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Evaluating the Role of TbTGT2 in the Formation of Queuosine tRNA Modification in the Bloodstream Stage of Trypanosomes

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Annotation:

The aim of this thesis was to investigate the role of the *TbTGT2* gene in the formation of queuosine modified tRNA by the preparation of the gene knock-out cell line of *TbTGT2* in the bloodstream stage of *Trypanosoma brucei.*

Sworn declaration:

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Abbreviations

bp	base pairs
BS	bloodstream stage
DNA	Deoxyribonucleic acid
EtBr	Ethidium bromide
EtOH	Ethanol
FBS	foetal bovine serum
HygR	Hygromycin resistance gene
IAA	Isoamyl alcohol
Ile	Isoleucine
KO	Knock-out
Min	minutes
$NaIO_4$	Sodium periodate
NaOAc	Sodium acetate
PCR	Polymerase chain reaction
PNK	Poly nucleotide kinase
PS	procyclic stage
QTRT1	queuine tRNA-ribosyltransferase 1
QTRTD1	queuine-tRNA ribosyltransferase domain containing 1
RNA	Ribonucleic acid
RT	room temperature
TAE	Tris-acetate- EDTA buffer
T. brucei	*Trypanosoma brucei*
Tm	melting temperature
tRNA	transfer RNA
Tyr	Tyrosine
UTR	Untranslated region

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1 Abstract

Trypanosoma brucei is a eukaryotic protozoan parasite causing African sleeping sickness in humans and nagana in domestic animals. In contrast with other eukaryotes, it lacks most of the transcriptional control with the bulk of regulation of gene expression occurring post-transcriptionally. tRNA modifications, which are responsible for an enormous amount of structural and functional diversity might provide an additional level of regulation of gene expression during the digenetic life cycle of these parasites. Queuosine (Q) is one of the most complex tRNA modifications. Despite its omnipresence in bacteria and eukaryotes, the physiological role of Q-tRNA modification is not clear. In this study, we generated gene knock out of the tRNA guanine transglycosylase (TGT), the enzyme responsible for Q-tRNA formation in eukaryotes, in order to better understand the role of this tRNA modification in trypanosomes. We demonstrate that knock out of the gene for TbTGT2, one of the TGT subunits, leads to a complete disappearance of Q-tRNA modification, however, no physiological effect of the bloodstream stage of cells was observed when cultured *in vitro*. Based on our data, we propose that TbTGT2 is the key protein connected with the formation of Q modification in *T. brucei*.

2 Introduction

2.1 *Trypanosoma brucei*

2.1.1 Global impact of trypanosomes

The protozoan order Kinetoplastida includes the genus Trypanosoma, uniflagellated parasitic species that are responsible worldwide for some of the most neglected human diseases. In South America, *Trypanosoma cruzi* causes Chagas' disease and in Africa two of the three subspecies of *T. brucei spp.* inflict humans leading to African trypanosomiasis also known as sleeping sickness. In west and central Africa, *T. brucei gambiense* is the cause for a chronic form of sleeping sickness, and in east and southern Africa *T. brucei rhodesiense* triggers an acute form of this disease. *T. brucei brucei* is not infectious to humans, however, it infects livestock with acute animal African trypanosomiasis (nagana) (1). Trypanosoma is a genus containing some of the deadliest parasites on earth. Human African trypanosomiasis alone causes more than 500,000 cases per year. It primarily affects the deprived rural population in some of the poorest developing countries of sub-Saharan Africa (2). If untreated it is invariably fatal (3), nevertheless, Chagas disease and Sleeping sickness have been considerably neglected, because it disproportionately affects the poor and marginalized populations (1). Furthermore, current treatments are inadequate, drugs for late-stage disease are highly toxic; there is no prophylactic chemotherapy and little or no chance for a vaccine (3). Additionally, agricultural development is restricted by nagana, contributing significantly to poverty in the affected regions (2). Consequently, diseases caused by trypanosomes have the biggest impact on those who cannot afford medical care.

2.1.2 Biology

The basic biology of trypanosomes has been the subject of intense research of the past 25 years (2). Studies on these early branching eukaryotic organisms bring many fascinating and unique molecular and biochemical phenomena to light (1). For example, trypanosomatids have complex life cycles, involving numerous developmental stages in different hosts (4). *T. brucei* proliferates in mammals as the bloodstream stage (BS) (Fig. 1), which fully depends on glycolysis as a main source of the energy because its mitochondrion is downregulated and doesn't produce ATP via oxidative phosphorylation (5). In the mammalian host, African trypanosomes remain exclusively extracellular (1) and their recognition by the immune system of the host is evaded by sequential expression of antigenically distinct variable surface glycoproteins (VSGs) (2). Upon blood meal and uptake into the tsetse fly (*Glossina* spp), procyclic stages (PS) are generated, which proliferate in the fly midgut (Fig. 1). The (PS) express a surface coat distinct from the one in the bloodstream stage, the VSG being

replaced by a coat of another protein called procyclin. After establishment in the fly midgut, trypanosomes reacquire a VSG coat, migrate to the tsetse salivary gland and prepare for the transmission into a new mammalian host (2).

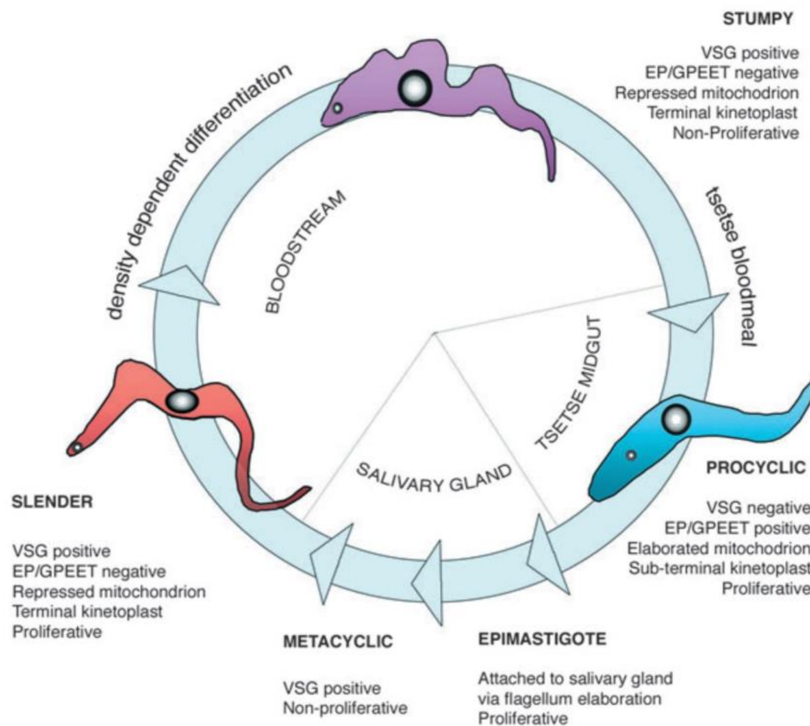

Figure 1:The complex life cycle of T. brucei (2)

Most of the research has focused on the bloodstream and procyclic stages, which can be easily cultured *in vitro*. Moreover, techniques such as RNA interference (RNAi) and other methods of forward and reverse genetics, are readily available and can be exploited for the functional analysis of genes. These methods are very useful to broaden our knowledge base on the function of specific genes, which might reveal an efficient strategy to control trypanosomes (2). However, countermeasures for one species are often not adoptable for related species, because basal differences in the host-parasite interactions complicate the efficiency of applicability (1).

2.1.3 Posttranscriptional processing

When compared to other eukaryotes, *T. brucei* shows unusual features in nuclear and organellar gene expression. Mature nuclear mRNAs are generated from primary polycistronic transcripts by trans-splicing and polyadenylation. During trans-splicing a capped 39-nucleotide spliced leader (SL) miniexon is added to the 5´termini of the mRNAs (6). In the trypanosomatids single mitochondrion, the process of RNA editing restores the otherwise untranslatable primary transcripts by posttranscriptional addition and/or deletion of uridylate residues to the mRNAs (7). In addition, complete lack of the mitochondrially encoded tRNA genes is compensated by the import of the cytosolic tRNAs in order to maintain mitochondrial translation (8). Consequently, gene expression in trypanosomes is controlled mostly by post-transcriptional pathways that has great importance for the transition between the mammalian and insect stage as a part of the complex life cycles of these parasites.

One good example of extremely heavily posttranscriptionally processed molecules is represented by tRNAs. In all organisms tRNAs are transcribed as precursor molecules containing extra sequences at their 5′ and 3′ ends and in a few cases with introns. These have to be removed by a series of maturation steps that generate a full-length tRNA required for protein synthesis (9). However, tRNA maturation is not limited to cleaving of these extra sequences but also includes the addition of numerous post-transcriptional chemical modifications. By far, tRNAs are appended with extremely abundant and diverse post-transcriptional chemical residues, which influence the structure, stability and abundance of tRNAs, as well as their recognition by aminoacyl synthetases, consequently affecting translation (10). In addition, several modifications have direct impact on protein synthesis by contributing to the strength and specificity of codon–anticodon selections during decoding (11). Despite the importance and variety of modified nucleosides in tRNA, the pathways and functions of specific modifications need to be further revealed and characterized (12).

