and the aqueous phase containing RNA was transferred to a new tube. RNA was precipitated with 3 volumes of ethanol, 0.1 volume of 3 M NaOAc and 1 µl of glycogen (20 mg/ml). The above mentioned extraction steps were repeated until the pellets were resuspended in 30 µl of water after the ethanol wash. To facilitate RNA dissolving in water, the samples were placed in a 56 °C heating block for 10 min as the last step of the extraction. The concentration of the isolated RNA was measured by NanoDrop Spectrophotometer.

3.6 Aminophenyl boronate affinity PAGE and northern blotting

3.6.1 APB affinity gel electrophoresis

The presence of queuosine-modified tRNAs was determined by northern blotting using modified affinity aminophenyl boronate gel electrophoresis (Igloi & Kössel 1985). Boronate gels were poured using 1x TAE gel solution (8 M Urea, 8% AA) with 10% APS, TEMED and 50 mg of aminophenylboronic acid.

1x TAE gel solution	
Compound	**Amount**
UREA	42 g
50x TAE	2 ml
Acrylamide	28.38 g
Bis-acrylamide	1.62 g
Water	up to 100 ml

Table 14: Composition of 1x TAE gel solution.

5 µg of total RNA was deacylated in 100 mM Tris-HCl (pH 9) for 30 min at 37 °C and precipitated with ethanol, NaOAc and glycogen (see Chapter 3.5). The deacylated RNA was washed with 70% ethanol and resuspended in 6 µl of Urea Load.

To prepare periodate oxidation control, 5 µg of WT RNA was deacylated and incubated in 50 mM NaOAc (pH 5) and 2.5 mM $NaIO_4$ in the dark at 37 °C for 2 hours. 2 mM glucose was added to inhibit the reaction and the sample was incubated in the dark at 37 °C for 30 min. The oxidized RNA was purified by Sephadex G-25 column, precipitated with ethanol, NaOAc and glycogen, and washed in 70% ethanol. The pellet was resuspended in 6 µl of Urea Load. This RNA sample acted as a negative control for APB affinity.

All samples were denatured at 70 °C for 10 min before loading on the gel. The gel was run in 1x TAE buffer at 75 V at 4 °C for 6 hours. RNA was visualized by staining the gel with ethidium bromide. The RNA was blotted on ZETA probe membrane (Bio-Rad#162-0165) by wet-transfer in 0.5x TAE at 150 mA for 90 min. The membrane was dried, UV-crosslinked for 1 min and stored at room temperature.

3.6.2 Oligonucleotide hybridization

The oligonucleotide probe complementary to $tRNA^{His}$ (Table 15) was 5' end labeled with [gamma-^{32}P]-ATP isotope. The labeling reaction (Table 16) was incubated at 37 °C for 1 hour. 50 µl of water was added and the reaction was purified by Sephadex G-25 column.

Number	tRNA	Anticodon	Sequence 5'→3'
ZP169R	Histidine	GTG	GAATCGAACCTGGGTCGCATCG

Table 15: The oligonucleotide probe for $tRNA^{His}$.

Compound	Volume [µl]
Oligonucleotide	1 (25 ng)
10x PNK buffer	1
10x T4 Polynucleotide kinase (NEB#M0201S)	1
[gamma-^{32}P]-ATP (Hartmann Analytic, 9.25 MBq)	1-5
Water	up to 10 µl

Table 16: Composition of the gamma labeling reaction.

The ZETA probe membrane was pre-hybridized in Hybridization solution (Table 17) at 48 °C for 1 h. The radioactive probe was denatured at 100 °C for 5 min, cooled on ice for 2 min and applied to the membrane overnight. The radiolabeled oligonucleotides were removed and the membrane was washed with Wash 1 (Table 19) for 20 min and afterwards, with Wash 2 (Table 21) for 20 min. The membrane was exposed to Phosphoimager screen (GE Healthcare™) overnight and developed using Typhoon Scanner 9410. After developing, the membrane was stripped by boiling in Stripping solution (Table 22) twice 20 min at 80 °C and stored in fresh Stripping solution at room temperature until next hybridization.

Hybridization solution	
Compound	**Amount**
20x SSC	125 ml
1 M P_i (pH 7.2)	10 ml
SDS	35 g
100x Denhardt's solution (see Table 18)	5 ml
Salmon sperm DNA (100 mg/ml)	5 ml
Water	up to 500 ml

Table 17: Composition of Hybridization solution.

100x Denhardt's solution	
Compound	**Concentration**
Ficoll 400	2%
Polyvinylpyrrolidone	2%
Bovine serum albumine (BSA)	2%

Table 18: Composition of Denhardt's solution.

Wash 1	
Compound	**Volume [ml]**
100x Denhardt's solution (see Table 18)	15
Pre-wash 1 (see Table 20)	135 (up to 150)

Table 19: Composition of Wash 1 solution.

Pre-wash 1	
Compound	**Volume [ml]**
20x SSC	75
20% SDS	125
1 M NaH_2PO_4 (pH 7.5)	12.5
Water	237.5 (up to 450)

Table 20: Composition of Pre-wash 1 solution.

Wash 2	
Compound	**Volume [ml]**
20x SSC	50
20% SDS	50
Water	900 (up to 1000)

Table 21: Composition of Wash 2 solution.

Stripping solution	
Compound	**Volume [ml]**
20x SSC	5
20% SDS	5
Water	990 (up to 1000)

Table 22: Composition of Stripping solution.

3.7 Preparation of denatured protein extracts from *S. pombe* cells

The yeast cells were stored in -80 °C freezer. Upon thawing, they were resuspended in 100 µl of 2 M NaOH with 7% β-mercaptoethanol. 1 ml of the working solution for 10 cell samples was prepared just before use from 930 µl of 1.85 M NaOH (stored in 4 °C) and 70 µl of β-mercaptoethanol. The tubes were vortexed for 2 min at 4 °C. 100 µl of 50% TCA was added, the samples were incubated on ice for 5 min and centrifuged at 12 000 rpm for 2 min at 8 °C. The supernatant was discarded and the pellets were resuspended in 500 µl of 1 M Tris-HCl with protease inhibitor (Roche#11697498001) added just before use. The samples were centrifuged at 12 000 rpm for 2 min at 8 °C and the supernatant discarded. The proteins were resuspended in 100 µl of 2x Laemmli buffer heated to 80 °C, incubated at 80 °C for 20 min and then cooled down on ice for 5 min. The tubes were centrifuged at 12 000 rpm for 3 min at

room temperature and the supernatant was transferred to a new tube, ready to be loaded on an SDS-PAGE gel or stored at -20°C.