2.2 Queuosine

Among all of the tRNA modifications located near the anticodon loop, queuosine (Q) is considered one of the most complicated modification (12). Discovered in 1968 at the wobble position 34 of *Escherichia coli* tyrosyl-tRNA ($tRNA^{Tyr}$) this new modification was designated as Q. The base form of Q was named queuine (q). Later, it was found that Q can be present at the anticodon with the sequence $G_{34}U_{35}N_{36}$,, which can be found in three other tRNAs for histidine, aspargine and aspartic acid respectively (13). Interestingly, it has been reported that Q modified tRNA has a higher affinity to the NAU codons than to NAC codons, therefore the modification may affect the efficiency of translation (14). The chemical structure of Q ,7-(5-[((1S,4S,5R)-4,5-dihydroxy-2-cyclopenten-1-yl)amino]methyl)- deazoguanosine (15), was described as a hypermodified guanosine analogue (16).

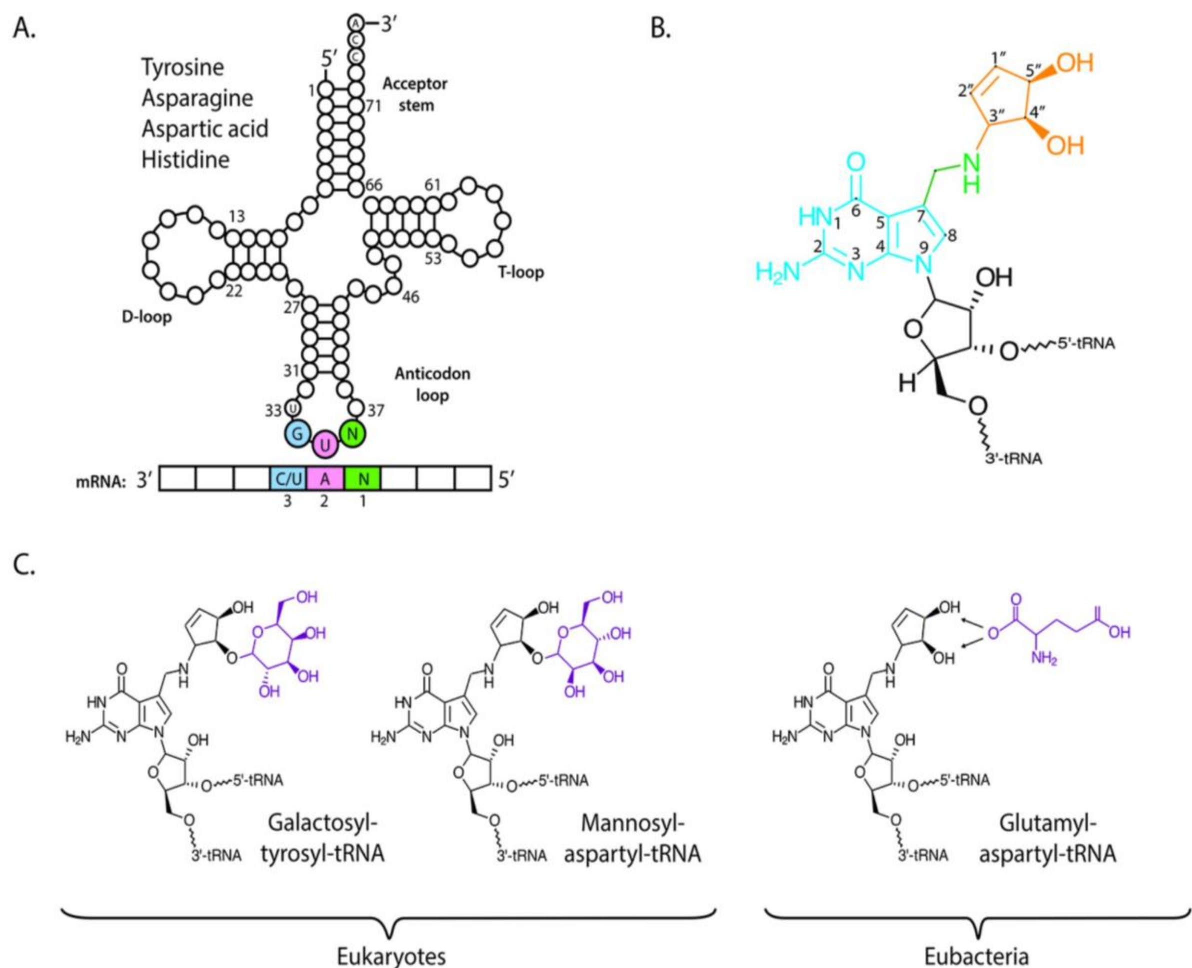

Figure 2: Structural information about Q and its derivatives. (A) The tRNA with the $G_{34}U_{35}N_{36}$ sequence can be modified with Q. (B) The Q molecule contains 7-deazaguanosine core with the carbon substituting the purine nitrogen at position seven (in blue). To this carbon is the amino-methyl side chain (green) and a cyclopentanediol group (orange) appended (C) In eukaryotes, Q can be further modified by sugar molecules or a glutamic acid residue in eubacteria.(12)

2.2.1 *De novo* synthesis of queuosine in Eubacteria

Although Q is ubiquitous in all three domains of life (Eukarya, Eubacteria and as archeosine in Archaea) (16), only Eubacteria are capable of *de novo* Q biosynthesis (17). First, the free cytosolic guanosine triphosphate nucleoside is converted in five enzymatic steps to the partially modified 7-aminomethyl-7-deazaguanine base (preQ1) (12), which is reversibly incorporated (16) by the bacterial tRNA guanine transglycosylase (TGT) (18) into the GUN wobble position of tRNAs (16). Eventually, the preQ1 tRNA is turned *in situ* by two additional enzymatic steps to the final Q modified tRNA (17), which participates in the normal cellular functions until tRNA turnover. The degradation products, Q nucleoside, nucleotide or q base are released to the extracellular space (16).

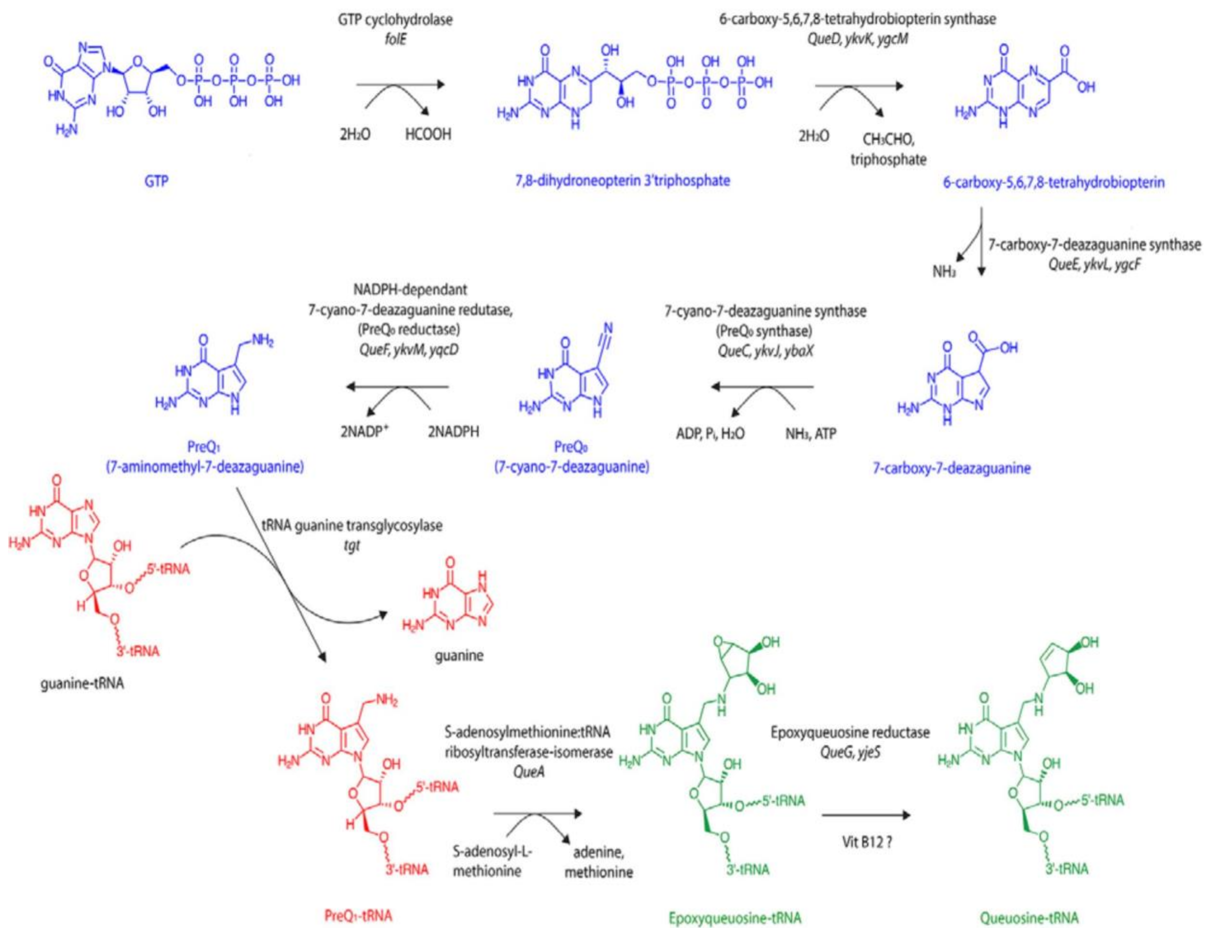

Figure 3: Q biosynthesis in Eubacteria (12).