3.8 Western blotting

Gels composed of 12% lower resolving and 5% upper stacking gel were cast using TGX Stain-Free™ FastCast™ Acrylamide Kit (Bio-Rad#161-0185). The protein lysates were denatured at 90 °C for 10 min, loaded on the gels and separated by SDS-PAGE at 90 V for the first 15 min, then at 120 V for 1 hour in 1x running buffer (Table 23). 8 µl of Precision Plus Protein™ marker (Bio-Rad#161-0373) was used to determine the mobility of the proteins. The resolved proteins were transferred to a PVDF membrane (GE Healthcare#10600023) by wet electro blotting. Before use, the PVDF membrane was activated in methanol for 3 min. The proteins were blotted at 200 mA for 90 min in 1x transfer buffer (Table 24).

10x SDS-PAGE running buffer	
Compound	**Amount**
Tris	30 g
Glycine	144 g
SDS	10 g
Water	up to 1L

Table 23: Composition of 10x SDS-PAGE running buffer solution.

10x SDS-PAGE transfer buffer	
Compound	**Amount**
Tris	116 g
Glycine	58 g
20% SDS	37 ml
Water	up to 2L

Table 24: Composition of 10x SDS-PAGE transfer buffer solution. 1L of 1x working buffer is prepared by mixing 100 ml of 10x SDS-PAGE transfer buffer, 200 ml of methanol and 700 ml of deionized water.

After the transfer, the membrane was blocked with 5% milk in 1x PBS-Tween for 45 min at room temperature. HA primary antibody (Sigma-Aldrich) was diluted 1:3000 in 5% milk in 1x PBS-Tween and applied to the membrane that was expected to contain TbTGT1 proteins. C-myc primary antibody (Sigma-Aldrich) diluted 1:2000 in 5% milk in 1x PBS-Tween was applied to the membrane that was expected to contain TbTGT2 proteins. The membranes were rotated in falcon tubes for 1 hour at room temperature. After washing three times with 1x PBS-

Tween for 20, 20 and 10 min, the secondary antibody was applied. Goat anti-rabbit HRP conjugated IgG secondary antibody diluted 1:2000 (GenScript) was used for HA primary antibody (TbTGT1). Goat anti-mouse HRP conjugated IgG secondary antibody diluted 1:2000 (GenScript) was used for c-myc primary antibody (TbTGT2). The membranes were rotated for 1 hour at room temperature. Following the final 1x PBS-Tween washing of 20, 20 and 10 min, ECL reagents (Clarity^{TM} Western ECL Substrate, Bio-Rad#170-5060) were incubated with the membrane for 1 min. The proteins were visualized by exposure on the Chemidoc MP Imager (BioRad) using the Image Lab^{TM} software. The membranes were stained with Ponceau S to verify whether the proteins were successfully isolated and transferred to the blot.

3.9 Northern blotting

Denaturing agarose gel was prepared by dissolving 1 g of agarose in 72 ml of deionized water and mixing the warm solution with 10 ml of 10x MOPS (Table 25) and 18 ml of 37% formaldehyde. Before pouring, the electrophoretic equipment was treated with RNase ZAP.
10 µg of each RNA sample was mixed with 1.5x formaldehyde sample buffer (Table 27) and boiled at 70 °C for 15 min. 0.5-10 kb ssRNA ladder (NEB#N0362S) was processed similarly. Before loading, the samples were mixed with 6x DNA loading dye. The gel was run in 1x MOPS buffer (Table 26) at 70 V for 3 hours in the chemical hood.

10x MOPS	
Compound	**Volume [ml]**
0.5 M MOPS (pH 7.0)	40
3 M Sodium acetate	1.67
0.5 M EDTA (pH 8.0)	2
DEPC water	56.33 (up to 100)

Table 25: Composition of 10x MOPS.

1x MOPS running buffer	
Compound	**Volume [ml]**
10x MOPS (see Table 25)	55
37% Formaldehyde	50
DEPC water	445 (up to 550)

Table 26: Composition of 1x MOPS running buffer.

1.5x formaldehyde sample buffer	
Compound	**Volume [µl]**
10x MOPS (see Table 25)	156
Ethidium bromide (10mg/ml)	5
37% Formaldehyde	210
Formamide	600 (up to 1000)

Table 27: Composition of 1.5x formaldehyde sample buffer.

The separated RNA was visualized by UV-exposure on the Chemidoc MP Imager (Bio-Rad). RNA was blotted to ZETA probe membrane (Bio-Rad#162-0165) using 20x SSC buffer for 16 hours using capillary transfer. The membrane was dried, UV-crosslinked at 120 mJoules for 1 min using UV Stratalinker 1800™ and stored at room temperature.
PCR amplicons of TbTGT1 and TbTGT2 ORFs (see Chapter 3.1) were randomly labeled with radioactive [alpha-^{32}P]-ATPs, using random decamer priming, according to the manufacturer's protocol (DecaLabel DNA Labeling Kit, ThermoScientific#K0622).
The ZETA probe membrane was pre-hybridized in Standard Hybridization Buffer (Table 28) at 65 °C for 1 hour. The radiolabeled probe was denatured at 100 °C for 5 min, applied to the membrane and incubated overnight at 65 °C. The membrane was washed with Standard Wash 1 (Table 29) twice 10 min, then with Standard Wash 2 (Table 30) twice 30 min. The membrane was exposed to Phosphoimager screen (GE Healthcare™) overnight and developed using Typhoon Scanner 9410. After developing, the signal was removed by boiling in Stripping solution (Table 22) twice, each time 20 min and stored in fresh Stripping solution at room temperature until next hybridization.

Standard Hybridization solution	
Compound	**Amount**
Na_2HPO_4 (pH 7.2)	0.5 M
SDS	7%

Table 28: Composition of Standard Hybridization solution.