2.2.2 Queuosine metabolism in Eukaryotes

Eukaryotes, in contrast to bacteria, are not capable of synthetizing Q *de novo* but rather acquire its modified base q through the salvage system from environment, diet or gut microbiome (19–21). The Q modification system in eukaryotes consists of three steps: uptake of q base from the surrounding into the cells, incorporation of q into the wobble position of the anticodon loop of tRNA and the salvage of q from Q, queuosine-5′-phosphate and queuosine-3′-phosphate after the tRNA degradation. This is done by the DUF2419 protein, which was recently identified as Q salvage protein (22). A specific uptake mechanism was proposed describing the absorption of q from the extracellular space (23–27). Compared with other biomonomers, the uptake mechanisms is relatively slow (28), on the other hand competitive experiments revealed a highly specific uptake, independent of other purines and purine derivatives (24).

Contrary to prokaryotes, eukaryotes insert the intracellular q directly and irreversibly in place of guanine by a single enzymatic step (16). This reaction is catalyzed by eukaryotic TGTase, a heterodimeric molecule consisting of two subunits (17). The first one, queuosine tRNA-ribosyltransferase 1 (QTRT1) was identified as the catalytic subunit by high protein sequence similarity to the eubacterial TGT. The second subunit without TGT-like catalytic activity, queuine-tRNA ribosyltransferase domain containing 1 (QTRTD1) (14) has been originally proposed as Q salvage enzyme that liberates free q from Queuosine monophosphates (29). It has a lower protein sequence homology to eubacterial TGT than QTRT1. However, sequence comparison between QTRT1, QTRTD1 and eubacterial TGT suggests a likely conservation of secondary structure elements, especially an irregular (β/α)8 triosephosphateisomerase (TIM) barrel structure, which is also found in the bacterial *Z. mobilis* TGT enzyme (30, 31). Individually, the two subunits are not functional, however, in a 1:1 molar ratio a guanine transglycosylase activity has been assayed (14, 17) . Therefore, the two subunits work together in a complex, with a possible involvement of other still unidentified factors. *In vitro*, the TGT complex has a very low efficiency compared to other enzymes, however, the low conversion rate may be compensated by the irreversibility of the formed bond (12).

2.2.3 Physiological role of queuosine in different model organisms

Despite a substantial number of studies in the last decade on Q modification, the physiological role of Q-modifications still remains largely unclear. Q has been frequently the object of research, especially in a number of deficiency studies and in cancer research. In mammals, normal diet supplies enough Q to fulfil physiological requirements and for the *in vitro* cultures of mammalian cells, a sufficient amount for Q comes from the animal serum present in the cultivation media (32, 33). In an attempt to uncover its physiological role, several studies have reported on the consequence

of Q depletion in various organisms. In unicellular organisms such as *Chlamydomonas* as well as in multicellular *Drosophila melanogaster*, *Caenorhabditis elegans*, no visible defects were observed if grown axenically in Q-free conditions. (34–37). A full Q-tRNA depletion was achieved in mice by maintaining them axenic and q free for the period of one year (20). After supplying these animals with exogenous q sources, the levels of Q modified tRNA renormalized to the levels before Q starvation (16). This proves that i) eukaryotic Q derives from eubacterial sources and ii) eukaryotes are not able to synthetize Q *de novo* (8,12). During all these experiments no physiological defects were recognised (38).

However, removal of the nonessential amino acid tyrosine from the diet leads to severe symptoms including squinting, stiffness, convulsion, lethargy and ultimately death after 18 days in mice (39). Hence in the absence of Q, tyrosine becomes an essential amino acid suggesting importance of Q in tyrosine biosynthesis in mammals (38). Another sign for the physiological importance of Q comes from two different studies, the first correlating the q abundance with the ability to tolerate hypoxic stress in HeLa cells (40) and the second reporting prokaryotic TGTase as the main enzyme regulating the bacterial virulence in *Shigella flexneri* by inserting Q in mRNA of the virulence factor virF (41, 42). Decrease of the TGT activity causes reduced virulence of this human pathogen proposing TGT as a putative drug target (43, 44).

In addition, Q hypomodified tRNA has been linked with clinical and *in vitro* studies to the development of malignancy in various cancers. These results have led to an increased interest to obtain a deeper understanding of the physiological roles of Q and its modifications in eukaryotes (14). Induced differentiation in leukemic cells caused and increased amount of Q modifications (45–47), in contrast in some neoplastic tissue and cancer cell lines a decline in Q modifications was determined (48, 49). Moreover, the level of Q modification of tRNA in cancerous tissues of human correlates with the grade of malignancy (49). This finding may be important for the development of new prognosis techniques for several types of cancer. Some studies documented even a positive correlation between a higher grade of Q modification in tRNA and a better chance of survival of the patients (16).

2.3 Queuosine in trypanosomes

It is apparent from literature that the physiological role of Q tRNA modification in the eukaryotic cell is far from being understood, and that some light was only recently shed on its novel role in the synthesis/conversion of the amino acid tyrosine in the mouse model (38). Thus, one of the goals of our laboratory is to address the principal question regarding the role of Q-tRNA modification with respect to biology and physiology of the protozoan parasite *T. brucei*. In our laboratory, two putative homologs of *T. brucei* TGT have been previously identified using the human TGT protein sequence as a bait in the BLAST search of the kinetoplastid genome database (Tritrypdb.org). TbTGT1 with a calculated molecular mass of 44.74 kDa possesses all conserved catalytic Asp residues required for transglycolase activity and shares 49% identity and 62% similarity to the human TGT enzyme. TbTGT2 with a calculated molecular mass of 33.17 kDa is more diverse with 24% identify and 38% similarity to the human TGT orthologue. In contrast to TbTGT1, there are no catalytic Asp residues in TbTGT2. However, the amino acids responsible for the Zn^{2+} binding are present in both TbTGTs and their occurrence may suggest similar subunit composition to the mouse and human TGTs.

In our laboratory, both *TbTGT*s have been previously down regulated by RNAi in the BS of trypanosomes. We observed significant decrease of Q-tRNA levels in the individual RNAi cell lines suggesting that these two proteins may function as a heterodimeric complex (data not shown). However, the obtained growth curves exhibit that only TbTGT1 is essential for cell growth, whereas down-regulation of *TbTGT2* did not affect growth in BS (data not shown). This can be explained by only partial downregulation of TbTGT2 mRNA by RNAi, as was documented by qPCR. Therefore, the main goal of this thesis was to generate a KO of *TbTGT2* in BS in order to facilitate its further phenotypic characterization.

3 Work aims

1) Preparation of the DNA knock-out construct of the *TbTGT2*.

2) Transfection of the construct into the bloodstream stage of *T. brucei* and selection of positive transformants.

3) Confirmation of the correct incorporation of the construct in the *T. brucei* genome.

4) Evaluation the level of Q-tRNA in the obtained *TbTGT2* knock-out cell line.

5) Evaluating the role of Q-tRNA depletion on the growth of the bloodstream stage of *T. brucei*.

4 Material and methods

4.1 Cell culture of the bloodstream stage of *T. brucei*

Bloodstream stage (BS) of *T. brucei* cells (strain Lister 427) was grown at 37°C, 5% CO_2 in HMI-9 medium usually supplemented with 10% foetal bovine serum (FBS) (Q+ medium), when indicated the medium was supplemented with 10% dialysed FBS (Q- medium). For growth curves, the cells were diluted to the starting concentration of $2x10^5$ cells/ml and the cell density was measured every 24 h with a Beckman Z2 Coulter counter over a period of 10 days. To maintain the cells in the exponential phase, the culture was tenfold diluted as soon as the concentration reached $1x10^6$ cells/ml.

4.2 Preparation of the TbTGT2 knock-out construct

The DNA knock-out construct for *TbTGT2* was prepared using the PCR fusion technique as described before by Merritt and Stuart (50). Four individual PCRs were necessary to yield the DNA construct. The first 3 PCRs served to amplify 3´and 5´untranslated regions (UTRs) of the *TGT2* gene and one to amplify the antibiotic resistance gene of Hygromycin (*HygR*). We designed the primers in a way that the reverse primer for amplification of 5´UTR of *TGT2* and forward primer for amplification of 3´UTR of *TGT2* have approximately 20 nucleotides extension complementary to the beginning or the end of the coding sequence of the *HygR* gene, respectively. Finally, those individually obtained PCR products were used as a template for the fusion PCR (Fig. 4).