Standard Wash 1	
Compound	**Volume [ml]**
20x SSC	50
20% SDS	5
Water	945 (up to 1000)

Table 29: Composition of Standard Wash 1 solution.

Standard Wash 2	
Compound	**Volume [ml]**
20x SSC	5
20% SDS	5
Water	950 (up to 1000)

Table 30: Composition of Standard Wash 2 solution.

3.10 Growth curve

The transformed *S. pombe* double cell line was used for a growth curve. An uninduced 50 ml preparatory culture (TbTGT1+2 medium, see Table 12) was cultivated in a shaking incubator (30° C, 110 rpm) until $OD_{600} = 0.958$. The cells were harvested by centrifugation and washed 3x with 1x PBS. Two pairs of 50 ml cultures (TbTGT1+2 medium) for the growth curve were prepared, one starting with 50 µl of the preparatory culture, the other starting with 100 µl of the prep. culture. One culture of the pair was uninduced, i.e. 15 µM thiamine was added, the other one was induced, i.e. without thiamine. OD_{600} was measured every 3 hours for the next 24 hours.

4 RESULTS

4.1 Preparation of the TbTGT1, TbTGT2 and double overexpression *S. pombe* cell lines

In order to transform *S. pombe*$^{TGT-}$ cells with *T. brucei* TbTGT1 and TbTGT2, the ORFs of the genes were cloned using PCR and primers containing appropriate restriction sites. The PCR products were ligated into yeast pREP41 and pREP42 plasmids digested with the same restriction endonucleases. The yeast expression plasmids were complete with tags upstream of the insert, so the proteins were to be expressed with fused N-terminal HA or myc-his tags. Complexes of pREP41+TbTGT1 and pREP42+TbTGT2 were electroporated into the *S. pombe*$^{TGT-}$ strain and positive transformants were confirmed by restriction analysis and sequencing. A co-expressing cell line was created by transforming a pREP41+TbTGT1 yeast clone with pREP42+TbTGT2 plasmid DNA. Thus, three different *S. pombe* cell lines were successfully created in TGT^{-} background to overexpress either **TbTGT1-HA** or **TbTGT2-myc-his** separately, or **both the proteins** simultaneously. The transformed *S. pombe* cell lines were cultivated in 50 ml of appropriate media and harvested at OD_{600} = 0.6-0.7, i.e. in the exponential phase. To provide positive and negative controls for further experiments, *S. pombe* cell lines of **WT** (TGT^{+}) and the original **KO** (TGT^{-}) were also cultivated under the same conditions. Overexpression of TbTGTs in the transformants was induced by removing the nmt promotor repressor thiamine from the culture media. The transformants were harvested at different time points.

4.2 Detection of Q modification in *S. pombe* tRNAs using aminophenyl boronate affinity gel

To find out whether the inserted *T. brucei* TGT proteins are able to recognize *S. pombe* tRNAs as substrate and modify them, aminophenyl boronate (APB) affinity PAGE and northern blotting was utilized. The harvested yeast cells from the above mentioned cultures were disrupted by sonication and total RNA was isolated by guanidine extraction. The samples were deacylated to remove any amino acids still bound to the tRNAs and the RNA was subjected to denaturing polyacrylamide-urea-APB gel electrophoresis.

Prior to pouring the gel, N-acryloyl-3-aminophenylboronic acid (APB) was added to 1x TAE gel solution. APB crosslinks with the acrylamide during polymerization of the gel and interacts

with the free *cis*-diol groups of the cyclopentene rings present in the Q-modified tRNAs (Figure 9), thus slowing down their progress through the gel. Consequently, tRNAs are specifically separated into two bands, the retarded one Q-modified, the faster one free of Q modification (Igloi & Kössel 1985). RNA treated with periodate served as negative control, since periodate oxidizes all the *cis*-diols (Figure 9). Unable to interact with APB, the oxidized tRNAs have the fastest mobility on the gel (Igloi & Kössel 1985).

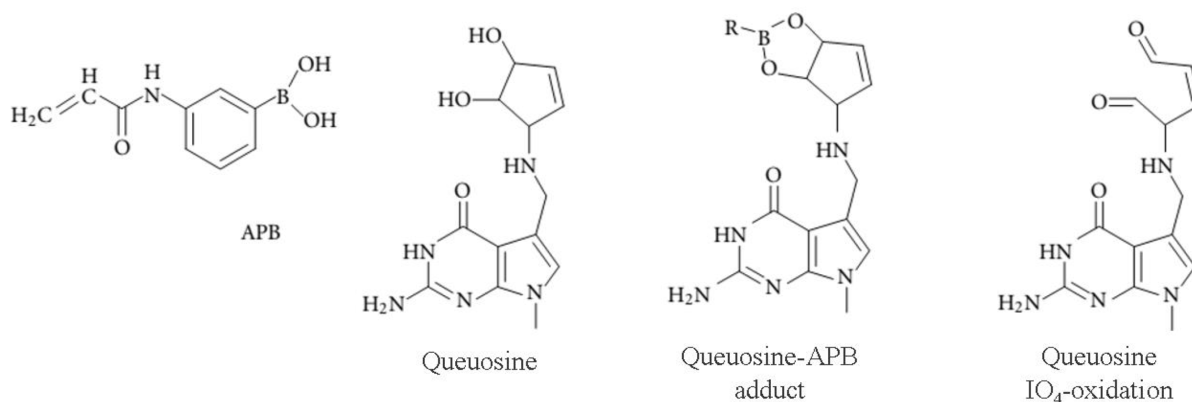

Figure 9: Aminophenylboronic acid, queuosine-APB adduct and periodate oxidation of queuosine (Behm-Ansmant et al. 2011).

The RNA was blotted on ZETA probe nylon membrane and specifically hybridized with gamma ^{32}P radiolabeled oligonucleotide probe, complementary to $tRNA^{His}$. The radioactive signal was fixed on a Phosphoimager screen and developed using Typhoon Scanner.
Q-modified tRNAs were detected in the *S. pombe* WT positive control (Figure 10). The lower band observed in the WT represents the tRNAs that contain guanosine, while the upper retarded band represents the tRNAs modified with queuosine. As was expected, the TGT knockout cell line contained no Q-modified tRNAs. Either TbTGT1 or TbTGT2 was overexpressed in this KO background to determine whether Q reappears in the tRNAs. Three different induction timepoints for each cell line were analyzed. However, no Q modification was observed in any of the samples. OX represents the periodate oxidation control.

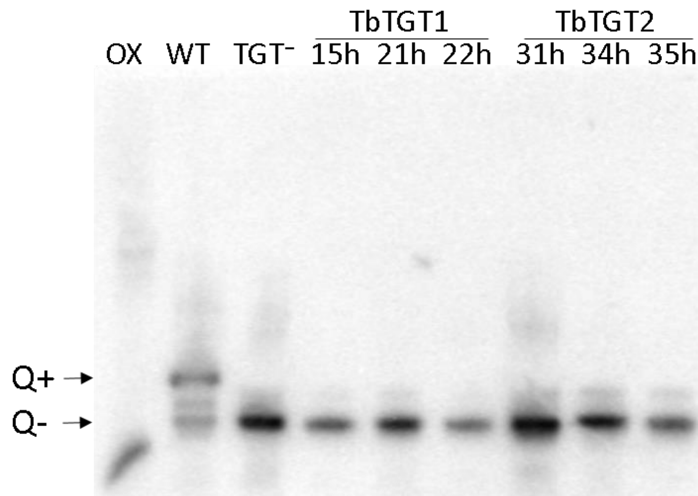

Figure 10: Northern blot analysis of *S. pombe* tRNA. OX represents the periodate oxidation control. *S. pombe* WT served as a positive control. *S. pombe* TGT knockout served as a negative control. Different induction timepoints from strains electroporated with TbTGT1 or TbTGT2.

The TbTGT proteins, when expressed independently, failed to modify *S. pombe* tRNAs. Therefore, the above mentioned northern blotting technique was used to test the co-expressing cell line expressing both TbTGT1 and TbTGT2 simultaneously.

However, no Q modification was observed in the double cell line for the three different induction times tested. The KO strain as well as the noninduced cell line served as negative controls showing no Q-modified tRNAs (Figure 11).

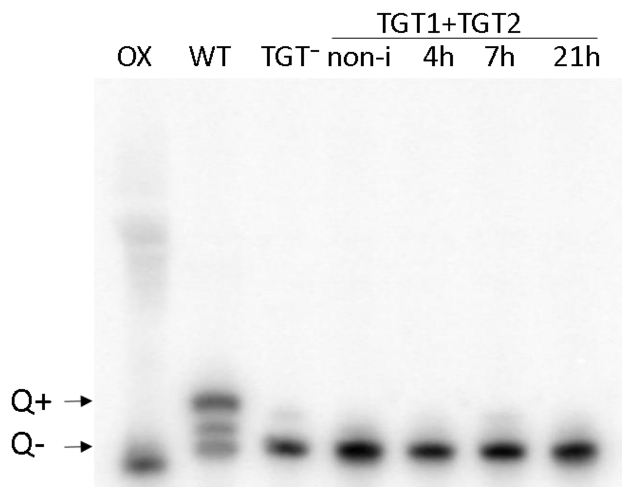

Figure 11: Northern blot analysis of *S. pombe* tRNA. OX represents the periodate oxidation control. *S. pombe* WT served as a positive control. *S. pombe* TGT knockout served as a negative control. Different induction timepoints from the co-expressing cell line electroporated with both TbTGT1 and TbTGT2.

Since no Q modification was detected in any of the transformants, it could mean that the *T. brucei* TGT proteins were not able to recognize *S. pombe* tRNAs as a substrate. There is also a distinct possibility that these proteins were not properly translated, folded or processed, in order to be functional. Therefore, we decided to find out whether the *T. brucei* TGT proteins were expressed in the *S. pombe* transformants using western blot analysis.

4.3 Confirmation of TbTGT1 and TbTGT2 protein expression by western blotting

Expression of *T. brucei* TbTGT1 and TbTGT2 proteins in *S. pombe* was analyzed using western blotting. *S. pombe* protein lysates were prepared using 2 M NaOH and 50% TCA. Proteins were separated by SDS-PAGE and blotted to a PVDF membrane. Since TbTGT1 was expressed with N-terminal HA tag, rabbit HA primary antibody was used to detect the proteins on the membrane. TbTGT2 was fused with N-terminal c-myc tag, therefore mouse c-myc primary antibody was used to detect the proteins. A *T. brucei* cell line overexpressing either an HA-tagged or a myc-tagged protein was used as positive control to verify the experiments. The membranes were incubated with anti-rabbit or anti-mouse goat secondary antibodies conjugated with HRP, and developed using ECL substrate. The proteins were visualized by chemiluminescence.

The expected sizes of HA-TbTGT1 and myc-TbTGT2 were **47.3 kDa** and **40.4 kDa**, respectively. However, neither TbTGT1 nor TbTGT2 proteins were detected on any of the membranes (Figure 12-15). WT, TGT^{-} and uninduced cell lines are negative controls showing no TGT bands. An HA-tagged protein overexpressed in *T. brucei* served as a positive control for the TbTGT1 blots and the band of the expected size 100 kDa was detected. A c-myc-tagged protein overexpressed in *T. brucei* served as a positive control for the TbTGT2 blots and the band of the expected size 70 kDa was detected.

Argument could be made against the results of the western blot analysis, that the protein extraction protocol used for *S. pombe* was suboptimal. Thus, we stained these membranes with Ponceau S stain, which binds to positively charged amino groups and non-polar regions of proteins. All the membranes showed that the proteins were successfully isolated and transferred on the blots. However, our proteins of interest were not detected in these cells.