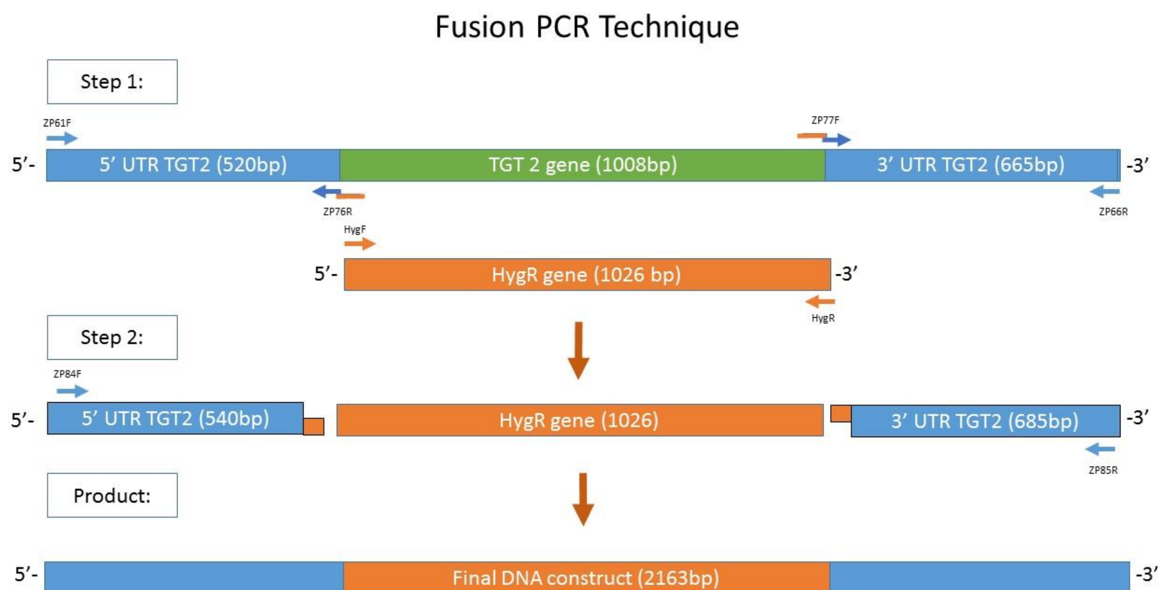

Figure 4: Scheme of the fusion PCR. Oligonucleotides used to amplify individual PCR products and the final construct are indicated by arrows and the complementary regions are highlighted in colour.

4.2.1 List of the used oligonucleotides

Name	Direction	Oligonucleotide sequence	Tm [°C]
ZP03	F	5'- CTTGTTGTTCATAATTTAGCCTTCC -3'	55
ZP61	F	5'-GTG GTG CAA AAG GTG GAG AG-3'	57
ZP62	F	5'-GAG AAA CTA TTC CAA CTA TCC TCT TTC C-3'	56
ZP76	R	3'-GTGAGTTCAGGCTTTTTCATTCCACTATTTGCTGCCACCT-5'	68
HygFW	F	5'-ATGAAAAAGCCTGAACTCACC-3'	52
HygRV	R	3'-CTATTCCTTTGCCCTCGGAC-5'	56
ZP77	F	5'-GTCCGAGGGCAAAGGAATAGGAACATTCAACTTAATGAAC-3'	65
ZP65	R	3'-CGA GAG ACA CAC TTC CTC CC-5'	57
ZP66	R	3'-CAG GGA ATG GAA GCC CAC AG-5'	58
ZP184	R	3'-TGGGGTTGCCTTCTAAAGCT -5'	56

Table 1: List of the used oligonucleotides. The overlapping sequences for the fusion are underlined.

4.2.2 Chemicals and conditions

The following PCR setting was used:

Compound	Volume [µl]
Q5 Buffer	10
10mM dNTP	1
Forward Primer	2.5
Reverse Primer	2.5
Template DNA	1
Q5 High fidelity DNA Polymerase (NEB#M0491S)	0.5
Q5 Enhancer	10
PCR water	22.5

Table 2: The composition of the PCR reaction.

Step	Temperature[°C]	Time [s]
Initial Denaturation	98	30
Denaturation	98	7
Annealing	55	30
Elongation	72	25
Final Elongation	72	600
Hold	4	

Table 3: The cycle settings for the PCR, the steps 2 -4 were repeated in 35 cycles.

4.2.3 DNA agarose gel

After each PCR a portion of 5 µl of the reaction was mixed with 1 µl of 6x loading dye and run on a 0.75% agarose/1xNNB gel in the presence of 0.1 µg/ml Ethidium Bromide (EtBr). The separated DNA and the 1kb Plus DNA ladder were visualized by the HP Alphaimager. The DNA of the correct size was cut out under a UV lamp, and eluted with Macherey-Nagel NucleoSpin® gel and PCR clean up kit, following the manufacturer's instructions. The concentration of the purified DNA was measured using NanoDrop 1000 spectrophotometer (Thermo Scientific) and the construct was confirmed by DNA sequencing (GATC).

4.3 Electroporation

For transfection 30 million of exponentially grown cells were harvested by centrifugation for 10 min, 2000 g at room temperature. The obtained pellet was re-suspended in 72 µl of Amaxa® T-cell solution, in which 5 µg of construct DNA was dissolved before. After addition of 18 µl of the supplement solution, the mixture was transferred into a 0.2 cm cuvette, which was subsequently inserted in the AMAXA® Nucleofector ®. The solution was electroporated with the program X-001 and diluted with the cultivation medium to 3 different concentrations, 10, 1, and 0.1 million cells per ml. For recovery of the cells, the transformants were incubated for 12 h without presence of the selective drug. To maintain and select for positive transformants, the medium was supplemented with 50 µg/ml hygromycin and the content of the cultivation flasks was plated in 1 ml aliquots on three 24 well-plates. The wells were daily examined for positive clones under bright field microscope.

4.4 Northern blot

4.4.1 Isolation of RNA by guanidine extraction

Solution D	
Compound	Concentration
Guanidin isothiocyanate extract	4M
Sodium citrate pH7	25mM
Sarcosyl	0.50%
Mercaptoethanol amine	0.1M

Table 4 Composition of the Solution D.

Total RNA was extracted from the cells using guanidine extraction (51). Total amount of $5x10^7$ *T. brucei* cells were spun at 2000 g at 14°C for 15 min. The medium was discarded and the pellet was washed twice with 1x PBS. 500 µl of solution D was added and the pellet was re-suspended by vigorous vortexing. To the obtained lysate 25 µl of 2M sodium acetate (NaOAc) pH 4, 500 µl of water saturated phenol and 75 µl of chloroform/isoamyl alcohol (IAA) 24:1 were added. The mixture was vortexed for 1 min and then cooled on ice for 10 min. To separate the two phases, the solution was centrifuged at 4000 g for 15 min and the upper RNA containing phase was precipitated with the same volume of isopropanol and 1µl of glycogen (20mg/ml) to enhance the RNA precipitation. The tube was incubated at least for 15 min at -20 °C and then spun at 21000 g for 30 min at 8°C. The liquid was discarded and the pellet was washed with 70% ethanol (EtOH), air-dried and resuspended in 100 µl of water. After the addition of 50 µl phenol pH 8 and 50 µl of chloroform/ IAA the mixture was vortexed for one minute, cooled on ice for 10 min and centrifuged at 4000 g for 15 min. The upper phase was collected and RNA was precipitated with 3 volumes of EtOH, 0.1 volume of 3M NaOAc and 1µl of glycogen at -20°C for at least 15 min. Then the solution was centrifuged at 21000 g for 30 min

at 8°C and the liquid was discarded. The pellet was washed twice with 70% EtOH, dried on air and dissolved in 50µl of pure water. To measure the amount and purity of the RNA a NanoDrop 1000 spectrophotometer was used.

4.4.2 Affinity RNA gel

TAE gel solution	
Compound	Amount
UREA	42g
50x TAE	2 mL
acrylamide	28.38 g
bis-acrylamide	1.62 g
water	up to 100 mL

Table 5. Composition of the TAE gel solution.

Detection of Q containing tRNA was carried out using the acrylamide RNA affinity gel containing aminophenyl boronate (APB), which has a high affinity to free *cis* diols present in Q (52). In brief, 50 mg of aminophenylboronic acid was dissolved in 10 ml of TAE gel solution. To trigger polymerization of the gel, 60 µl of 10% APS and 10 µL of TEMED were added. The solution was mixed and immediately poured into a Biorad mini gel apparatus. The comb was inserted and the gel was left to polymerize.

Prior loading, 5 µg of RNA was deacetylated in 50 µl 10 mM Tris HCl pH 9 for 30 min and subsequently precipitated in EtOH as described above. For the oxidation control (Ox), deacetylated RNA was mixed with 60 µl of water, 1 µl 3M NaOAc and 1.5 µl 100 mM sodium periodate ($NaIO_4$) and incubated for 2 h at 37°C in darkness. After desalting by Sephadex G-25 column, the RNA was precipitated with ethanol.

Each RNA pellet was resuspended in 6 µl of UREA Load, denatured at 70°C for 10 min and loaded on the gel, which was run for 5 h at 4°C with 75V in a 1x Tris-Acetate-EDTA (TAE) buffer until the bromophenol dye reached the end. After removing the glass plates, the gel was stained with EtBr for 15 min and visualised by HP Alpha Imager.

4.4.3 Northern blotting and oligonucleotide hybridization

The resolved RNA was blotted on a ZETA Probe membrane (Biorad) in a 0.5x TAE buffer at 150 mA for 90 min. The RNA was fixed to the membrane by UV crosslinking and stored dry at RT prior the oligonucleotide hybridization.

Hybridization Solution	
Compound	Amount
20x SSC	125ml
1M Pi Ph7.2	10ml
SDS	35g
100x Denhards solution	5ml
Salmon sperm DNA	500 mg
water	up to 500ml

Table 6: Composition of the Hybridization solution.

100x Dennardt's solution	
Compound	Amount
Ficoll 400	2%
Polyvinylpyrrolidone	2%
Bovine Serum Albumine	2%

Table 7: Composition of the Dennardt's solution.