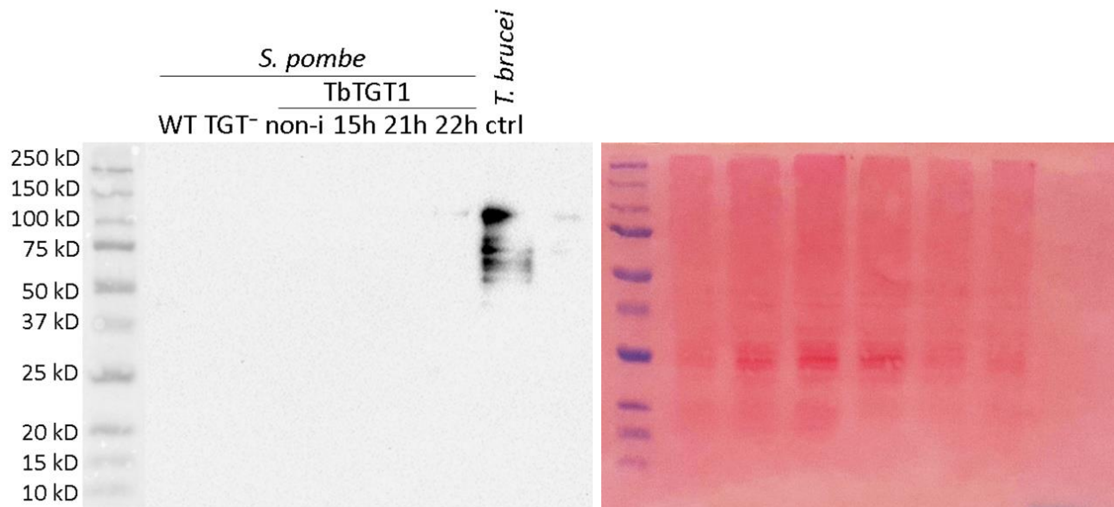

Figure 12 (A): Western blot analysis of *S. pombe*$^{TGT-}$ containing TbTGT1. *15h, 21h* and *22h* is the time of induction in hours. (B): The Ponceau S stain of the same membrane.

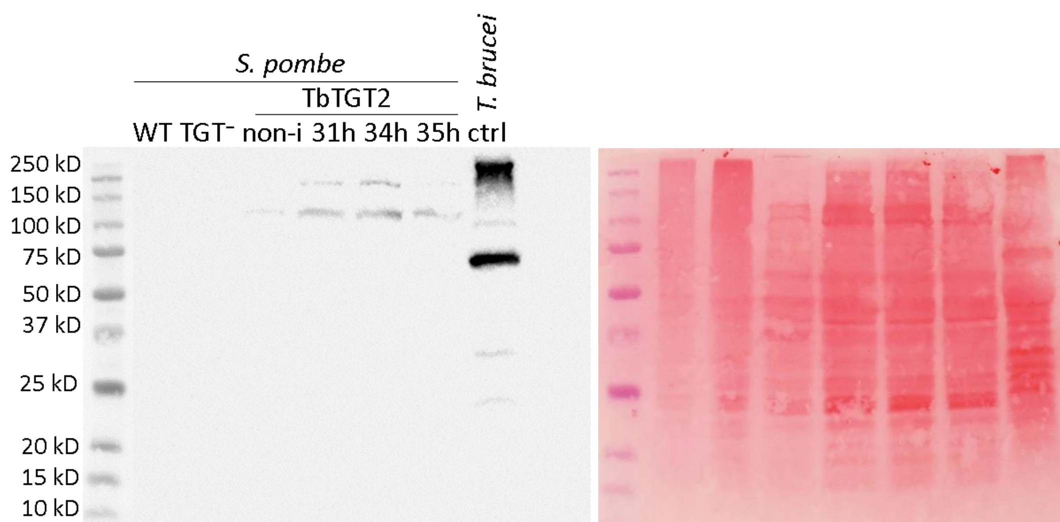

Figure 13 (A): Western blot analysis of S. pombe^{TGT-} containing TbTGT2. *31h, 34h* and *35h* is the time of induction in hours. (B): The Ponceau S stain of the same membrane.

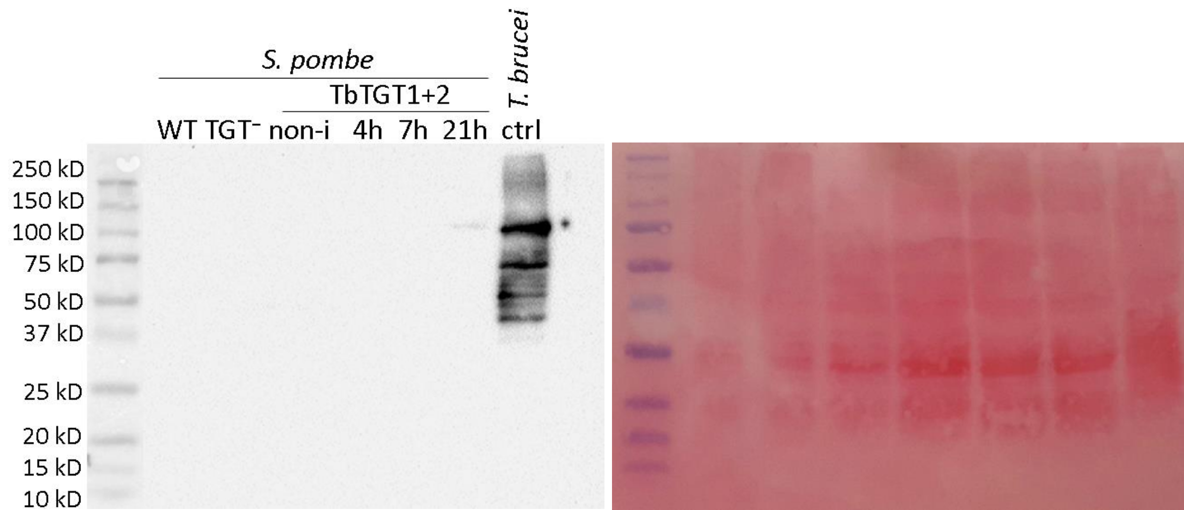

Figure 14 (A): Western blot analysis of the *S. pombe*$^{TGT-}$ **double cell line** containing both TbTGT1 and TbTGT2 probed with the **HA** antibody. *4h, 7h* and *21h* is the time of induction in hours. (B): The Ponceau S stain of the same membrane.

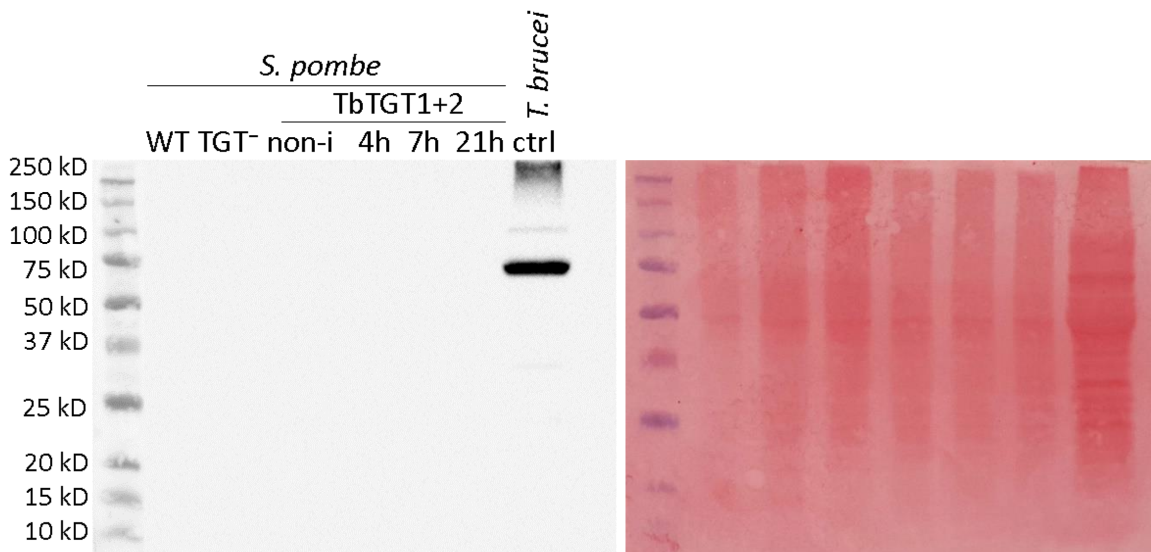

Figure 15 (A): Western blot analysis of the *S. pombe*$^{TGT-}$ **double cell line** containing both TbTGT1 and TbTGT2 probed with the **c-myc** antibody. *4h, 7h* and *21h* is the time of induction in hours. (B): The Ponceau S stain of the same membrane.

Since TbTGT1 and TbTGT2 proteins were not detected by western blotting, we decided to analyze whether the mRNAs of TbTGT1 and TbTGT2 were transcribed.

4.4 Confirmation of the production of TbTGT1 and TbTGT2 mRNAs by northern blotting

The presence of TbTGT1 and TbTGT2 mRNAs was analyzed by northern blotting. Total RNA was resolved on a formaldehyde denaturing gel and blotted on a ZETA probe membrane. The membrane was hybridized with alpha ^{32}P radiolabeled probes against TbTGT1 or TbTGT2 transcripts at 65 °C.

When the RNA separated on the denaturing gel was visualized under UV light, a low molecular weight extended smear was visible (Figure 16 and 17). This might indicate that the purity or quality of the RNA was compromised.

Upon exposure to the Phosphoimager screen, no TGT mRNA was detected on the membranes (Figure 16 and 17). The *T. brucei* positive controls were smeared which could have been caused by non-specific binding of the probe due to sub-optimal hybridization conditions.

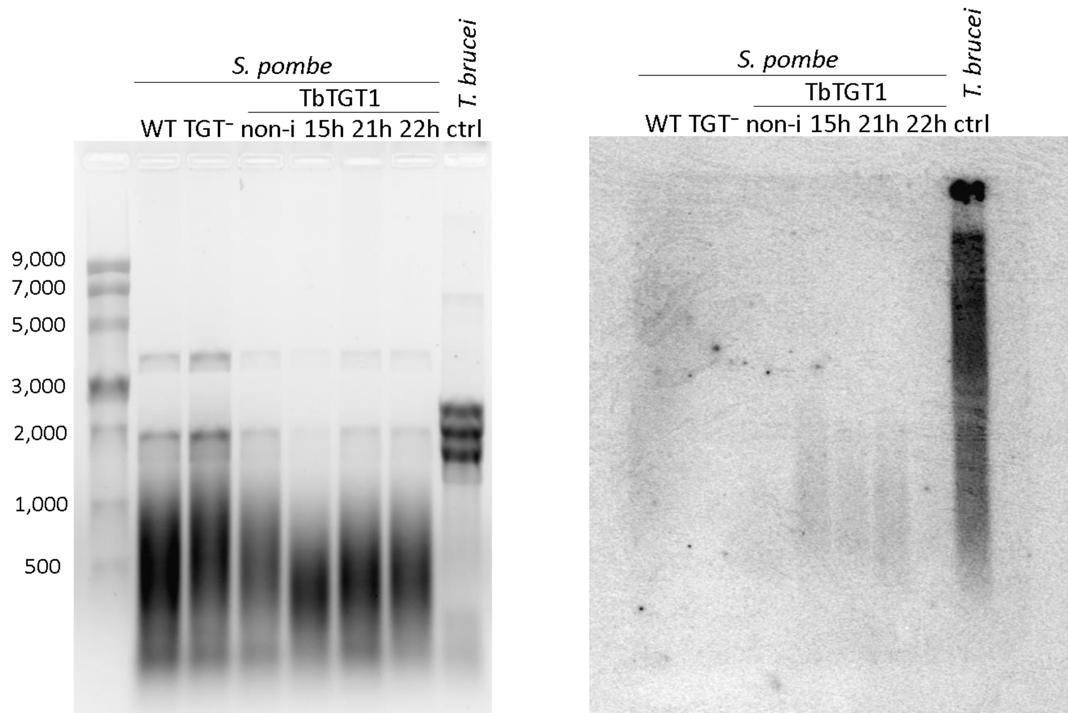

Figure 16: The denaturing gel and northern blot analysis of *S. pombe*$^{TGT-}$ RNA isolated from the cell line containing TbTGT1. *15h, 21h* and *22h* is the duration of induction in hours.