Pre wash 1	
Compound	Volume [ml]
20x SSC	75
20% SDS	125
NaH_2PO_4 pH7.5	12.5
Water	237.5

Table 8: Composition of the pre wash 1 solution.

Wash solution 1	
Compound	Volume [ml]
100x Dennardt's solution	15
Pre wash 1	135

Table 9: Composition of the wash 1 solution.

Wash solution 2	
Compound	Volume [ml]
20x SSC	50
20% SDS	50
Water	900

Table 10: Composition of the wash 2 solution.

Stripping solution	
Compound	Volume [ml]
20x SSC	5
20% SDS	5
Water	990

Table 11: Composition of the stripping solution.

Oligos			
Number	tRNA	Anticodon	Sequence 5´-3´
ZP13R	Tyrosine	GUA	GTGGTCCTTCCGGCCGGAATCGAA
ZP6R	Isoleucine	AAU	CCAACAGGGGTCGAACCTGTGACC

Table 12: Oligonucleotides used for probing of specific tRNAs.

4.4.3.1 Method

Compound	Volume [µl]
Oligo	1 (25ng)
10x T4 Polynucleotide Kinase (PNK) (NEB#M0201S)	1
10x T4 PNK buffer	1
[gamma-32P]-ATP	1-5
water	Up to 10

Table 13: Kinase reaction mixture for the radio-labelling of the oligo.

To visualize various tRNAs, specific oligonucleotide probes were labelled with a radioactive [gamma-^{32}P]-ATP isotope. To insert the P^{32} into the 5'-end of the DNA oligo, the kinase reaction mixture was incubated at 37 °C for 2h, then 90 µl of water was added and the sample was purified through a Sephadex G25 column. The membrane was pre-hybridized for 1 h at 47°C in the hybridisation solution, the radiolabelled oligo was added and it was hybridised for 12 h at the same temperature. The free oligos were removed by two washing steps, each for 20 min at 47°C and the membrane was exposed to Phosphoimager cassette and subsequently developed using the Typhoon scanner.

5 Results

5.1 Queuosine starvation experiment

In our *in vitro* culturing conditions, the q base serves as a Q precursor in the medium and comes from the supplemental foetal bovine serum (FBS). Thus, we used commercially available dialysed FBS, which is free of all molecules with low molecular weight including q base. BS trypanosomes were grown in the media with dialysed FBS for a period of 10 days without significant change in doubling time (Fig. 5). The minimal effect on growth of trypanosomes can be explained by the small amount of Q-tRNAs, which were detected using APB (data not shown). To further reduce the amount of Q modification and to fully understand its physiological role, *TGT2* KO cell line was prepared.

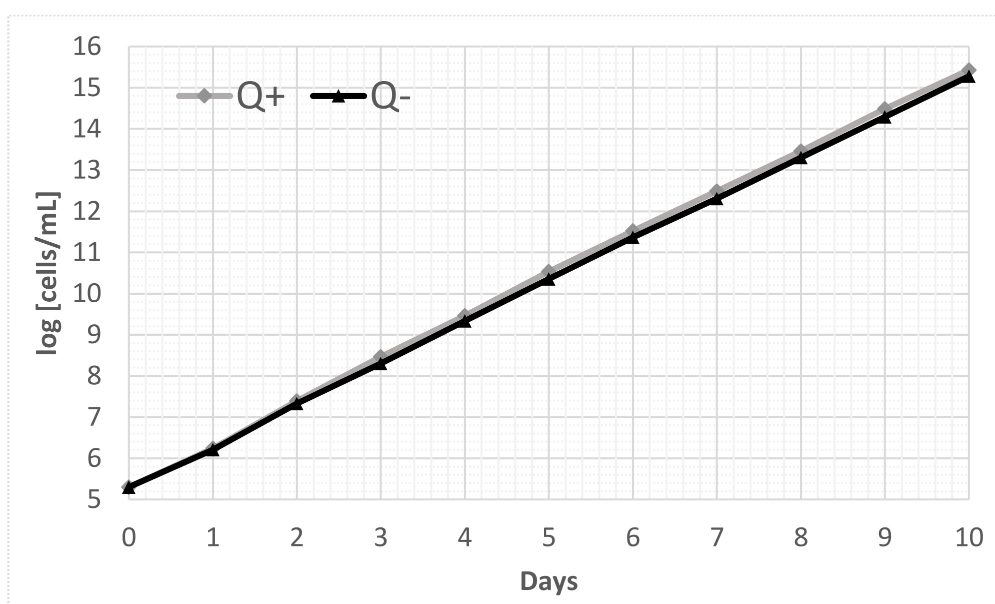

Figure 5: Effects of Q starvation on the growth of T. brucei. Growth curves of the BS wild type cells in media with regular FBS (Q+; squares) and with dialysed FBS (Q-; triangles).

5.2 Preparation of the *TbTGT2* knock-out cell line

To obtain a KO of *TbTGT2*, a DNA construct was prepared by fusion PCR strategy. To check for a successful amplification of the first three individual PCRs, the products were analysed by 0.75% agarose gel electrophoresis. The separated DNA was visualized in the HP Alphaimager and the correct sizes of the PCR products (5’ 540bp, HygR 1026bp, 3’ 685bp) were determined by comparison with the DNA ladder. In the final fusion PCR, these products were merged into the final DNA construct with nested primers. The correct size of the construct was determined by DNA agarose gel electrophoresis. Additionally, the construct was confirmed by sequencing.

During electroporation, a membrane weakening electric pulse enables the DNA to enter the nucleus. The KO construct, containing the gene for the hygromycin resistance, was transfected into *T. brucei* cells (strain 427). After the cells recovered, a selective pressure was applied with hygromycin

and the medium was plated in 1 ml aliquots. With a bright field microscope, the wells were examined for positive transformants. Four days after the transfection, one positive clone was observed and cultivated.

5.3 Confirmation of the knock-out cell line by PCR

To confirm the correct integration of the DNA construct in the genome of T. *brucei* diagnostic PCR was performed. Genomic DNA isolated from the WT strain and the KO cell line served as a template in PCR with two primer combinations, which either anneal to the specific regions of *TbTGT2* or the *HygR* sequence. As shown in Figure 6 *HygR* presence was confirmed in the KO, while *TGT2* was detected only in the WT gDNA. The oligo “ZP184R” was deigned to anneal further downstream of the 3’ (outside of the electroporated construct), to exclude the possibility of integration into different location within the *T. brucei* genome. Since *T. brucei* is a diploid species, we expected to remove only one allele per electroporation. However, the results of the diagnostic PCR confirm the correct integration of the HygR and the removal of all *TGT2* alleles. Therefore, the resulting cell line was considered to be a *TbTGT2* double KO and was further examined for its role in formation of Q-tRNA modification.

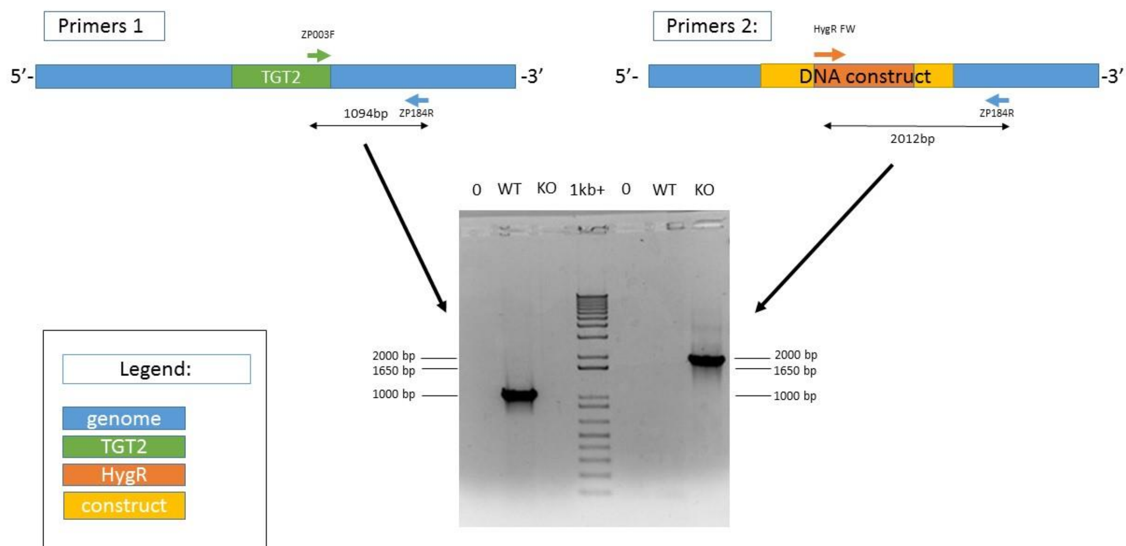

Figure 6:The diagnostic PCR to test the correct integration of the DNA construct in the T. brucei genome.