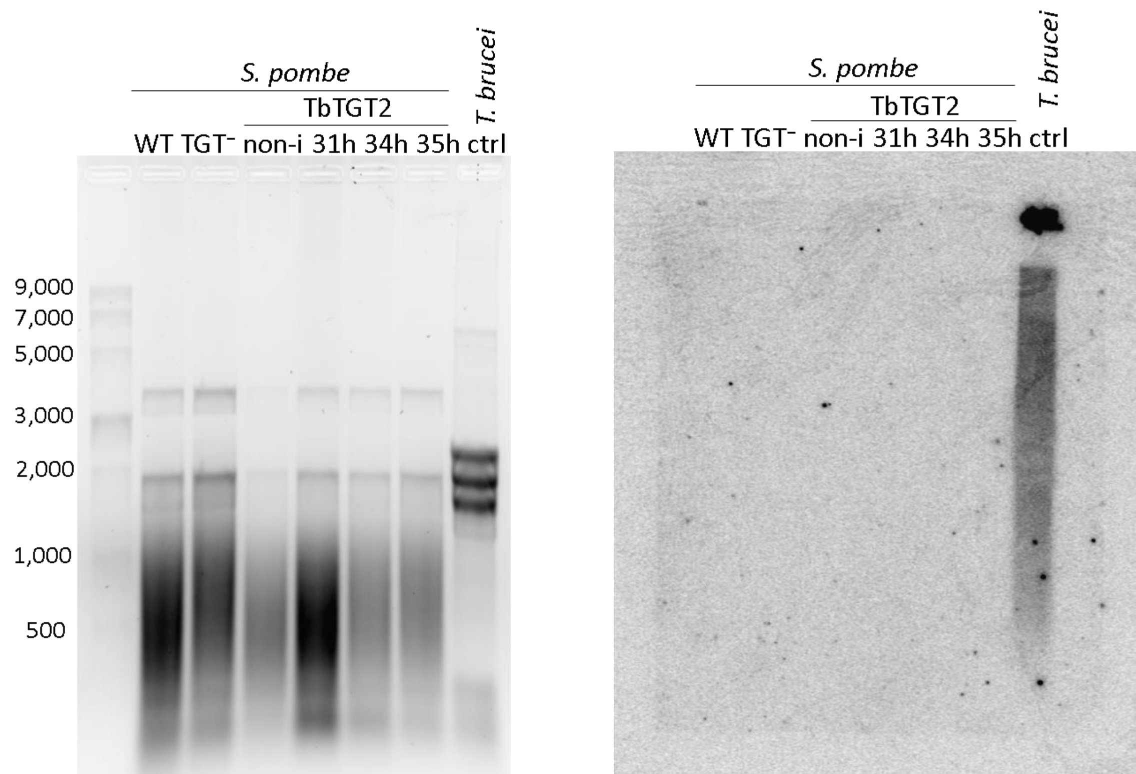

Figure 17: The denaturing gel and northern blot analysis of *S. pombe*$^{TGT-}$ RNA isolated from the cell line containing TbTGT2. *31h, 34h* and *35h* is the duration of induction in hours.

If the overexpressed foreign proteins are toxic to the yeast cells, cells specifically expressing the proteins in very low amounts, or not expressing at all, are predominantly selected in order to combat the toxicity, and this ultimately results in lack of protein production. This might be the case for *T. bruce*i TGT proteins, and hence it was worthwhile to check the effect of induction of these proteins on the growth of yeast cells.

4.5 Comparing the growth phenotype of the uninduced and induced double cell lines

To determine the possible influence of TbTGT overexpression on the viability of the yeast cells, the growth of the uninduced and induced **double cell line** was measured in OD units at regular intervals for 24 hours (Table 31). Two initial seeding densities were used, either 50 µl or 100 µl of the preparatory culture, which had been cultivated to OD_{600} = 0.958.

Induced *S. pombe* cells overexpressing both TbTGT1 and TbTGT2 proteins didn't show any growth phenotype changes compared to the uninduced controls. In fact, the induced cells were

growing slightly quicker than the uninduced ones, which might have been caused by slight differences in the initial cell concentrations.

	50 µl		**100 µl**	
Time (h)	***Ind***	***Non-i***	***Ind***	***Non-i***
0	0.021	0.02	0.022	0.022
3	0.03	0.011	0.014	0.033
6	0.015	0.012	0.043	0.04
9	0.041	0.04	0.052	0.069
12	0.082	0.058	0.154	0.135
15	0.214	0.167	0.442	0.402
18	0.565	0.466	1.05	0.959
21	1.243	1.117	1.983	1.807
24	2.258	2.021	2.814	2.741

Table 31: OD_{600} measurements.

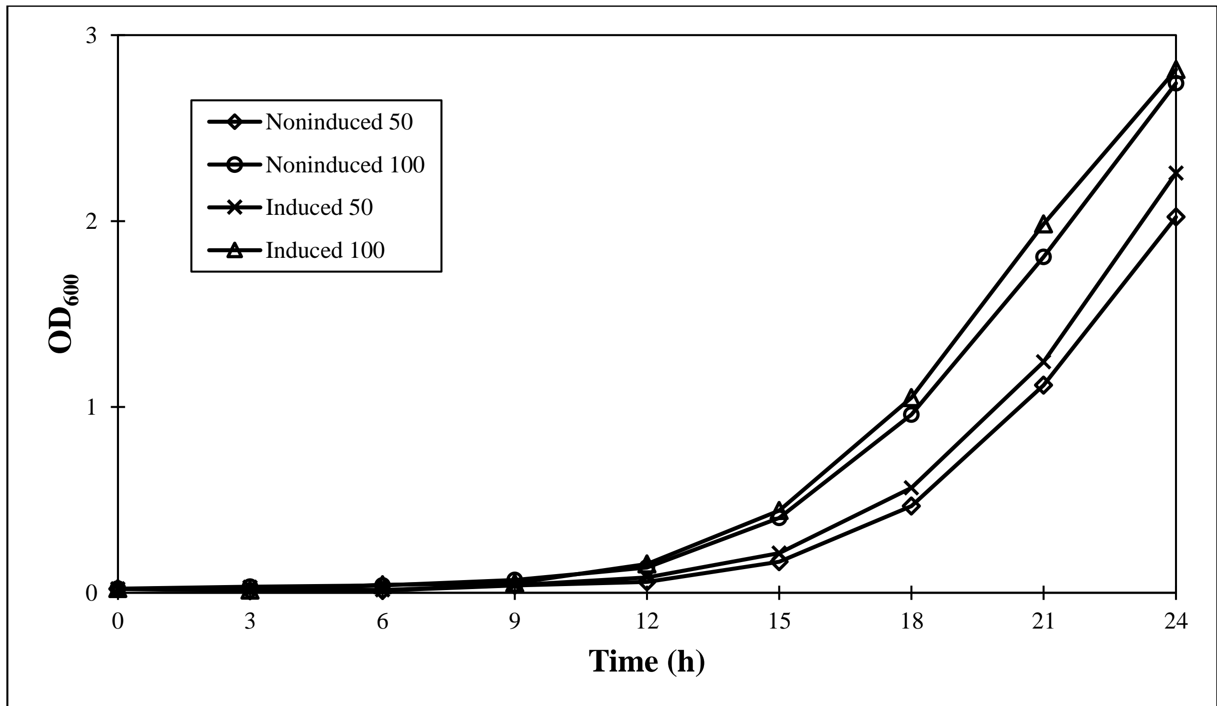

Figure 18: Growth curves of the uninduced and induced double cell lines, starting with two different seeding densities: 50 µl and 100 µl of the preparatory culture.

5 DISCUSSION

The aim of this thesis was to elucidate the interaction between two trypanosomatid TGT proteins via overexpression in a *S. pombe* experimental system. We aimed to determine whether they are able to perform the queuosine modification separately, possibly substituting each other, or they are functional as a heterodimer only. In many animals, QTRT1 and QTRTD1 are reported to form a heterodimer (see Chapter 1.5, Table 1) including humans (Chen et al. 2010), whereas plant TGT is reported to form a homodimer (Walden et al. 1982). We identified homologs of the two proteins in *Trypanosoma brucei* and our laboratory is currently employing a number of strategies to characterize the functionality and interaction of the two putative subunits. The characterization of the proteins and their interaction would lead to further understanding of TGT and new research avenues in *Trypanosoma* biology.

In this research project, we used a strategy routinely employed by many research groups, to utilize the molecular biology tools available for *S. pombe,* in order to elucidate the function of previously uncharacterized proteins. We decided to perform a rescue experiment to test the identified trypanosomatid TGT proteins. The experiment involved complementing an *S. pombe* TGT KO cell line with *T. brucei* TGT, which could be able to recognize the yeast tRNAs and rescue the Q modification. We prepared yeast constructs containing either TbTGT1 or TbTGT2 ORF and transformed *S. pombe*$^{TGT-}$ cells with these constructs. We proceeded to create three yeast cell lines, the first containing only the TbTGT1 construct, the second containing only the TbTGT2 construct, and the third containing both TbTGT1 and TbTGT2 constructs. We planned to utilize aminophenyl boronate PAGE and subsequent northern blotting to confirm whether the inserted protein(s) are able to catalyze the Q modification. Furthermore, the overexpression of the proteins was to be verified by western blotting. If the proteins needed to form a heterodimer to be functional, we expected to see no tRNAs being modified in the single cell lines and Q-modified tRNAs in the co-expressing cell line. We also expected to confirm the production of TbTGT proteins in all *S. pombe* cell lines by western blotting.

Our initial hypothesis was that if the TbTGTs also need to form a heterodimer in order to be functional, as seen in several eukaryotes, we would see the rescue of the Q modification only in the cell line co-expressing both the TGTs and not in the individual cell lines. On the other hand, if we were to detect the transglycosylase activity within the separate cell lines as well as the double cell line, it would indicate that the proteins could substitute each other without the necessity to form any kind of dimer. And if the Q modification was detected in only one of

the separate cell lines, confirming the functional catalytic unit, the role of the other protein would then require further investigation.

During this project, the *T. brucei* TGT genes were successfully cloned and the TbTGT1 and TbTGT2 constructs successfully created. The electroporation of the *S. pombe* cells with the constructs was also successful including the creation of the co-expressing cell line. However, the rescue of the Q modification within the *S. pombe* tRNAs was not detected in any of the cell lines. There was a possibility that the proteins were expressed, but not catalytically active, due to their inability to recognize *S. pombe* tRNAs as substrates, or on account of incorrect post-translational processing and modifications. If the proteins were being expressed only in the double cell line and not in the single cell lines, it could indicate that the individual proteins might be unstable and require the formation of a heterodimer for stability. It has been observed in certain cases; (one of the most recent being human mitochondrial complex 1) that loss of one subunit affects the stability of other subunits belonging to the same complex (Stroud et al. 2016). We could also not discount the possibility that the proteins were not expressed at all, which was unfortunately confirmed, as the western blot analysis failed to detect the proteins in any of the cell lines. Trying to further investigate these results, we decided to find out whether the TGT mRNAs are being transcribed using formaldehyde northern blotting but the results of this experiment were inconclusive. Taking into consideration the experience of the supervisor of this thesis, we studied whether these proteins were toxic to the cells by monitoring if the growth of the yeast cells is influenced by the TGT constructs. We compared the growth phenotypes of the uninduced and induced yeast cells but found no differences.

Our current results are open to various interpretations, needing further in depth analysis of this yeast expression system and standardization of induction protocols. If one of the protein subunits or both had been successfully identified and the transglycosylase activity had been confirmed, the proteins could have been purified for use in further experiments, e.g. to confirm their physical interaction by *in vitro* co-immunoprecipitation, or to study their substrate specificity, structural requirements of the catalytic subunit, as well as various other parameters of enzyme kinetics, using *in vitro* reaction system. In addition, the proteins could have been purified and used as antigens to produce antibodies, since there are no commercially available antibodies against *T. brucei* TGTs at the moment.

6 CONCLUSION

In order to investigate the role of TbTGT subunits, we generated two yeast constructs containing TbTGT1-HA and TbTGT2-myc-his with the different selection markers LEU2 and URA4+, respectively. These constructs were confirmed by sequencing and electroporated into *S. pombe* TGT knockouts individually and/or simultaneously. Selected positive clones were analyzed for a rescue of Q-tRNA formation and subsequently tested by western blot for the overexpression of the tagged proteins. Unfortunately, the levels of Q-tRNA in the yeast knockout could not be complemented and using western blot we could not detect any of the overexpressed proteins in *S. pombe*. This was most likely because of the cytotoxicity of the *T. brucei* proteins to yeast. Thus, we decided not to proceed further with the complementation and instead we focus on the validation of the subunit composition by immunoprecipitation.

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