5.4 Detection of the Q-tRNA levels in the *TGT2* knock-out cell line using RNA affinity gel

In order to determine Q-tRNA levels, total RNA was isolated from the WT and KO cells, deacylated and subsequently resolved on an affinity gel containing the N-acryoyl-3-aminophenylboronic acid (APB). This principle of Q detection approach was developed by Igloi and Kössel (52). *Cis*-diol groups located at the free ribose at the 3´end of every tRNA exhibit a slower migration through gels containing polyacrylamide covalently linked to APB. The hypermodified Q contains two neighbouring hydroxyl groups in the *cis* conformation (Fig. 7). Consequently, the migration of Q-modified tRNAs is slower in comparison to G-tRNAs, resulting in two bands on an APB gel. The periodate treatment causing oxidation of *cis*-diol groups eliminates the differential mobility shift, which results in a single band.

Prior the Northern blot transfer, the gel was stained with ethidium bromide (EtBr) in order to check the integrity of the extracted RNA (Fig. 8A). Sharp bands of the rRNA and tRNA indicated good quality of the resolved RNA, which was subsequently electro blotted on a positively charged nylon membrane and fixed by UV crosslinking.

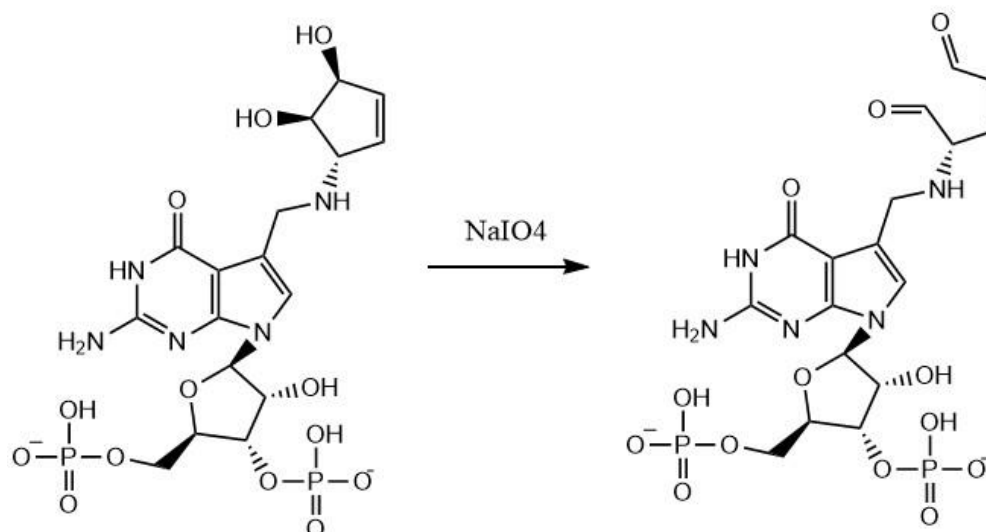

Figure 7: The sodium periodate oxidizes the cis diol of the Q modification into two aldehyde groups. Since the APB gel has an affinity to diols, the complete oxidation of the Q tRNA leads to one distinct bend. This allows the comparison of other samples to a Q free tRNA sample.

The membrane was hybridised with a radiolabelled oligo, which specifically binds to the tyrosyl-tRNA (tRNA$^{Tyr)}$, one of four Q modified tRNAs in *T. brucei* (previous data of the laboratory). Following exposure of the membrane in the Phosphoimager cassette (which has the ability to record the energy from radioactive decay) the signal was obtained using the Typhoon scanner.

The obtained results showed the complete disappearance of the shifted band for tRNA^{Tyr} in the case of *TbTGT2* KO in comparison to WT RNA, in which most of the tRNA^{Tyr} contains Q modification (Fig. 8B). Periodate oxidation (Ox) served as a control to show complete oxidation of the *cis*-diols, eliminating the mobility shift. For a loading control the membrane was stripped and re-hybridized with the oligo for isoleucyl tRNA (tRNA^{Ile}), which lacks Q modification (Fig 8C).

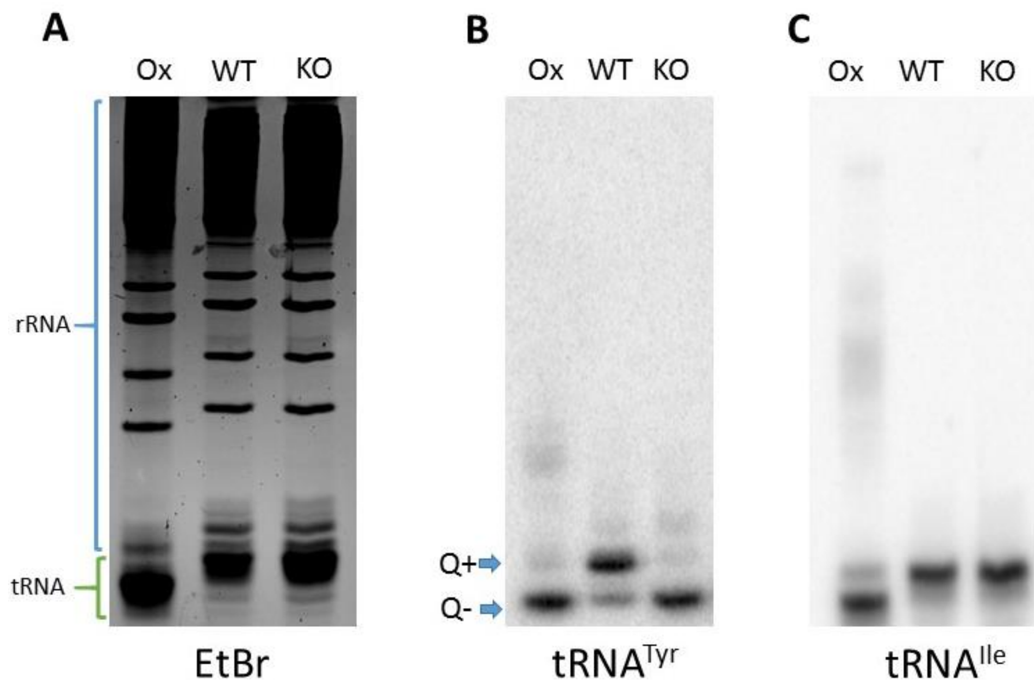

Figure 8: A) The EtBr stained gel serves as loading control, showing the presence and separation of RNA. The upper region contains ribosomal RNA(rRNA) and the lower tRNA. B) The Northern blot with the oligo specific for tRNA^{Tyr} to visualize Q modified tRNA. Two main bends can be distinguished in the gel. The upper Q+ bend represents Q modified tRNA and the lower Q- bend corresponds to G-tRNA. Periodate oxidation (Ox) served as a control to show complete oxidation of the cis-diols, eliminating the mobility shift. C) Detection of the isoleucyl-tRNA (tRNA^{Ile}), which doesn't contain Q, served as loading control.

5.5 Evaluation of the role of Q-tRNA depletion on the growth of the bloodstream stage of *T. brucei*:

Since the almost complete elimination of the Q modification was confirmed by the Northern analysis, we further examined the physiological relevance of the TbTGT2 in the cell culture by measuring cell densities of the WT and KO strains respectively. However, cells ablated for TGT2 grow very well in the HMI medium, their growth curve being virtually indistinguishable from those of the wild type 427 cells (Fig. 9).

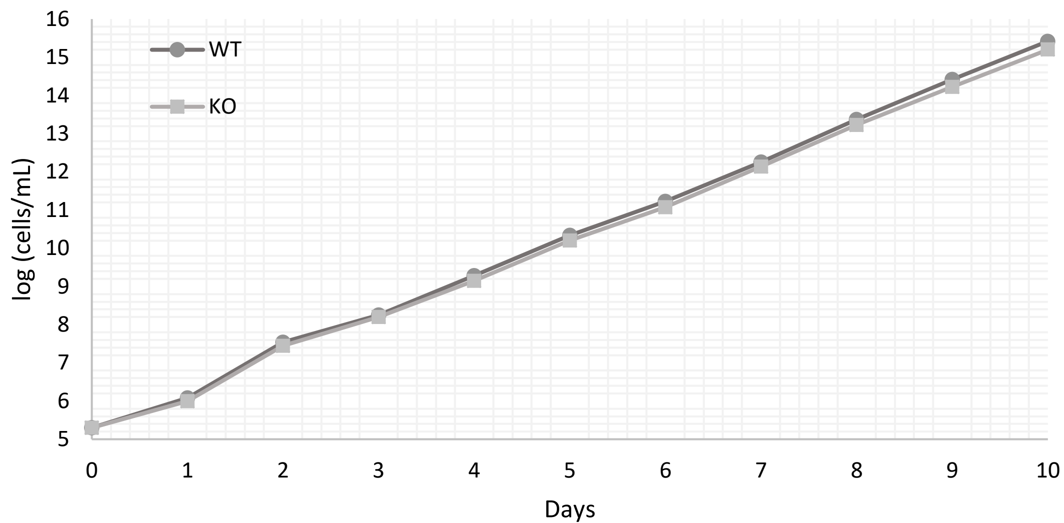

Figure 9: Effects TbTGT2 KO on the growth of the BS of T. brucei. Growth curves of wild type (WT;circles), KO (KO-; squares) cell line of TbTGT2 in Q+medium. The y axis is labelled by a log scale and represents the products of the measured cell densities and total dilutions. Cell densities were measured using the Beckman Z2 cell counter.

6 Discussion

Queuosine (Q), a hypermodified guanosine, is one of the most intricate tRNA modifications that have been described to date. The existence of Q was documented in nearly all kingdoms of life. Nevertheless, while bacteria can synthetize Q *de novo*, eukaryotes lack this pathway and rely on the salvage of Q or its free base q form the environment or gut microbiome. The omnipresence of Q and its location in the anticodon of tRNA suggest that the modification is most likely of a great importance. Nevertheless, the physiological role of Q deficiency was subject of several studies with no obvious defects if grown axenically in Q free conditions (34–37). Recent studies provided some evidence by showing that either Q deficiency or disruption of the mouse *TGT* (gene responsible for Q-tRNAs in eukaryotes) impairs the conversion of phenylalanine to tyrosine by phenylalanine hydroxylase (PAH). This occurs supposedly due to an uncontrolled oxidation of the tetrahydrobiopterin (BH4), an essential cofactor of PAH (38). However, the exact role of Q in oxidation of BH4 is still not clear. Additionally, it has been shown that Q-deficient *D. melanogaster* are more sensitive to increased cadmium concentrations (36).

In this study, we were investigating the physiological role of the Q-tRNA modification in the bloodstream stage of *T. brucei*. We achieved Q starvation of trypanosomes by growing them in the media with dialysed FBS, which has a significant lower concentration of molecules with low molecular weight including Q or q. Based on the results and similar to starvation experiments performed in other organisms (35, 37), *T. brucei* cells cultivated in the media with reduced Q sources show no pathological symptoms. Although, we observed almost complete disappearance of Q-tRNA in dialysed FBS, there are inconsolable traces of q, which together with everyday dilution of the cell culture may be sufficient to provide enough of the q micronutrient for the normal physiological status of the cells.

With a goal to further challenge the cells, knock-out cell line of the previously identified *TbTGT2* was generated in order to completely reduce levels of Q-tRNAs and to perform phenotypic analysis of this studied protein. The obtained data demonstrated almost complete disappearance of Q-tRNA in the KO, which resulted only in an insignificantly slower growth of the cells for 10 days. Perhaps, the measured period was not long enough to exhibit a potential translation problems considering the very high stability of tRNAs (53). In our opinion, the long half-life is not responsible for the lack of the growth phenotype in Q-depleted cells, because the exponential growth of the BS with generation time of 8-12 h would certainly dilute out the remaining Q-tRNAs. Alternatively, minor Q pathways may exist, which incorporate enough Q into tRNA for the cell to be viable (54). This could be done by the other subunit TbTGT1, modifying only small portion of the tRNA pool, which are under detection

levels of the Northern blot analysis. Nevertheless, it has to be considered that the growth curve experiments were done *in vitro*, where the organism will never experience any stress situations similar to those in the host. In order to determine the functional significance of Q-tRNA modification in trypanosomes, we propose additional phenotypic *in vivo* characterization of the generated knock-out cell line directly in the bloodstream of the mammalian host with the goal to face real environment during parasite infection.

7 Conclusion

With the help of a successfully created *TbTGT2* KO cell line, the role of TGT2 in the formation of Q modified tRNA in bloodstream stage of *T. brucei* was proved. Additionally, the physiological effect of the absence of Q -tRNA in *in vitro* cultured cells was observed with no immediate physiological consequences.

8 References

1. Barrett, M. P., Burchmore, R. J. S., Stich, A., Lazzari, J. O., Frasch, A. C., Cazzulo, J. J., and Krishna, S. (2003) The trypanosomiases. *The Lancet* **362**, 1469–1480

2. Matthews, K. R. (2005) The developmental cell biology of *Trypanosoma brucei*. *Journal of cell science* **118**, 283–290

3. Berriman, M., Ghedin, E., Hertz-Fowler, C., Blandin, G., Renauld, H., Bartholomeu, D. C., Lennard, N. J., Caler, E., Hamlin, N. E., Haas, B., Bohme, U., Hannick, L., Aslett, M. A., Shallom, J., Marcello, L., Hou, L., Wickstead, B., Alsmark, U. C. M., Arrowsmith, C., Atkin, R. J., Barron, A. J., Bringaud, F., Brooks, K., Carrington, M., Cherevach, I., Chillingworth, T.-J., Churcher, C., Clark, L. N., Corton, C. H., Cronin, A., Davies, R. M., Doggett, J., Djikeng, A., Feldblyum, T., Field, M. C., Fraser, A., Goodhead, I., Hance, Z., Harper, D., Harris, B. R., Hauser, H., Hostetler, J., Ivens, A., Jagels, K., Johnson, D., Johnson, J., Jones, K., Kerhornou, A. X., Koo, H., Larke, N., Landfear, S., Larkin, C., Leech, V., Line, A., Lord, A., Macleod, A., Mooney, P. J., Moule, S., Martin, D. M. A., Morgan, G. W., Mungall, K., Norbertczak, H., Ormond, D., Pai, G., Peacock, C. S., Peterson, J., Quail, M. A., Rabbinowitsch, E., Rajandream, M.-A., Reitter, C., Salzberg, S. L., Sanders, M., Schobel, S., Sharp, S., Simmonds, M., Simpson, A. J., Tallon, L., Turner, C. M. R., Tait, A., Tivey, A. R., van Aken, S., Walker, D., Wanless, D., Wang, S., White, B., White, O., Whitehead, S., Woodward, J., Wortman, J., Adams, M. D., Embley, T. M., Gull, K., Ullu, E., Barry, J. D., Fairlamb, A. H., Opperdoes, F., Barrell, B. G., Donelson, J. E., Hall, N., Fraser, C. M., Melville, S. E., and El-Sayed, N. M. (2005) The genome of the African trypanosome *Trypanosoma brucei*. *Science (New York, N.Y.)* **309**, 416–422

4. Bringaud, F., Riviere, L., and Coustou, V. (2006) Energy metabolism of trypanosomatids: adaptation to available carbon sources. *Molecular and Biochemical Parasitology* **149**, 1–9

5. Lai, D.-H., Hashimi, H., Lun, Z.-R., Ayala, F. J., and Lukeš, J. (2008) Adaptations of *Trypanosoma brucei* to gradual loss of kinetoplast DNA: *Trypanosoma equiperdum* and *Trypanosoma evansi* are petite mutants of *T. brucei*. *PNAS* **105**, 1999–2004

6. Martinez-Calvillo, S., Vizuet-de-Rueda, J. C., Florencio-Martinez, L. E., Manning-Cela, R. G., and Figueroa-Angulo, E. E. (2010) Gene expression in trypanosomatid parasites. *Journal of biomedicine & biotechnology* **2010**, 525241

7. Read, L. K., Lukes, J., and Hashimi, H. (2016) Trypanosome RNA editing: the complexity of getting U in and taking U out. *Wiley interdisciplinary reviews. RNA* **7**, 33–51

8. Schneider, A., and Maréchal-Drouard, L. (2000) Mitochondrial tRNA import: Are there distinct mechanisms? *Trends in Cell Biology* **10**, 509–513

9. Paris, Z., Fleming, I. M. C., and Alfonzo, J. D. (2012) Determinants of tRNA editing and modification: avoiding conundrums, affecting function. *Seminars in cell & developmental biology* **23**, 269–274

10. Agris, P. F. (2008) Bringing order to translation: the contributions of transfer RNA anticodon-domain modifications. *EMBO reports* **9**, 629–635

11. Gustilo, E. M., Vendeix, F. A., and Agris, P. F. (2008) tRNA's modifications bring order to gene expression. *Current opinion in microbiology* **11**, 134–140

12. Fergus, C., Barnes, D., Alqasem, M. A., and Kelly, V. P. (2015) The Queuine Micronutrient: Charting a Course from Microbe to Man. *Nutrients* **7**, 2897–2929

13. Harada, F., and Nishimura, S. (1972) Possible Anticodon Sequences of $tRNA^{His}$, $tRNA^{Asn}$, and $tRNA^{Asp}$ from *Escherichia coli*. Universal Presence of Nucleoside Q in the First Position of the Anticodons of These Transfer Ribonucleic Acids. *Biochemistry* **11**, 301–308

14. Chen, Y.-C., Kelly, V. P., Stachura, S. V., and Garcia, G. A. (2010) Characterization of the human tRNA-guanine transglycosylase: Confirmation of the heterodimeric subunit structure. *RNA (New York, N.Y.)* **16**, 958–968

15. Phillipson, D. W., Edmonds, C. G., Crain, P. F., Smith, D. L., Davis, D. R., and McCloskey, J. A. (1987) Isolation and Structure Elucidation of an Epoxide Derivative of the Hypermodified Nucleoside Queuosine from *Escherichia coli* Transfer RNA. *J. Biol. Chem.* **262**, 3462–3471

16. Vinayak, M., and Pathak, C. (2010) Queuosine modification of tRNA: its divergent role in cellular machinery. *Bioscience Reports* **30**, 135–148

17. Boland, C., Hayes, P., Santa-Maria, I., Nishimura, S., and Kelly, V. P. (2009) Queuosine Formation in Eukaryotic tRNA Occurs via a Mitochondria-localized Heteromeric Transglycosylase. *The Journal of biological chemistry* **284**, 18218–18227

18. Okada, N., and Nishimura, S. (1979) Isolation and Characterization of a Guanine Insertion Enzyme, a Specific tRNA Transglycosylase, from *Escherichia coli*. *J. Biol. Chem.* **254**, 3061–3066

19. Nishimura, S. (1983) Structure, Biosynthesis, and Function of Queuosine in Transfer RNA. In Waldo E. Cohn, Ed. *Progress in Nucleic Acid Research and Molecular Biology,* Academic Press

20. Reyniers, J. P., Pleasants, J. R., Wostmann, B. S., Katze, J. R., and Farkas, W. R. (1981) Administration of Exogenous Queuine Is Essential for the Biosynthesis of the Queuosine-containing Transfer RNAs in the Mouse. *J. Biol. Chem.* **256**, 11591–11594

21. Katze, J. R., Gunduz, U., Smith, D. L., Cheng, C. S., and McCloskey, J. A. (1984) Evidence That the Nucleic Acid Base Queuine Is Incorporated Intact into tRNA by Animal Cells. *Biochemistry* **23**, 1171–1176

22. Zallot, R., Brochier-Armanet, C., Gaston, K. W., Forouhar, F., Limbach, P. A., Hunt, J. F., and Crecy-Lagard, V. de (2014) Plant, Animal, and Fungal Micronutrient Queuosine is Salvaged by Members of the DUF2419 Protein Family. *ACS chemical biology* **9**, 1812–1825

23. Elliott, M. S., Katze, J. R., and Trewyn, R. W. (1984) Relationship between a Tumor Promoter-induced Decrease in Queuine Modification of Transfer RNA in Normal Human Cells and the Expression of an Altered Cell Phenotype. *Cancer Res* **44**, 3215–3219

24. Elliott, M. S., Trewyn, R. W., and Katze, J. R. (1985) Inhibition of Queuine Uptake in Cultured Human Fibroblasts by Phorbol-12,13-didecanoate. *Cancer Res* **45**, 1079–1085

25. Morris, R. C., Galicia, M. C., Clase, K. L., and Elliott, M. S. (1999) Determination of Queuosine Modification System Deficiencies in Cultured Human Cells. *Molecular Genetics and Metabolism* **68**, 56–67

26. Elliott, M. S., and Crane, D. L. (1990) Protein kinase C modulation of queuine uptake in cultured human fibroblasts. *Biochemical and Biophysical Research Communications* **171**, 393–400

27. Morris, R. C., Brooks, B. J., Hart, K., and Elliott, M. S. (1996) Modulation of queuine uptake and incorporation into tRNA by protein kinase C and protein phosphatase. *Biochimica et Biophysica Acta (BBA) - Molecular Cell Research* **1311**, 124–132

28. Elliott, M. S., and Katze, J. R. (1986) Inhibition of Queuine Uptake in Diploid Human Fibroblasts by phorbol-12,13-didecanoate. Requirement for a factor derived from early passage cells. *J. Biol. Chem.* **261**, 13019–13025

29. Chen, Y.-C., Brooks, A. F., Goodenough-Lashua, D. M., Kittendorf, J. D., Showalter, H. D., and Garcia, G. A. (2011) Evolution of eukaryal tRNA-guanine transglycosylase: insight gained from the heterocyclic substrate recognition by the wild-type and mutant human and Escherichia coli tRNA-guanine transglycosylases. *Nucleic acids research* **39**, 2834–2844

30. Romier, C., Reuter, K., Suck, D., and Ficner, R. (1996) Mutagenesis and crystallographic Studies of *Zymomonas mobilis* tRNA-Guanine Transglycosylase Reveal Aspartate 102 as the Active Site Nucleophile. *Biochemistry* **35**, 15734–15739

31. Xie, W., Liu, X., and Huang, R. H. (2003) Chemical trapping and crystal structure of a catalytic tRNA guanine transglycosylase covalent intermediate. *Nature structural biology* **10**, 781–788

32. Katze, J. R., and Farkas, W. R. (1979) A factor in serum and amniotic fluid is a substrate for the tRNA-modifying enzyme tRNA-guanine transferase. *PNAS* **76**, 3271–3275

33. Katze, J. R. (1985) Queuosine Metabolism: Possible Relation to B-Cell Activation by C8 Derivatives of Guanosine. *Exp Biol Med (Maywood)* **179**, 492–496

34. Ott, G., Kersten, H., and Nishimura, S. (1982) *Dictyostelium discoideum:* A useful model system to evaluate the function of queuine and of the Q-family of tRNAs. *FEBS Letters* **146**, 311–314

35. Gaur, R., Björk, G. R., Tuck, S., and Varshney, U. (2007) Diet-dependent depletion of queuosine in tRNAs in *Caenorhabditis elegans* does not lead to a developmental block. *J Biosci* **32**, 747–754

36. Siard TJ, Jacobson KB, and Farkas WR (1991) Queuine metabolism and cadmium toxicity in *Drosophila melanogaster*. *Biofactors* **3**, 41–47

37. Kirtland, G. M., Morris, T. D., Moore, P. H., O'Brian, J. J., Edmonds, C. G., McCloskey, J. A., and Katze, J. R. (1988) Novel Salvage of Queuine from Queuosine and Absence of Queuine Synthesis in *Chlorella pyrenoidosa* and *Chlamydomonas reinhardtii*. *J. Bacteriol.* **170**, 5633–5641

38. Rakovich, T., Boland, C., Bernstein, I., Chikwana, V. M., Iwata-Reuyl, D., and Kelly, V. P. (2011) Queuosine deficiency in eukaryotes compromises tyrosine production through increased tetrahydrobiopterin oxidation. *The Journal of biological chemistry* **286**, 19354–19363

39. Marks, T., and Farkas, W. R. (1997) Effects of a Diet Deficient in Tyrosine and Queuine on Germfree Mice. *Biochemical and Biophysical Research Communications* **230**, 233–237

40. Reisser, T., Langgut, W., and Kersten, H. (1994) The nutrient factor queuine protects HeLa cells from hypoxic stress and improves metabolic adaptation to oxygen availability. *Eur J Biochem* **221**, 979–986

41. Hortner, S. R., Ritschel, T., Stengl, B., Kramer, C., Schweizer, W. B., Wagner, B., Kansy, M., Klebe, G., and Diederich, F. (2007) Potent Inhibitors of tRNA-Guanine Transglycosylase, an Enzyme Linked to the Pathogenicity of the *Shigella* Bacterium: Charge-Assisted Hydrogen Bonding. *Angewandte Chemie (International ed. in English)* **46**, 8266–8269

42. Klebe, G. (2000) Recent developments in structure-based drug design. *J Mol Med* **78**, 269–281

43. Brenk, R., Gerber, H.-D., Kittendorf, J. D., Garcia, G. A., Reuter, K., and Klebe, G. (2003) From Hit to Lead: *De Novo* Design Based on Virtual Screening Hits of Inhibitors of tRNA-Guanine Transglycosylase, a Putative Target of Shigellosis Therapy. *HCA* **86**, 1435–1452

44. Meyer, E. A., Furler, M., Diederich, F., Brenk, R., and Klebe, G. (2004) Synthesis and *In Vitro* Evaluation of 2-Aminoquinazolin-4(3H)-one-Based Inhibitors for tRNA-Guanine Transglycosylase (TGT). *HCA* **87**, 1333–1356

45. Shindo-Okada, N., Terada, M., and Nishimura, S. (1981) Changes in Amount of Hypo-Modified tRNA Having Guanine in Place of Queuine during Erythroid Differentiation of Murine Erythroleukemia Cells. *Eur J Biochem* **115**, 423–428

46. Lin, V. K., Farkas, W. R., and Agris, P. F. (1980) Specific changes in Q-ribonucleoside containing transfer RNA species during Friend leukemia cell erythroid differentiation. *Nucl Acids Res* **8**, 3481–3489

47. Chen, Y.-L., and Wu, R.-T. (1994) Altered Queuine Modification of Transfer RNA Involved in the Differentiation of Human K562 Erythroleukemia Cells in the Presence of Distinct Differentiation Inducers. *Cancer Res* **54**, 2192–2198

48. Gündüz, U., Elliott, M. S., Seubert, P. H., Houghton, J. A., Houghton, P. J., Trewyn, R. W., and Katze, J. R. (1992) Absence of tRNA-guanine transglycosylase in a human colon adenocarcinoma cell line. *Biochimica et Biophysica Acta (BBA) - Molecular Basis of Disease* **1139**, 229–238

49. Emmerich, B., Zubrod, E., Weber, H., Maubach, P. A., Kersten, H., and Kersten, W. (1985) Relationship of Queuine-lacking Transfer RNAs to the Grade of Malignancy in Human Leukemias and Lymphomas. *Cancer Res* **45**, 4308–4314

50. Merritt, C., and Stuart, K. (2013) Identification of Essential and Non-essential Protein Kinases by a Fusion PCR Method for Efficient Production of Transgenic *Trypanosoma brucei*. *Molecular and Biochemical Parasitology* **190**, 44–49

51. Chomczynski, P., and Sacchi, N. (1987) Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Analytical Biochemistry* **162**, 156–159

52. Igloi, G. L., and Kössel, H. (1985) Affinity electrophoresis for monitoring terminal phosphorylation and the presence of queuosine in RNA. Application of polyacrylamide containing a covalently bound boronic acid. *Nucl Acids Res* **13**, 6881–6898

53. Engelke, D. R., and Hopper, A. K. (2006) Modified view of tRNA: stability amid sequence diversity. *Molecular cell* **21**, 144–145

54. Reader, J. S., Metzgar, D., Schimmel, P., and Crécy-Lagard, V. d. (2004) Identification of Four Genes Necessary for Biosynthesis of the Modified Nucleoside Queuosine. *J. Biol. Chem.* **279**, 6280–6285